SIDEROPHORE AND PIGMENT PRODUCTION BY *CANDIDA ALBICANS*

By

ALTAHER IBRAHIM ALTABET

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in the Faculty of Science,

University of Glasgow

Institute of Biomedical and Life Sciences

Division of Infection and Immunity

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

PREFACE

This thesis is the original work of the author.

Altaher I. Altabet

DEDICATION

I should like to dedicate this thesis to my wife "Mrs. Samia I. Abulgasem" for her enthusiasm encouragement through my academic career, my family and to the memory of my father.

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LIST OF ABBREVIATIONS

A	Absorbance
ATCC	American Type Culture Collection
B.C.	Before Christ
BHIA	Brain heart infusion agar
°C	Degrees Celsius
CAS	Chrome Azural S
cm	Centimetre
CO_2	Carbon dioxide
Conc.	concentration (s)
CY	Casein-yeast extract medium
d	Day (s)
DNA	Dioxyribonucleic acid
dYNB	Deferrated yeast nitrogen base agar
et al.	*et alios* (and others)
EDDA	Ethylene diamine di-orthohydroxy phenyl acetic acid
EDTA	Ethylenediaminetetra-acetic acid
Fe^{2+}	Ferrous, iron (II) compounds
Fe^{3+}	Ferric, iron (III) compounds
g	Gram
Gal.	Galactose
GDH	Glasgow Dental Hospital, Scotland UK
Glc.	Glucose
GGB	Glucose glycine broth
GRI	Glasgow Royal Infirmary, Scotland UK
h	Hour (s)
HDTMA	Hexadecyltrimethyl ammonium bromide
HY^-	*C. albicans* mutants producing only yeast forms
l	Litre
LF	Lactoferrin-iron binding proteins
M	Molar
M_r	Relative Molecular Mass
µg	Microgram
µl	Microlitre
µM	Micromolar
min	Minute (s)
ml	Millilitre (s)

mM	Millimolar
NCTC	National Collection of Type Cultures
NSB	Neopeptone starch broth
NYP	N-acetylglucosamine-yeast nitrogen base-proline medium
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
Rf	Relative Mobility
RNA	Ribonucleic acid
r.p.m	Revolutions per minute
SDA	Sabouraud dextrose agar
SEM	Standard error of the mean
Sid.	Siderophore
spp.	Species
SRBCs	Sheep Red Blood Cells
TF	Transferrin-iron binding proteins
TLC	Thin layer chromatography
UV	Ultraviolet
vol.	Volume
v/v	Volume/volume ratio
wt.	Weight
w/v	Weight/volume ratio
Y	Yeast
Y^-	*C. albicans* mutants producing only hyphal forms
YNB	Yeast nitrogen base medium

SUMMARY

The aim of this project was to study siderophore and pigment production by yeast and hyphal forms of *Candida albicans* .

The ability to form true hyphal cells is virtually unique to *C. albicans* and the first objective was to identify the best liquid medium capable of yielding pure yeasts or pure hyphae for use in this study. Seven liquid media were tested and overall the results demonstrated that the optimal growth conditions for hyphae are high temperature (37°C) and neutral pH. On the other hand, pure-yeast form cultures were obtained by growing at a lower temperature (25°C). Three media (glucose glycine broth, NYP medium and Lee's medium) gave a high percentage of hyphae or yeasts by simply a change in incubation temperature.

One mechanism of iron acquisition is the secretion of ferric-specific chelators termed siderophores, whose function is to solubilize and transport iron into the cell. Generally, two types of siderophores exist: the phenolate and hydroxamate types. In this study, four strains of *C. albicans* were examined for the production of siderophores after growth in glucose glycine broth at 37°C (for hyphae) or 25°C (for yeasts). Siderophore production in both liquid and solid media was determined by the universal chemical assay for siderophores. This assay utilises a dye complex of Chrome azurol S (CAS) and hexadecyltrimethyl ammonium bromide (HDTMA) that has a high affinity for iron. The iron-dye complex is blue with an absorption maximum at 630 nm. When a strong chelator removes the iron from the dye, its colour turns from blue to red. The dye was incorporated into yeast nitrogen base agar to detect siderophore production via the formation of pink zones around *Candida* colonies. Specific assays for phenolate- or hydroxamate-type siderophores were also used. Phenolate compounds were determined by the Arnow method with catechol as a positive control. Hydroxamates were detected by the method of Holzberg and Artis (1983) with desferal as a positive control. The results showed that siderophores are produced by both yeast

and hyphal forms of *C. albicans*. When siderophore production was considered in relation to cell dry weight, secretion by hyphal forms was greater than that of yeast forms. Only hydroxamate-type chelators were found; phenolate-type siderophores were not detected.

Green pigment production by *C. albicans* on plates of blood agar was first observed by Jones and Peck in 1940. A later report (McCourtie and Douglas, 1985) indicated that the pigment was produced by *C. albicans* during prolonged incubation in medium containing 500 mM galactose. Subsequently, Sweet and Douglas (1991) showed that green pigment synthesis was regulated by the availability of iron.

In this study, green pigment production by strains of *C. albicans* was affected by various environmental factors. All strains tested produced green pigment; however, the amount produced varied from strain to strain. Green pigment synthesis was affected by factors such as carbon source, and increased when galactose was used instead of glucose. However, glucose induced pigment synthesis under conditions of iron limitation whereas galactose did not. Pigment production was also increased at a growth temperature of 37°C as compared with 25 or 30°C; by yeast morphology rather than hyphal morphology; by prolonged incubation periods; and finally, by shaking rather than static growth conditions. All strains inoculated on to Sabouraud dextrose blood agar showed a very dark grey zone around the colonies after incubation at 37°C for 24h.

Green pigment production was not induced by growing *C. albicans* in medium with low concentrations of phosphate , magnesium, manganese, zinc and copper, in contrast to the induction seen with a low concentration of iron. The highest pigment production was noted when the organism was grown at normal concentrations of these components with either carbon source (glucose or galactose). Generally, very low pigment production was observed in media which had no added phosphorus, magnesium, manganese, zinc and copper and which

therefore contained only traces of these elements associated with the other chemicals which comprise yeast nitrogen base.

When cultured in a chemically defined medium having tryptophan as a major nitrogen source, *C. albicans* 'Outbreak' strain produced a pink pigment; this was only noted with media containing iron. No pigment was observed with media containing only proline as a nitrogen source even in the presence of exogenous iron. These results indicate that tryptophan is the constituent responsible for the production of a pink pigment and that iron is necessary for pigment formation.

The two pigments (green and pink) are quite different from each other. The pink pigment consists of an indole derivative whereas the green pigment comprises two unusual fluorescent materials which give it the yellow-greenish colour. The green pigment shows absorption at 360 nm and 440 nm, but no absorption at 520 nm. Exposure to ordinary light had a marked effect on the green pigment, which is unstable and becomes colourless after few days. Exposure of the pink pigment to light for four months caused a change in colour eventually to light orange. The addition of either acid or alkali had no effect on the green pigment in culture supernates which had an initial pH value of 2.29; the yellow-greenish colour was unchanged. On the other hand, a substantial effect was observed with the pink pigment in culture supernates with an initial pH 3.5 ; the pink pigment was sensitive to alkali and on addition of sodium hydroxide it converted to a light orange, returning to pink with the addition of acid

Fluorescence spectra of green and pink pigments confirmed that the green pigment consists of two components, one yellow and the other blue; the yellow component fluoresced at 520 nm (in the middle of the green, not yellow, colour zone) and the blue component fluoresced at 440 nm. The fluorescence spectra of the green and pink pigments showed no similarities. There were also no similarities between the spectra of those of chemicals which comprise YNB medium.

INTRODUCTION

1. The Genus *Candida*

1.1 Historical aspects

Candida albicans is generally considered the most important species in the genus *Candida* ; it causes disease when the host immune system is locally or systemically impaired. Superficial infections of the skin, nails and particularly the mucosal surfaces of the oral cavity and vagina (often called thrush) now rank among the most common of all infectious diseases (Odds, 1988, 1987).

The earliest recorded descriptions of thrush as a clinical entity were made by Hippocrates in his "Epidemics" (circa 460-377 B.C.) when he described two cases of oral aphthae associated with underlying diseases (Adams, 1939). The disease was also mentioned in the diary of Samuel Pepys for 17th June 1665 (Winner and Hurley, 1964). However, the initial discovery of the organism causing thrush was not made until 1839 when Langenbeck described a fungus in buccal aphthae in a case of typhus (Odds, 1988) and he wrongly thought the fungus was the cause of this disease. The thrush fungus was the subject of confused taxonomic studies for a century thereafter. Gruby placed the fungus in the genus *Sporotriticum* in 1842, while Robin, in 1853, named it *Oidium albicans*. Reess proposed the name *Saccharomyces albicans* in 1877 and *Monilia albicans* was suggested by Zopf in 1890. This name was retained until 1923 when Berkhout proposed the name *Candida* for the genus which was later adopted internationally. Since " *Candida* " derives from the Latin name (*Toga candida*) for the special white robe worn by candidates for Senate, and "*albicans*" is the present participle of the Latin albicare (to whiten), *Candida albicans* means "Whitening white" (Odds, 1988).

During the past two decades, the organism has assumed increasing prominence as major pathogen in immunocompromised hosts (Wade, 1993). It has also become a serious pathogen of persons infected with the human immunodeficiency virus (HIV) who develop acquired immunodeficiency syndrome

(AIDS). Full accounts are provided by Dupont *et al.* (1992) and Coleman *et al.* (1993).

1.2 Ecological niche

Yeasts are found commonly in association with plants and insects (Barnett *et al.*, 1983; Meyer *et al.*, 1984). *C.albicans* is the principal opportunistic yeast pathogen in man, warm-blooded animals and birds, and has been recovered from a far wider range of animal hosts than other yeast species (Odds, 1988). *C. albicans* has been isolated from samples of soil, plants, air and water. Indeed, efforts to detect *C.albicans* in sewage and water have focused on the value of this species as an indicator of faecal pollution (Robertson and Tobin, 1983; Cook and Schlitzer, 1981). *C.albicans* is found commonly in bedding and wash-basins of nurseries (Kashbur *et al.*, 1980), and in the hospital environment (Barnett *et al.*, 1983). The organism survives poorly on dry surfaces such as skin or glass (Kashbur *et al.*, 1980). *C.albicans* is known to colonise humans more frequently than other *Candida* species. *C. glabrata* and *C. tropicalis* can be found in the normal flora of the oral cavity, gastro-intestinal tract and vagina, but less frequently than *C. albicans*. *C. krusei*, *C. guilliermondii* and *C. parapsilosis* are found more frequently as part of the skin flora. Colonisation rates increase dramatically for all species in patients receiving cytotoxic drugs that alter the gastrointestinal mucosa, and / or broad spectrum antibiotics (Odds, 1988).

2. Biology of *Candida*

2.1 Classification

A yeast may be defined as a unicellular fungus reproducing by budding or fission (Kreger-Van Rij, 1984a). Nearly 600 species of yeasts are known; however, only about 25 are commonly associated with man or have been documented as pathogenic for man (Ahearn, 1978). The taxonomic position of

Candida is as follows: Kingdom *Fungi*, Division *Fungi Imperfecti*, Class *Blastomycetes*, Order *Cryptococcales*, Family *Cryptococcaceae*, Genus *Candida.*

At present there are 196 species accepted in the genus *Candida* which can be separated by biochemical reactions (Meyer *et al.*, 1984). Among these species, *C. albicans* isolates are consistently the most virulent. *C. tropicalis* is also pathogenic but is quantitatively less so than *C. albicans*. Less pathogenic still are *C. parapsilosis*, *C. pseudotropicalis*, *C. glabrata. C. kefyr*, *C. guilliermondii* and *C. krusei.* Another two species, *C. lusitaniae* and *C. viswanathii*, are possibly to be regarded as pathogenic but only in the immunocompromised host (Odd, 1988). *C.albicans* is classified as an imperfect yeast because of the absence of the taxonomically important character of sexual reproduction (Kreger-van Rij, 1984b).

2.2 Identification

There are two simple tests for identifying *Candida albicans.* In the first one, the germ-tube test, *Candida albicans* cultures can be identified within 90 min to 4 h by the formation of short germ tubes upon inoculation into 0.5 ml of human or animal serum substitutes, and incubation at 37°C to 42°C (Taschdjian *et al.*, 1960). The other test, for chlamydospore production by *Candida albicans*, is performed by growing the organism on corn meal agar (Benham, 1931).

Several differential isolation media have been proposed for the pathogenic *Candida* species. CHROMagar Candida is a new differential medium that allows selective isolation of yeasts and simultaneously identifies colonies of *C. albicans*, *C. tropicalis*, *C.glabrata* and *C. krusei* on the basis of colony morphology and pigmentation (Pfaller *et al.,* 1996). Other media, Albicans ID and Fluoroplate are commercially available media for the rapid identification of *C. albicans*. They contain, respectively, a chromogenic substrate and a fluorogenic substrate hydrolysed by the hexosaminidase of *C. albicans*, leading to a rapid identification of *C. albicans* on the basis of colony colour (Rousselle *et al.*, 1994). Recently,

Haynes and Westerney (1996) reported rapid identification of *Candida* spp. by using species-specific PCR within 3 hours of primary isolation.

2.3 Growth and Nutrition

Candida albicans can grow in the pH range 2.5 to 8, and at temperatures ranging from 20°C to 40°C, although growth has also been reported at pH values below 2 (Odds and Abbott, 1980). The organism can grow on simple defined media containing a source of carbon (eg. glucose), nitrogen (eg. ammonium salts), phosphate and other salts. Biotin, thiamine or other vitamins of the B complex may be required for growth, but folic acid has no stimulatory or inhibitory effect (Littman and Miwatant, 1964; Pitillo and Narkates, 1964 ; Kockova *et al.*,1964). *Candida* species grow best under aerobic conditions but they will grow under elevated concentrations of CO_2 in air (Odds, 1988). On the other hand, an atmosphere of pure CO_2 is inhibitory to *C.albicans* growth (Eklund and Jarmud, 1983). Anaerobic growth of *C.albicans* has been reported on agar media in clinical laboratory anaerobic jars, where the organism formed stellate colonies. This has been confirmed by Webster and Odds (1987). *C. albicans* typically ferments glucose and maltose more rapidly than other sugars (Zsolt *et al.*, 1963), while acid only is produced from galactose and sucrose. Glucose, galactose, sucrose and maltose can be assimilated as the sole carbon source (Lodder, 1970).

3. Morphogenesis

Candida albicans can exist in different morphological forms: budding yeast cells or blastospores, pseudohyphae (elongated yeast cells which appear as filamentous cell chains) and true hyphae with septa. *C. albicans* can also form chlamydospores; these are larger than blastospores, and are refractile, thick- walled cells that are often subtended from hyphae or pseudohyphae. *C.albicans* forms chlamydospores only *in vitro* (Odds, 1988). However, a few papers describe chlamydospores observed in host tissues *in vivo* (Ho and O'Day, 1981; Montes

and Wilborn, 1985). Chlamydospores are generally rich in lipid material (Kreger-Van Rij, 1984b), and seem to be essentially the product of a starvation growth environment for *C.albicans* . They are usually regarded as a " storage " form of the fungus (Odds, 1985).

Hyphae are branches from blastospores (Fig. 1); a microscopic tube contains multiple fungal cell units divided by septa. These can be in two forms, true hyphae or pseudohyphae. True hypha formation, a cylindrical outgrowth of new cellular material, is initiated from any point on the surface of blastospores by germination to give germ tubes, which are distinguishable from buds by their longer length and narrower width. Pseudohypha formation arises from a blastospore or a hypha by a budding process; the cells are very elongated, and are microscopically distinguishable from a true hypha only by conspicuous constrictions of the pseudohypha at septal junctions (Winner and Hurley, 1964). The production of hyphae is the only reproducible property known to belong exclusively to *C. albicans* among *Candida* species, and is therefore indicative of this species.

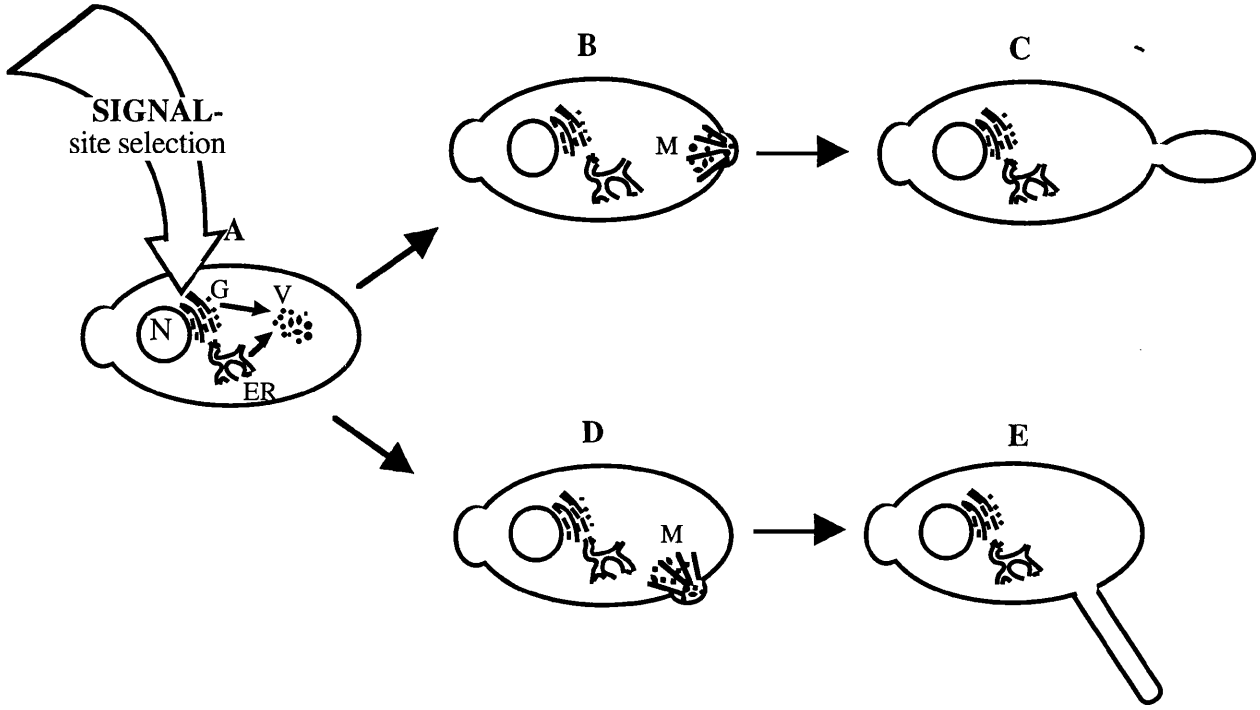
3.1 Dimorphism and pathogenicity

Candida albicans is capable of growing as yeasts or as elongate hyphae. Hypha formation has long been assumed to be a process of pathological significance. Several publications attempt to compare the pathogenicity of different morphological forms; these are reviewed below. However, it is now generally recognised that both yeasts and hyphae are present in infected tissue, but that production of filamentous forms aids penetration of host cells.

3.1.1 Pathogenicity depends on blastospores

Olsen and Birkeland (1977) found that the presence *C. albicans* hyphae was not pathognomic for denture stomatitis. Lethality in mice and rabbits was reported

Figure 1. A model for yeast-to-hyphal transformation. **(A)** A signal is received from the environment (temperature, pH, nutrient). The decision on yeast or hyphal growth is made and **(B,D)** a site for evagination is selected. **(C,E)** Actin fibrils move to the site of evagination and apical growth begins through the directed secretion of vesicles. The wall polysaccharides are synthesized through activation of the synthases in the plasma membrane. **(E)** With hyphal growth, the rigid cyclindrical shape is maintained through secondary wall formation; apical growth is continuous. **(C)** In yeast cells, the final size of the bud is achieved through generalized growth. The formation of a septum occurs through the controlled activation of chitin synthase. Intracellular organelles are: N, nucleus; G, membranous assembly similar to similar to Golgi; ER, endoplasmic reticulum; V, vesicles; and M, actin microfibrils. Adapted from Shepherd (1991).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to be more evident with yeast forms than with pseudohyphae (Evans and Mardon, 1977). Yeast forms were claimed to be more pathogenic because of less effective host defence mechanisms against the yeast phase (Evans, 1980). Higher mortality rates and invasion of deep organs with yeast-form inocula as compared with hyphal-form inocula have been observed in experimental disseminated candidosis (Evans,1980 ; Mardon *et al.*, 1975; Simonetti and Strippoli, 1973 ; Vaughn and Weinberg, 1978). *C. albicans* yeast cells form hyphal outgrowths when they escape from macrophages and neutrophils *in vitro* (Arai *et al.*, 1977; Stanley and Hurley, 1967). Hyphal forms are more sensitive to azole drugs, whereas growth in the yeast phase tends to be more resistant (Ryley and Ryley, 1990).

3.1.2 Pathogenicity depends on hyphal forms

Large numbers of publications state that only the hyphal form is pathogenic in human disease. Hyphal forms appear to play a pathogenetic role in the initial processes of tissue invasion. Hyphae have been found to adhere better than yeast cells to human buccal and vaginal epithelia (Kimura and Pearsall, 1978 ; Kimball and Pearsall, 1980 ; Sobel and Obedeanu, 1983 ; Anderson and Odds, 1985) and to vascular endothelia (Rotrosen *et al.*, 1985). Russell and Jones (1973a,b) found a slightly greater amount of *C. albicans* in the mouth of rats fed on high carbohydrate diets when they were inoculated intraorally with hyphal form *C. albicans* instead of blastospores. In experimental oral (Martin *et al.*, 1984), vaginal (Sobel *et al.*, 1984), and disseminated (Richardson and Smith, 1981) *Candida* infections in rodents, various abnormal *C. albicans* strains deficient in hypha formation were shown to be less virulent than strains that do form hyphae. Recently, Sherwood-Higham *et al.* (1995) have demonstrated that *C. albicans* hyphae are produced as helices when they are grown on cellophane surfaces. This phenomenon may be of significance in the penetration and colonization of tissue by *C. albicans.*

3.1.3 No differences in pathogenicity between the two forms

Shepherd (1985) tested the virulence for mice of several morphological and auxotrophic mutants of *C. albicans*, and concluded that yeast and hyphal forms were both pathogenic. Strippoli and Simonetti (1973) found no differences in mortality between mice inoculated intraperitoneally with *C. albicans* yeasts and hyphae. Also, Dastidar *et al.* (1971) found no differences in cutaneous infectivity of the two forms in rabbits and humans. *C. albicans* hyphae and yeast cells appear to stimulate quantitatively similar humoral and cellular immune responses *in vivo* (O'Grady *et al.*, 1967; Ponton *et al.*, 1985; Ponton *et al.*, 1986).

In summary, there are many accounts of experimental studies which associate hypha formation with infection. Therefore, the capacity of *C. albicans* to produce hyphae appears to be an important but not essential virulence factor. No-one has provided unarguable scientific proof that either *C. albicans* yeasts or hyphae consistently play the major role in the pathogenesis of candidosis (Odds, 1988).

3.2 Factors affecting dimorphism of *Candida albicans*

A wide range of environmental factors have been established as favouring morphological change in *C. albicans* (Table 1). pH has been reported to influence the ability of *C. albicans* to form hyphae or germ-tubes. Evans *et al.* (1974) showed that three isolates developed hyphae in the early stages of growth. The proportion of hyphae produced was highest in complex media with pH values of 7.5-8.6. Later, the same group established pH 7.4 and a temperature of 40°C as optimal for hypha production in Sabouraud broth. At 40°C very few blastospores multiplied by budding , and this number was reduced essentially to zero at pH 7.4 (Evans *et al.*, 1975b).

The importance of temperature in relation to morphology has been recognized by a number of workers. Evans *et al.* (1975b) showed that maximum filamentation occurred at 40°C, but almost no filamentation occurred at a

temperature only 8°C lower. However, six *C. albicans* isolates produced a higher mycelium count at 40°C, as compared with 36°C and 42°C. None of the isolates grew significantly at 44°C. Another study showed that environmental temperature has no influence on dimorphism after the initial stages of growth. Within 1 h the cells have become committed to filamentation without any subsequent change in their morphological behaviour (Evans *et al.*, 1975a).

A chemically defined medium composed of 6 amino acids, biotin, inorganic salts and glucose had the capacity to support growth of *C. albicans* in pure hyphal form for up to 27h, when incubated at 37°C, and in pure yeast form if incubated at 25°C (Lee *et al.*, 1975). Shepherd and Sullivan (1976), who studied the relationship between morphology of *C.albicans* and the carbon source, found that maltose produced a hyphal morphology, whereas with lactate a yeast culture was obtained. Fructose or glucose as a carbon source produced a mixed morphology of yeasts, pseudohyphae and true hyphae. Glucose concentration in the Sabouraud broth (pH 7.4) had little effect on growth and filamentation in *C. albicans* until raised to 20% at which point growth and hypha formation were depressed (Evans *et al.*, 1975b). The addition of glucose stimulates the growth of *Candida* in saliva even in the presence of the normal bacterial flora which cause inhibition of fungal growth in unsterilized saliva due to depletion of salivary glucose. Patients with diabetes mellitus tend to have increased concentrations of glucose in saliva, which is associated with an increase of candidal growth in their saliva (Knight and Fletcher, 1971).

Yields of *C. albicans* after 24h growth are maximal with 3 μM Zn^{2+}; zinc above 10^{-5}M is inhibitory to growth. Zinc-deficient cultures consist almost entirely of hyphal cells but the proportion of yeast cells increases with increasing concentrations of zinc, reaching a maximal level at 9μM Zn^{2+} (Yamaguchi, 1975). Other factors that have been claimed to stimulate hyphal production include a controlled CO_2 to O_2 ratio in the incubation atmosphere (Mardon *et al.*, 1969), low concentrations of vitamins (especially biotin) (Yamaguchi, 1974), an initial

blastospore concentration of about 10^6 cells/ml (Chattaway *et al.*, 1971; Mardon *et al.*, 1971), and finally, particular formulation of growth media (Table 1).

4. Candidosis

Candidosis is a primary or secondary infection involving a member of the genus *Candida.* The clinical appearance of the disease is extremely varied, and it can be acute, subacute, or chronic. It may be localized in the mouth, throat, skin, scalp, vagina, fingers, nails, bronchi, lungs or the gastrointestinal tract, or become systemic as in septicemia, endocarditis, and meningitis (Rippon, 1982).

4.1 Factors predisposing to candidosis

The severity and extent of *Candida* infection tends to increase with the number and severity of predisposing factors (Odds, 1988). Apart from the debilitating effects of serious illness in general, there are certain diseases and disorders that have for years been especially associated with candidosis. Some of these factors, which are discussed in more detail below, are listed in Table 2.

4.1.1 Debilitating diseases and disorders

High blood and tissue glucose levels favour the growth of *Candida* in diabetics (Kandhari *et al.*, 1969). Several investigators have reported higher than normal frequencies of yeast carriage in samples from the vagina (Segal *et al.*, 1984a), rectum (Barlow and Chattaway, 1969), urine (Ahearn *et al.*, 1966) and mouth (Tapper-jones *et al.*, 1981 ; Odds *et al.*, 1987) among diabetic patients. Candidosis is also associated with other infections. Haralabidis (1984) showed that the level of anti-*C. albicans* antibodies was high in patients infected with *Giardia lamblia* . In animal models *C. albicans* found to interact synergistically with *Staphylococcus aureus*, *Serratia marcescens* and *Streptococcus faecalis* (Carlson, 1983). Oral and oesophageal candidosis are common conditions in AIDS patients (Stenderup and Schonheyder, 1984 ; Fauci *et al.*, 1984), and vaginal candidosis is

References for Table 1

1. Bell *et al.*, (1980)

2. Bernander and Edebo (1969)

3. Chattaway *et al.*, (1968)

4. Chattaway *et al.*, (1971)

5. Muerkoester *et al.*, (1979)

6. Evans *et al.*, (1974)

7. Evans *et al.*, (1975b)

8. Johnson *et al* ., (1954)

9. Pollack and Hashimoto (1987)

10. Land *et al* ., (1975)

11. Lee *et al* ., (1975)

12. Mackenzie (1962)

13. Mardon *et al* ., (1969)

14. Mardon *et al.*, (1971)

15. Marichal *et al* ., (1986)

16. Marriott (1975)

17. McClary (1962)

18. Nickerson and Mankowski (1953)

19. Nishioka and Silva-Hutner (1974)

20. Scherr and Weaver (1953)

21. Shepherd and Sullivan (1976)

22. Skinner (1947)

23. Soll and Bedell (1978)

24. Soll and Herman (1985)

25. Soll *et al* ., (1981)

26. Walker *et al.*, (1984)

27. Widra (1964)

28. Yamaguchi (1974)

29. Yamaguchi (1975)

Table 1. Environmental factors favouring the conversion of *C. albicans* from yeast to hyphal phase.

Factors	Reference
General	
High temperature	3,7,14,22
Low temperature	17
pH above 7	4,6,7,8,14,22
pH below 7	9,17
An initial blastospore concentration of about 10^6 cells/ml	2,4,12,14
Reduced oxygen tension	8,20,22
A controlled CO_2:O_2 ratio	13
Starvation conditions	8,22,24
Conversion induced after yeasts enter stationary phase	1,23
"Strain variation" in response to the growth environment	7,21
Specific chemicals	
Polysaccharides	9,18,20,21
Serum albumin	2
Inorganic phosphate	17,26,27
Nitrogen source	14
Low zinc concentration	25,29
Proline	10
Low concentrations of biotin	28
Glutamate as carbon and nitrogen source	19
Particular growth media	
Glucose glycine broth	5
Glucose salts medium	4
Neopeptone-starch broth (NSB)	3
Casein-Yeast Extract (CY)	15
N-acetylglucosamine-Yeast nitrogen base-Proline medium (NYP)	15
Lee's medium	11
Starch, salts and vitamins medium (SSV)	16

probably very common among women with AIDS (Rhoads *et al.*, 1987). Boggs *et al.* (1961) described the incidence of thrush in 15% of patients with malignant disease. *Candida* carriage is very common among cancer patients, especially those with leukaemia (Ghannoum *et al.*, 1985 ; Gentle *et al.*, 1984).

4.1.2 Digressions from normal physiological status

Several reports show that most cases of oral infection within the first few days of life arise primarily because of maternal contamination of babies with *Candida* from the birth canal (Taschdjian and Kozinn, 1957). *Candida* carriage in the adult mouth has been shown to rise with age (Marples, 1960; Smits *et al.*, 1966), but old age itself cannot be considered an important factor predisposing to candidosis. Vaginal candidosis has almost always been found to be greater in pregnant than in non-pregnant women (Odds,1988). The numbers of epithelial cells to which *C. albicans* can adhere *in vitro* was higher among pregnant women than in any other group (Segal *et al.*, 1984b).

4.1.3 Dietary factors

Horowitz *et al.* (1984) detected elevated quantities of glucose, arabinose and ribose in urine samples from women with recurrent *Candida* vaginitis and claimed to reduce the occurrence of candidosis among patients who reduced their intakes of dietary sugar, milk products and artificial sweeteners. The presence of high carbohydrate levels in the gut may tend to favour multiplication of *Candida* (Cormane and Goslings, 1963), and promote oral colonisation (Samaranayake, 1986). Denture adhesives mixed with sucrose support *C. albicans* growth *in vitro* (Stafford and Russell, 1971). Nutritional deficiencies, such as vitamin C deficiency, predispose to candidosis. Rogers *et al.* (1983) showed that vitamin C deficiency reduced the resistance of guinea pigs to renal candidosis. Other work showed that hypovitaminosis A increased, and hypervitaminosis A decreased the susceptibility of rats and mice respectively to infection with *C. albicans*; there were

higher death rates due to *C. albicans* in mice pretreated with vitamin K_3 (Odds, 1988).

Iron-deficiency anaemia has been proposed as an important factor in candidosis. Jenkins *et al.* (1977) showed that in some susceptible individuals iron deficiency could facilitate invasion of the oral epithelium by hyphae of *C.albicans*. Iron deficiency is thought to affect cellular immune responses, which can lead to candidosis (Higgs and Wells, 1972).

4.1.4 Mechanical factors

Several case reports show that severely burned patients are particularly susceptible to colonization and deep-seated infection with *Candida* (Zanini *et al.*, 1983). Some of the authors of these papers have suggested that the incidence of candidosis as a complication of burns has risen with improvements in burn management (Law *et al.*, 1972; Richards *et al.*, 1972).

Local occlusion and maceration are factors that predispose to yeast overgrowth and infection. *Candida albicans* is rarely found on the skin, but the probability of its recovery from moist areas such as the axillae, groins and toe webs is far higher than from the general skin surface (Macura *et al.*, 1984; Marks *et al.*, 1975). Several workers have detected a higher oral yeast occurrence among denture wearers of various types than among non-wearers (Berdicevsky *et al.*, 1980; Mitchell, 1982).

4.1.5. Treatment with drugs

Some studies claim that antibiotics are important factors in candidosis, including tetracyclines (Odds, 1988), penicillin (Meads *et al.*, 1951), erythromycin (Heimdahl and Nord, 1982) and ampicillin (Fitzpatrick and Topley, 1966). Corticosteroids are compounds with anti-inflammatory and immunosuppressive effects on mammalian hosts (Mims, 1987), and several investigations have shown a higher oral yeast occurrence in patients receiving corticosteroid therapy (Johnston *et*

Table 2. Factors commonly found to predispose humans to candidosis

Predisposing factors	Examples
Debilitating diseases and disorders	Diabetes Infectious diseases Immune deficiencies Malignant diseases
Digressions from normal physiological status	Infancy Genetic factors Old age Pregnancy
Dietary factors	Carbohydrate-rich diet Vitamin deficiency Iron deficiency
Mechanical factors	Wearing dentures Local occlusion and maceration Burns
Treatment with drugs	Antibiotics Corticosteroids Hormonal contraceptives
Surgical procedures	Abdominal surgery Indwelling catheters Heart valve replacement

al., 1967; Shastry *et al.*, 1969). A number of case reports suggest that steroid therapy was the major factor predisposing to superficial candidosis (Gale, 1982; Katoh and Takahashi, 1974) or systemic candidosis (Date *et al.*, 1983). Wied *et al.* (1966) found a significant increase in vaginal *Candida* among users of hormonal contraceptives. Toppozada *et al.* (1986) demonstrated a rise in vaginal *Candida* carriage from 2% of 50 women to 44% after the women had used hormonal contraceptives for 12 months.

4.1.6 Surgical procedures

Systemic candidosis has emerged as a clear hazard of surgical procedures including bone marrow transplantation (Berkowitz *et al.*, 1983), renal transplantation (Howard *et al.*, 1978), pancreas transplantation (Hesse *et al.*, 1986), heart-lung transplantation (Brooks *et al.*, 1985) and liver transplantation (Wajszczuk *et al.*, 1985).

5. Iron in Biological Systems

Iron is essential for a wide variety of metabolic processes in the body; like many other transition elements, iron is an important biological catalyst (Bullen and Griffiths, 1987). The crucial role of iron in biological systems has been revealed by observations on the effects of iron deprivation. In iron-starved bacteria, the growth rate typically is reduced, and morphological changes such as filament formation suggest an inhibition of DNA synthesis or cell division (Shelley, 1988). In vertebrates, iron absorbed through the intestinal wall must be transfered to tissues requiring iron for biosynthesis or maintenance (Beveridge and Doyle, 1989). Neutrophil function, T-and B-lymphocyte activity and natural killer cell function are also dependent on iron (Dallman, 1986). In the human body, iron is maintained in a soluble form by being complexed to carrier molecules or by reduction to ferrous ion within cells. There is plenty of iron in the body (about 4.5g for the average man; Bell *et al.*, 1953), but the body fluids of vertebrates contain only 10^{-8} M Fe^{3+} as

free ionic iron (Bullen *et al.*, 1978). Most of the body's iron is found intracellularly, in ferritin, or haem. That which is extracellular in body fluids is attached to high affinity iron-binding proteins: transferrin in plasma or lymph, and lactoferrin in external secretions such as saliva, tears, nasal secretions, intestinal fluids, seminal fluids, cervical mucus, colostrum and milk (Aisen, 1980; Bezkorovainy, 1980; Morgan, 1981).

5.1 Iron - binding proteins

The iron-binding and transport proteins, transferrin and lactoferrin, play a major role in resistance to infection in vertebrate species. Their discovery was associated with the observation of Schade and Caroline (1944) that raw egg white (conalbumin) was inhibitory to bacterial growth, and that addition of iron reversed the inhibition. Schade and Caroline (1946) also obtained the first transferrin preparation from a human plasma fraction and tested its ability to inhibit growth of *Shigella dysenteriae* . The fraction was bacteriostatic and growth inhibition was reversed by the addition of iron.

5.1.1 Transferrin (TF)

The vertebrate transferrins are serum glycoproteins with M_r values of approximately 80,000. Each TF molecule can band tightly two molecules of iron (Shelley, 1988). TF in the blood consists of iron-free and iron-containing protein, and the concentration of iron-binding sites normally exceeds the concentration of iron. In the adult human, TF is typically 20 to 40% saturated with iron (Laurell, 1947). Unsaturated TF also appears to contribute to the bactericidal activity of normal human plasma. TF serves as a transporter of iron and binds to specific receptors found on haemoglobin synthesizing cells. A wide range of bacteria such as *Haemophilus influenzae*, *Neisseria* spp. and *Bordetella pertussis* are capable of obtaining iron from TF by directly binding TF-iron complexes (Wooldridge and Williams, 1993).

5.1.2 Lactoferrin (LF)

Lactoferrin was first identified in human milk (Groves, 1960). It has also been found in saliva, sweat, tears , bronchial secretions, gastrointestinal secretions, cervical mucus and urine (Weinberg, 1984). The LFs have M_r values in the range 75,000-80,000, with two specific iron-binding sites on each molecule. They have a high affinity for iron but differ in their ability to bind iron at more acidic pH values (Aisen and Leibman, 1972). Bullen *et al.* (1972) showed that human milk containing unsaturated LF was bacteriostatic for *Escherichia coli* . Valenti *et al.* (1986) studied the mechanism of antifungal activity of LF and ovotransferrin towards *C. albicans*. They found that antifungal activity was not simply related to iron deprivation, but involved interaction of the protein with the fungal surface. However, Sweet and Douglas (1991b) were unable to demonstrate growth inhibition of five strains of *C. albicans* by TF, LF, or ovotransferrin and suggested that this might be due to instability of the iron-chelator complex at the initial pH value (5.4) of the growth medium. TF has two iron-binding sites which respond differently to pH changes; iron is lost from one site at neutral pH whereas the other site is rather more stable, losing its iron at pH 5-6 (Bezkorovainy, 1987). Human LF in its iron-free state has been shown to kill *C. albicans* ; the lethal effect was stronger at pH 7.0 than at pH 5.5 and Fe^{3+}-saturated LF had no fungicidal activity. Killing was also temperature-dependent with enhanced inhibition at higher temperatures (37, 42°C; Soukka *et al.*, 1992). Another recent finding of anticandidal activity was by Bellamy *et al.* (1993), who reported that the active peptides of bovine LF (named lactoferrin B) generated upon digestion with gastric pepsin have a lethal effect on *C. albicans* , causing a rapid loss of colony-forming capability.

5.1.3 Haemoglobin

Haemoglobin is considered to be of relatively little importance to microbes. It is only accessible to bacteria when it is released from red cells. However, recent

reports indicate that some pathogenic species such as *Campylobacter jejuni*, *Haemophilus influenzae*, *Neisseria* spp., *Vibrio cholera* and *Yersinia* spp. are capable of using haemoglobin as an iron source (Wooldridge and Williams, 1993). Haemoglobin consists of four haem complexes, each linked to a polypeptide chain; each haem complex can bind one molecule of oxygen and contains one atom of iron. The black-pigmented obligate anaerobe *Porphyromonas gingivalis* can utilize haemin-containing compounds, transferrin and haemoglobin *in vitro*. However *P. gingivalis* utilizes haemoglobin as an iron source much more effectively than other iron-containing compounds in an iron-limited environment (Shizukuishi *et al.*, 1995). The growth of *C. albicans* is effectively inhibited in the presence of transferrin. It can utilize iron derived from either ferritin, haemin, or haemoglobin. However, haemoglobin-derived iron restores *Candida* growth more efficiently than do the other two compounds (Manns *et al.,* 1994).

5.1.4 Ferritin and Phosvitin

Ferritin is the main soluble iron-storage protein in mammals and provides the major non-toxic store of iron in the liver, spleen and other organs which can be mobilized when needed. Phosvitin is also an iron-storage protein, and occurs in the egg yolk of numerous species including birds, fish, reptiles and amphibians (Bullen and Griffith, 1987).

5.1.5 Bacterioferrin

It is important to note that ferritin-like molecules have been found in several bacterial species (Harrison, 1979). Bacterioferrin, first reported by Stiefel and Watt (1979), was isolated from the nitrogen-fixing bacterium *Azotobacter vinelandii. Proteus mirabilis* and *Mycoplasma capricolum* have also been reported to possess similar proteins, as has *E.coli* (Harrison, 1979; Harrison *et al.*, 1987).

5.1.6 Iron-sulphur proteins

Iron-sulphur proteins are found in both the plant and animal kingdoms and their function is largely to act as electron carriers. The simpler iron-suphur proteins are termed ferredoxins and rubredoxins. The former contain inorganic sulphur, which is bonded with iron. The inorganic sulphur can be released from ferredoxins as H_2S by acid. Rubredoxins, present only in microorganisms, do not contain inorganic sulphur (Bezkorovainy , 1987).

5.1.7 Ovotransferrin

This iron-binding protein is found in the white of hen's egg and inhibits the growth of certain bacteria (Schade and Caroline, 1946). Nearly all of the iron of the egg is found in the yolk. The egg is protected from microbial infection by the shell, shell membranes, and the chemical composition of the egg white; many studies have shown that ovotransferrin is the main defence factor present in egg white (Tranter *et al.*, 1982; Weinberg, 1984).

6. Role of Iron in Microorganisms

Iron has an essential role in many diverse biological systems. Indeed, with the exception of the lactic acid bacteria (Archibald, 1983), all biological systems have an absolute requirement for iron in the range 0.4-4.0 μM. Iron is an essential micronutrient involved in many biochemical processes in microorganisms, as shown in Table 3.

6.1 The significance of iron in infection

The involvement of iron in biological processes such as respiration and DNA synthesis make it an essential element for all life-forms (Wrigglesworth and Baum, 1980). Although there is an abundance of iron present in body fluids of humans and animals, the amount of free iron in equilibrium with iron-binding proteins has been calculated to be of the order of 10^{-18}M, which is essentially zero

Table 3. Role of iron in microorganisms

Affected function	Effect
Cell composition	Iron starvation can cause: growth inhibition, decrease in RNA and DNA synthesis, inhibition of sporulation , change in cell morphology.
Intermediary metabolism	Processes requiring iron: tricarboxylic acid cycle (aconitase), electron transport, oxidative phosphorylation, nitrogen fixation, aromatic biosynthesis, photosynthesis.
Metabolic products	Biosynthesis of the following products is regulated by iron : porphyrins, toxins, vitamins, antibiotics, hydroxamates, cytochromes, pigments, siderophores, aromatic compounds, DNA and RNA.
Proteins and enzymes requiring iron	Peroxidase, superoxide dismutase, nitrogenase, hydrogenase, glutamate synthase, ribonucleotide diphosphate reductase, aconitase, DAHP synthetase cytochromes, ferredoxin, flavoproteins, ferritin or ferritin-like iron storage compound iron-sulfur proteins

(From Messenger and Ratledge, 1986).

(Bullen *et al.*, 1978; Griffiths, 1987). Puschmann and Ganzoni (1977) showed that reduced serum iron levels resulted when mice were fed an iron-deficient diet. The mice were less susceptible to infection with *Salmonella typhimurium* than were littermates fed an iron-replete diet. Enhanced resistance was not observed if the iron-deficient mice were injected with sufficient iron to restore normal iron levels. Similarly, the ability of *Proteus mirabilis* to cause pyelonephritis in rats was related to the nutritional iron status (Hart *et al.*, 1982). Jackson and Burrows (1956) reported that many strains of *Yersinia pestis* formed pigmented colonies by absorption of hemin when grown on a suitable medium. These workers isolated a nonpigmented mutant from a fully virulent strain. The lethal dose of the virulent organism for mice was `100 bacteria, whereas even 10^8 organisms of the mutant strain failed to kill all the animals. Virulence of the mutant was fully restored when $FeSO_4$ was injected with the bacteria. Only iron was effective; equivalent amounts of cobalt, nickel, copper and zinc were inactive (Jackson and Burrows, 1956; Bullen, 1981). Additional reports have suggested a correlation between the level of iron saturation of iron-binding proteins and human susceptibility to disease . In acute leukaemias, the serum iron saturation is also increased. Caroline *et al.* (1969) demonstrated that sera obtained from patients with acute leukemia could support the growth of *C. albicans*, but growth was inhibited if purified transferrin was added to the serum

6.2 Iron and virulence of *C. albicans*

Growth of several fungi, including *C. albicans* , is inhibited *in vitro*, in the presence of iron-binding proteins or serum, and this inhibition can be abolished by adding iron (Caroline *et al.*, 1964; Esterly *et al.*, 1967; Kirkpatrick *et al.*, 1971; Elin and Wolff, 1973). Elin and Wolff (1974) showed that injection of *E.coli* endotoxin into mice resulted in a fall in the percentage iron saturation of the serum transferrin; this was accompanied by an increased resistance to infection with *C.albicans*, but the effect was overcome by injecting ferric ammonium sulphate.

There is a relationship between systemic candidiasis in patients with acute leukaemia and increased saturation of their serum transferrin with iron (Caroline *et al.*, 1969). Sweet and Douglas (1991b) showed that adhesion to buccal epithelial cells of five strains of *C. albicans* was maximal after growth in 0.2-0.4 μM-iron. In addition, germ-tube formation in serum was near-maximal after growth in 0.2-0.4 μM iron but was inhibited at lower concentrations. These results indicate that iron is an essential element and important in the virulence of *C. albicans*. It is known that this organism can exist in one of two morphological forms. However, the significance of the one form compared to the other in relation to pathogencity is not clear. Whether iron restriction plays a part in morphogenesis remains to be seen.

7. Acquisition of iron

Since all known pathogens need iron to multiply, they must, initially, be able to adapt to the iron-restricted environment usually found *in vivo* and develop mechanisms for assimilating protein-bound iron or for acquiring it from liberated haem. Under normal physiological conditions micro-organisms might be expected to assimilate the iron bound by the high affinity iron-binding glycoproteins of the host in one of four ways:

7.1 Through proteolytic cleavage

Bacteria such as black-pigmented *Bacteroides* species are able to degrade either transferrin or lactoferrin and remove the iron; they can also degrade haemopexin (a haem-binding protein) and haptoglobin (a plasma glycoprotein) (Carlsson *et al.*, 1984).

7.2 By reduction of Fe^{3+} complex to Fe^{2+}

Cowart and Foster (1985) showed that *in vitro Listeria monocytogenes* secretes a soluble reductant that effectively removes iron from the Fe^{3+}-transferrin

complex. Deneer and Boychuk (1993) confirmed these results with other species of *Listeria* and with *Clostridium perfringens*. *Streptococcus mutans*, which does not produce a phenolate or a hydroxamate siderophore, also appears to transport only reduced (ferrous) iron (Evans *et al.*, 1986).

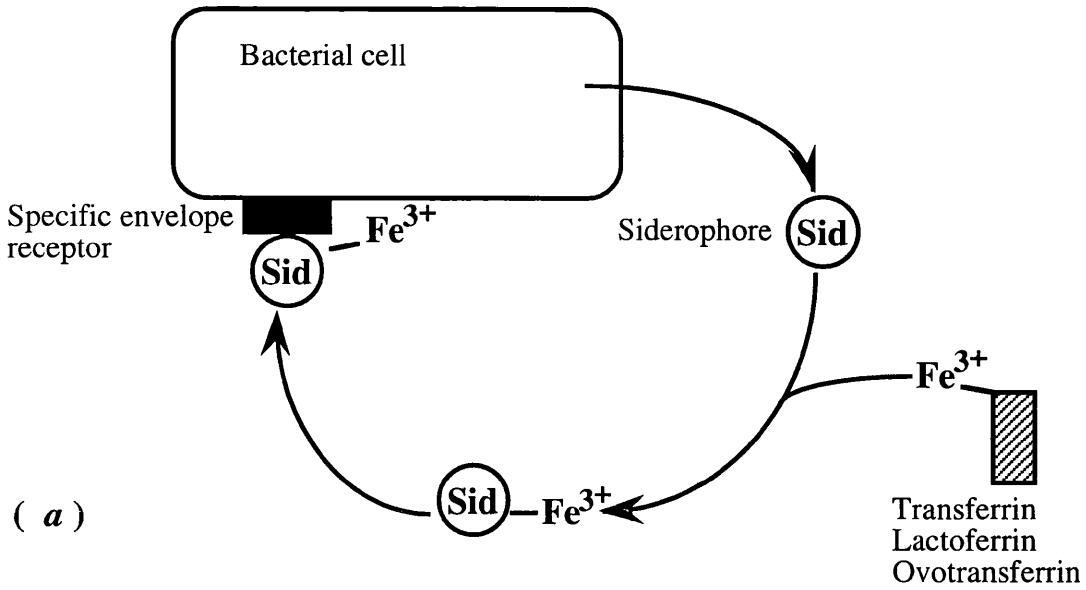
7.3 By direct interaction (at cellular level)

Acquisition of iron by direct interaction with iron-binding proteins has been reported for *Bordetella* species. *B. pertussis* has two mechanisms for obtaining iron. The first is via siderophores, identified as hydroxamate compounds. The second and apparently more effective system involves direct binding of chelator proteins to the bacteria (Redhead and Hill, 1991). This system may be similar to that demonstrated for *Neisseria* spp. (Simonson *et al.*, 1982). Generally it is now accepted that neither the gonococcus nor the meningococcus synthesizes siderophores, but both organisms can utilize siderophores provided exogenously (Mickelson and Sparling, 1981; Simonson *et al.*, 1982). West and Sparling (1985) showed that aerobactin (hydroxamate-type) siderophores can be used by the gonococcus. Isolates of *Neisseria* were able to use haemin as a sole source of iron and could also remove iron from haemoglobin (Mickelson and Sparling, 1981). In addition, *Pasteurella piscicida* and *Pasteurella multocida* do not produce siderophores but are able to obtain iron from transferrin by a mechanism mediated by direct binding to cell surface receptors (Magarinos *et al.*, 1994; Ogunnariwo *et al.*, 1991). A schematic representation of iron uptake by direct interaction with host iron-binding proteins is shown in Figure 2 (b).

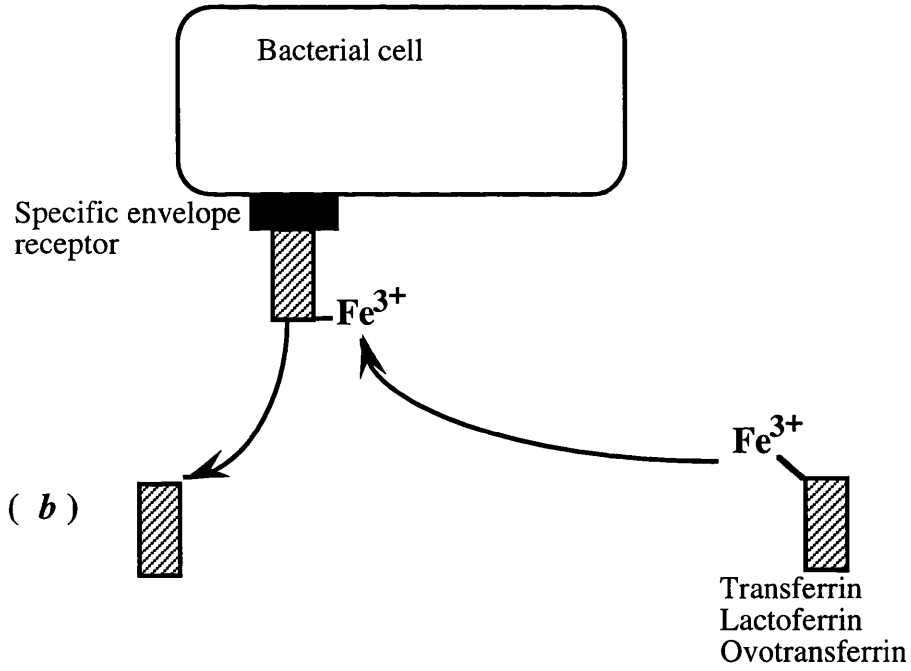
7.4 By production of an iron-chelating compound

Low molecular mass iron-chelating compounds, known as siderophores, are produced by some micro-organisms. These compounds are able to remove iron from the Fe^{3+} glycoprotein complex and deliver the iron to the microbial cell.

Figure 2. Schematic representation of two ways by which pathogenic bacteria obtain iron from iron-binding proteins: (***a***) siderophore-mediated iron uptake; (***b***) iron uptake by direct interaction with host iron-binding glycoproteins.


Bacterial cell
Specific envelope receptor
Fe3+
Sid
Siderophore
Sid
Fe3+
Sid
Fe3+
Transferrin
Lactoferrin
Ovotransferrin

(*a*)


Bacterial cell
Specific envelope receptor
Fe3+
Fe3+
Transferrin
Lactoferrin
Ovotransferrin

(*b*)

8. Siderophores

Pathogenic microorganisms synthesize a large number of different iron-chelating compounds known as siderophores (Lankford, 1973; Neilands, 1981a,b) (see Tables 4,5 and Figurs 4, 5 and 6). In early reports, siderophores were called siderochromes or sideramines (Raymond *et al.*, 1984). Siderophores, as defined by Lankford, are "microbial products that bind iron in soluble organic chelates and present the iron to the microorganism's envelope in a form suitable for active transport" (Lankford, 1973).

During the period 1949-52, four different siderophores were isolated and identified as growth factors. Mycobactin and arthrobactin (Terregens factor) were isolated as free ligands (Francis *et al.*, 1949; Lockhead *et al.*, 1952), while ferrichrome (Neilands, 1952) and coprogen (Hesseltine *et al.*, 1952) were isolated as iron complexes. A key observation concerning the mode of action of these growth factors was made by Garibaldi and Neilands (1956), who demonstrated that the production of ferrichrome A was enhanced by growing *Ustilago sphaerogena* in medium deficient in iron (Hider, 1984).

Siderophores (Greek, "iron carriers") are compounds of relatively low molecular mass (500-1000 Daltons), possessing a high affinity for iron (III). They include virtually ferric-specific coordination compounds excreted under low iron stress by fungi for the purpose of securing iron from the environment (Beveridge and Doyle, 1989). Siderophores belong to two distinct classes: the phenolates (or catechols) and the hydroxamates (Griffths, 1987). A given organism may produce siderophores of either one or both classes. These low molecular mass chelators are part of what is termed the "high affinity " iron transport system. This pathway, which has been identified in virtually all aerobic and facultative anaerobic microbes, comprises two parts:

1) Relatively low M_r (55-1000 Daltons) ferric ion-specific ligands generally termed siderophores (Neilands, 1984).

2) A membrane-bound system for transport and utilization of the chelated iron.

A "low affinity " iron transport system is thought to operate when iron is freely available. This process is relatively insensitive and non-specific and does not require specific carriers. The schematic representation of low- and high-affinity systems is shown in Figure 3.

8.1 The structure of microbial siderophores

Siderophores can be classified into two main chemical structural groups: the hydroxamates and phenolates (Neilands, 1981b, 1984a, 1989).

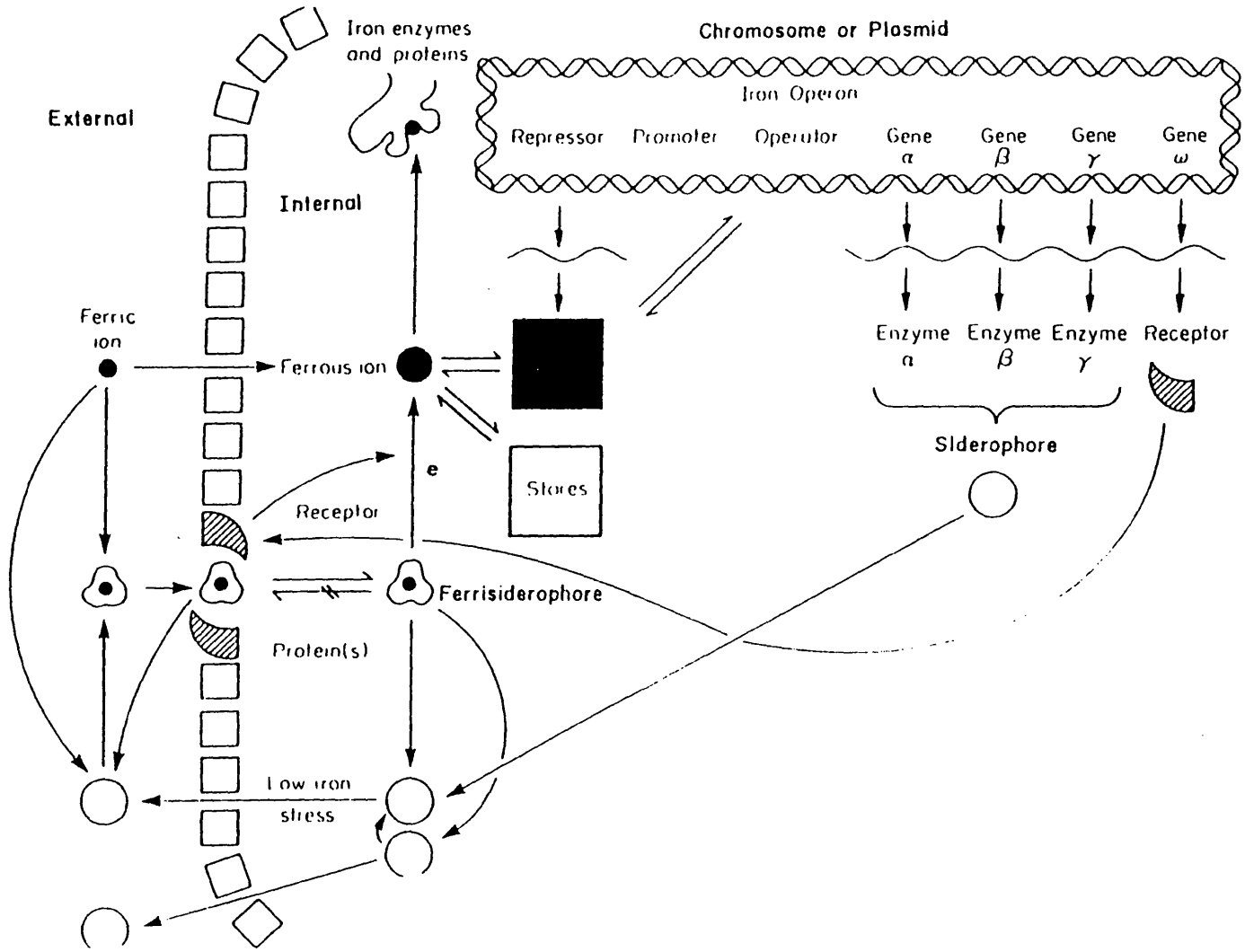
8.1.1 Phenolate-type siderophores

Perhaps the best studied "high affinity" iron transport systems are those produced by the bacteria *E.coli*, *Klebsiella pneumoniae* and *Salmonella typhimurium*. Enterobactin (also called enterochelin) is a phenolate-type siderophore and an amino acid conjugate of 2,3-dihydroxbenzoic acid. This compound, which is the cyclic triester of 2,3-dihydroxybenzoylserine, has been isolated from *E.coli*, *K. pneumoniae* and *S. typhimurium.* It is made only under conditions of iron restriction, and it removes iron from iron-binding proteins efficiently thus promoting bacterial growth (O'Brien and Gibson, 1970; Pollack and Neilands, 1970; Bullen and Griffiths, 1987; Wooldridge and Williams, 1993).
Each enterochelin molecule complexes with one atom of ferric iron. A hexadentate complex is formed between the ferric iron atom and the six phenolic hydroxy groups of enterochelin. The structure of enterochelin is shown in Figure 4(a).

8.1.2 Hydroxamate-type siderophores

The principal property of hydroxamate-type siderophores is their capacity to chelate ferric iron; ferrous iron is weakly bound. The hydroxamate function produces a stable five-membered ring with one iron atom. At neutral pH, three hydroxamate ligands will bind with trivalent iron to yield a neutral chelate. Iron transport cofactors containing two hydroxamates per molecule have been isolated;

Figure 3. Schematic representation of low- and high-affinity iron assimilation in aerobic and facultative anaerobic microorganisms. In the former, iron (III) crosses the cell envelope, not co-ordinated to specific ligands and without mediation by membrane-associated receptor protein. The high-affinity pathway consists of siderophores (circles), outer-membrane receptor proteins of ferric siderophores, and inner-membrane and periplasmic proteins. Four mechanisms of siderophore-mediated iron (III) uptake are possible. Only the iron of the ferric siderophore may be deposited in the envelope, or conversely, the intact complex may be incorporated. If the latter is the case, the iron may be removed without processing of the ligands; conversely, the ligand may be either reversibly or irreversibly (broken circles) processed.


Iron enzymes
and proteins
Chromosome or Plasmid
Iron Operon
External
Repressor
Promoter
Operator
Gene α
Gene β
Gene γ
Gene ω
Internal
Ferric ion
Ferrous ion
Enzyme α
Enzyme β
Enzyme γ
Receptor
Siderophore
e
Stores
Receptor
Ferrisiderophore
Protein(s)
Low iron
stress

in these chelators, oxygen atoms forming other chemical groups probably participate in chelation. The fungi *Schizonella melanogramma, Sphacelotheca andropogonis* and *Ustilago sphaerogena* produce ferrichrome A which is a hydroxamate-type siderophore (Garibaldi and Neilands, 1956; Winkelmann and Huschka, 1987). The structure of ferrichrome is shown in Figure 4(b). In the ferrichrome molecule three residues of N-hydroxy-1-ornithine form the basic structure. Acetylation of the three hydroxy amino groups produces the hydroxamic acid function. The molecule forms a stable ring structure in which all three hydroxamic acid ligands will be oriented about a ferric iron atom. The siderophore-active dihydroxamic acid, rhodotorulic acid (Fig. 5a), is formed by *Rhodotorula pilimanae*, a basidiomycetous yeast, and related fungi. It can be regarded as a dipeptide fragment of ferrichrome and has been shown to form a chelate containing 2/3 atom of Fe(III) (Neilands and Leong, 1986). Coprogen is the predominant siderophore of *Neurospora crassa, Microsporum gypseum* and some *Penicillium* spp. The structure (Figure 5b) has been elucidated and shown to be a linear trihydroxamate ligand, containing three moles of trans-anhydromevalonic acid and three moles of N^5-hydroxy-ornithine, one of which is acetylated at the α-amino group (Matzanke, 1987; Winkelmann and Huschka, 1987).

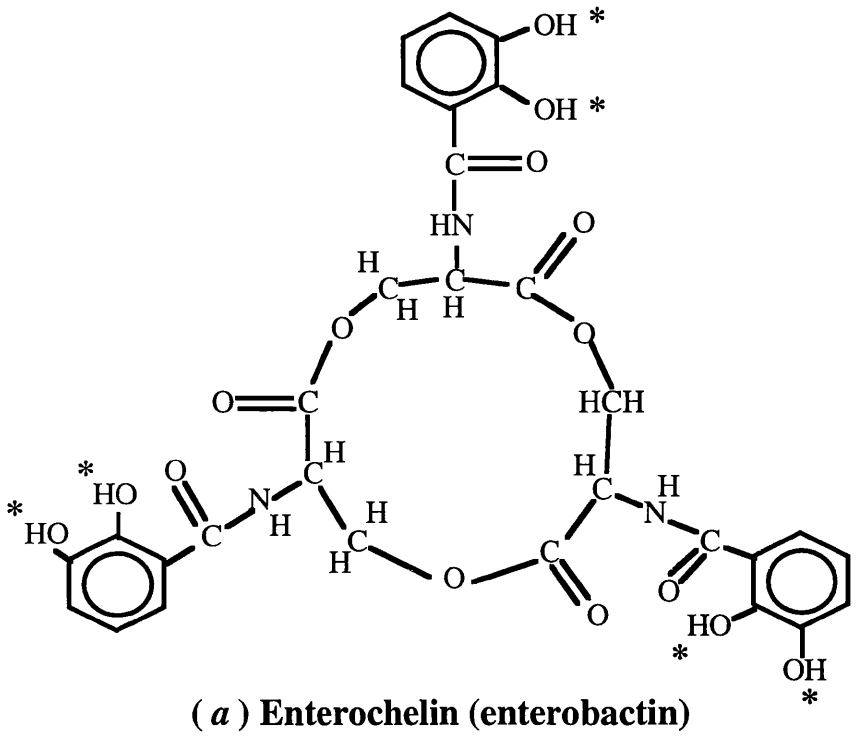
8.2 Detection and isolation of siderophores

Different testing methods have been used for detecting the production of siderophores. These include chemical assays, measurement of absorption spectra, and biological methods (Table 4).

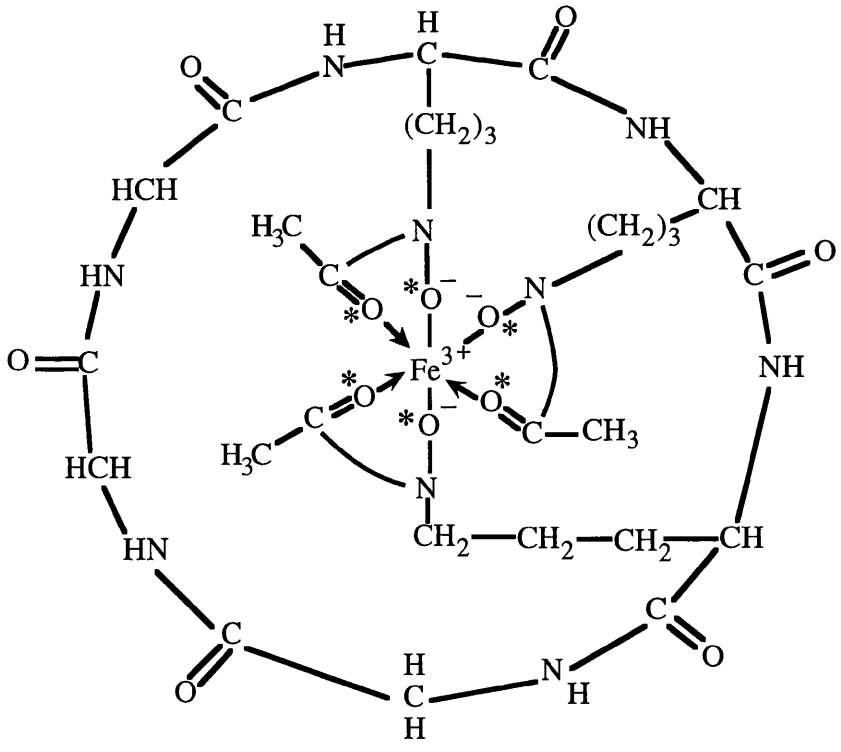
8.2.1 Chemical assays

The universal chemical assay, the most widely used assay for detection of siderophores, was developed by exploiting the high affinity of these compounds for iron (III). The complex chrome azurol S/iron(III)/hexadecyltrimethylammonium bromide serves as an indicator. When a strong chelator removes iron from the dye,

Figure 4. Structures of (***a***), enterochelin (enterobactin), a phenolate-type siderophore; (***b***), ferrichrome, a hydroxamate-type siderophore. Iron chelation sites are indicated by asterisks.

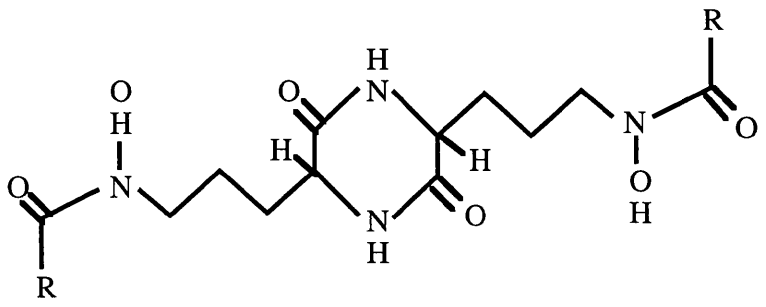


(*a*) **Enterochelin (enterobactin)**

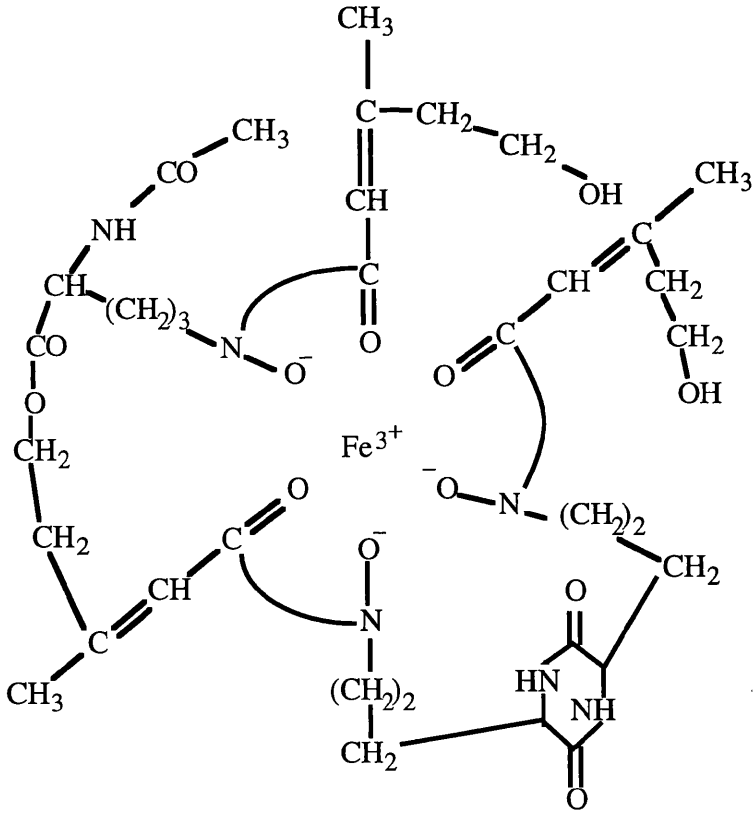


(*b*) **Ferrichrome**

Figure 5. Structures of two hydroxamate-type siderophores. (***a***), Rhodotorulic acid produced by *Rhodotorula pilimanae*, *Leucosporidium scottii*, *Sporobolomyces pararoseus*, *Sporobolomyces roseus* and *Ustilago major*; (***b***), Coprogen produced by *Neurospora crassa, Penicillium camemberti,* and *Microsporum gypseum.*



(*a*) **Rhodotorulic acid**



(*b*) **Coprogen**

its colour turns from blue to orange. The method is also applicable to agar plates. Orange halos around the colonies on blue agar are indicative of siderophore excretion (Schwyn and Neilands, 1987). Phenolates are most conveniently assayed with the nitrite-molybdate reagent of Arnow (1937). With this assay, if the siderophore is a phenolate, it will first form a yellow product when reacted with nitrous acid that will then turn orange-red when it is made basic by the addition of NaOH. Hydroxamates may be detected with acidified ammonium vanadate (Snow, 1969) or with I_2 (Csaky, 1948; Tomlinson, 1971). The most sensitive method used for detection of hydroxamate-type siderophores is that described by Holzberg and Artis (1983) who demonstrated that oxidation (using periodic acid) of certain hydroxamic acids yields material with a very strong absorption at 264 nm (Ismail *et al.*, 1985b; Sweet and Douglas, 1991a).

8.2.2 Biological methods (Bioassays)

Bioassays for siderophores are, in some cases, more sensitive than the best chemical methods. The organism most commonly used for assay of hydroxamate is the soil isolate *Arthrobacter flavescens* (Neilands, 1984a). A mutant enteric bacterium can also be employed for the assay (Lucky *et al.*, 1975; Wayne *et al.*, 1976), and recently a novel bioassay with a ketohydroxy bidentate ligand and *Morganella morganii* was described (Thieken and Winkelmann, 1993). A bioassay for siderophore utilization by *C. albicans* has been reported (Minnick *et al.*, 1991); this allows compounds to be examined for their ability to stimulate the growth of *C. albicans* under iron-deficient conditions. The addition of a chelator (EDDA) with a high affinity for Fe^{3+} to a chemically defined agar medium (Lee's agar) allows such a state of iron deficiency to be obtained. A growth promotion effect will then be observed under these conditions as long as the compound being tested can (i) compete effectively with the EDDA iron-chelator for Fe^{3+} in the medium, and (ii) be recognized by the existing or induced microbial iron assimilation system. In this way, the culture conditions are somewhat equivalent to circumstances found in

mammalian systems in which iron is bound tightly to proteins such as transferrin and lactoferrin (Bullen and Griffiths, 1987).

Biological assays were used to confirm the production of two chemically different classes of siderophores by *C. albicans*. Ismail *et al.* (1985b) showed that the candidal phenolate extract and enterobactin (a phenolate siderophore from *S. typhimurium*) supported the growth of the phenolate-dependent auxotroph (*A. flavescens*) but not the hydroxamate auxotroph. Growth of the hydroxamate auxoutroph was promoted by the candidal hydroxamate extract and the known hydroxamate, desferroxamine.

8.2.3 Absorption spectrum assays

The production of siderophores in liquid media has also been investigated spectrophotometrically. Kumar and Dube (1991) recorded the UV-visible spectrum of cell-free culture filtrates of a fluorescent *Pseudomonas* isolate on a spectrophotometer and noticed that a peak at or around 405 nm indicated the presence of a siderophore. Xu and Gross (1986) found a peak at 400 nm on similar examination of *Pseudomonas putida* and *P. fluorescens* ; this is typical of the pyoverdin class of siderophore, and was also recently noted by Manninen and Sandholm (1994), who showed that cell-free culture supernates of *P. fluorescens, P. putida* and *P. aureofaciens* had absorption maxima at 400 nm. These cultures only exhibited an absorbance peak when grown without added iron.

The various methods available for detection of siderophores are shown in Table 6. Extraction into an organic solvent, such as ethyl acetate in the case of phenolates (Rogers, 1973), or either benzyl alcohol or chloroform-phenol (1:1) for hydroxamates (Simpson and Oliver, 1983), can be used for concentration and purification of siderophores.

Table 4. Methods for detection of siderophores in low-iron culture supernates.

Methods	Reference(s)
Chemical assays	
Arnow reaction for catechols.	Arnow, 1937; Barnum, 1977
Csaky reaction for bound hydroxylamine	Csaky, 1948; Tomlinson *et al.*, 1971
Ferric perchlorate reaction	Atkin. *et al.*, 1970
Hydroxamic acids assay (Emery and Neilands method)	Holzberg and Artis, 1983
Universal chemical assay	Schwyn and Neilands, 1987
Colorimetric determination of catechol (Rioux method)	Rioux *et al.*, 1983
Biological methods (Bioassays)	
Bioassay with *Arthrobacter flavescens* JG-9 as siderophore auxotroph	Neilands, 1984 a
Bioassay with *Salmonella typhimurium* against citrate	Miles and Khimji, 1975
Bioassay with two mutants of *E. coli* K12 against Deferrichrome A as a chelating agent	Wayne *et al.*, 1976
Bioassay with wild-type strains against EDDA as deferration agent	Miles and Khimji, 1975; Manninen and Sandholm, 1994
A novel bioassay with *Morganella morganii*	Thieken and Winkelmann, 1993
Absorption spectrum assays	Manninen and Sandholm, 1994; Kumar and Dube, 1991

8.3 Production of siderophores by bacteria

A wide range of bacteria produce siderophores under iron-limiting conditions (Table 5). However, not all bacteria appear to have this ability. Some anaerobic bacteria utilize ferrous (Fe^{2+}) iron which is freely soluble and available in the environment and they do not require to synthesise siderophores. A species of *Legionella* does not appear to form siderophores of any kind when cultured in laboratory media and it is not known how the pathogen acquires iron in the host (Reeves *et al.*, 1983). Certain strains of *Lactobacillus* have gone to extraordinary lengths to avoid a need for this element. Such species may qualify as the sole forms capable of life without iron (Archibald, 1983). *Bacteroides* species have the ability to obtain iron from iron salts, haem compounds and transferrin under iron-limited conditions *in vitro*, but there is no evidence for the production of chelators by these bacteria (Vught *et al.*, 1988). *Helicobacter pylori* does not produce siderophores; human lactoferrin supported full growth of the bacteria in media lacking other iron sources, but neither human transferrin, bovine lactoferrin, nor ovotransferrin served as a source of iron (Husson *et al.*, 1993).

It has been demonstrated that under iron-restricted conditions *Bordetella pertussis* can take up iron from human transferrin within 30 min of exposure. *B. pertussis* utilizes two mechanisms for acquiring iron from human transferrin: a direct contact method and a siderophore-mediated system. Both systems are shown to result in bacterial internalization of iron from transferrin. However, direct contact between *B. pertussis* and transferrin provides for more effective iron uptake than siderophore activity alone (Redhead and Hill, 1991). *Pseudomonas aeruginosa* produces two siderophores, pyochelin and pyoverdin, and can also transport Fe(III) complexed with siderophores produced by other species, including enterobactin, one of the major siderophores produced by *E.coli*. This ability to use other siderophores may improve survival prospects in certain situations, for example, when different species are competing for the same nutrients (Poole *et al.*, 1990).

Table 5. Siderophore production by bacteria.

Organism	Hydroxamate-type	Phenolate-type	Siderophore-like compound	Ref.
Aeromonas hydrophila		Amonobactins		*a*
Bordetella bronchiseptica	Not named			*b*
B. pertussis	Not named			*c*
Citrobacter spp.	Aerobactin			*a*
Erwinia spp.	Ferrioxamine-type			*a*
E. coli	Aerobactin	Enterochelin		*a*
Hafnia spp.	Ferrioxamine-type			*a*
Klebsiella pneumoniae	Aerobactin Ferrioxamine-type	Enterochelin		*a*
Morganella spp.			α-Keto acids, α-Hydroxycarboxylic acids	*a*
Mycobacterium neoaurum	Mycobactin		Exochelin	*d*
M. smegmatis	Mycobactin		Exochelin	*d*
M. leprae			Exochelin	*d*
Proteus ssp.	Aerobactin		α-Keto acids, α-Hydroxycarboxylic acids	*a*
Pseudomonas aeruginosa	Pyoverdin		Pyochelin	*e*
Salmonella spp.	Aerobactin, Not named	Enterochelin		*a*
Serratia spp.	Aerobactin			*a*
Shigella spp.	Aerobactin	Enterochelin		*a*
Staphylococcus hyicus			Staphyloferrin A,B	*a*
Vibrio cholerae		Vibriobactin		*a*
V. vulnificus	Structure unknown	Structure unknown		*a*
V. parahaemolyticus			Structure unknown	*f*
V. anguillarum			Structure unknown	*f*
Yersinia enterocolitica			Yersiniophore	*g*
Y. frederiksenii	Aerobactin			*g*
Y. intermedia	Aerobactin			*g*

Ref. (*a*), Wooldridge and Williams, 1993; (*b*), Foster and Dyer, 1993; (*c*), Redhead and Hill, 1991; (*d*), Ratledge, 1987; (*e*), Meyer *et al.*, 1987; (*f*), Payne, 1987; (*g*), Chambers and Sokol, 1994.

8.4 Production of siderophores by fungi

Studies on siderophore production by pathogenic yeasts and fungi are limited. The first indication of siderophores produced by pathogenic fungi was in *Histoplasma capsulatum* (Burt *et al.*, 1981). Fungi in general do produce siderophores under iron-limiting conditions (Lankford, 1973; Neilands, 1981b, Winkelmann and Winge, 1994) (Table 6). Investigations have shown that a number of opportunistic fungi, including *C. albicans*, can secrete siderophores of the hydroxamate type (Holzberg and Artis, 1983; Ismail *et al.*, 1985b; Sweet and Douglas, 1991a). Some reports indicated that only one type of siderophore was produced by *C. albicans, C. lusitaniae, C. glabrata* and *C. parapsilosis* ; phenolate-type siderophores were not detected (Sweet and Douglas, 1991a ; Holzberg and Artis, 1983). Other reports have suggested that certain isolates of *C. albicans* are capable of simultaneous production of both hydroxamate- and phenolate-type siderophores (Ismail *et al.*, 1985b). The chemical structures of the *Candida* chelators are unknown, as is their role, if any, *in vivo* during infections (Griffiths and Bullen, 1987). Generally, fungi produce siderophores of the hydroxamate type, and the production of both classes of siderophores by yeasts has not been confirmed. A reason for the production of two types of siderophore is not clear (Ismail *et al.*, 1985b).

Fungi synthesize a variety of siderophores with different structural backbones such as ferrichromes (Figure 4b), coprogens (Figure 5b) and fusigens (fusarinins); these vary depending on the strain and medium and cultivation conditions used (Huschka *et al.*, 1986). Until recently, it was generally assumed that fungal siderophores are exclusively hydroxamate-type siderophores. This, however, is not true for the Mucorales (Zygomycetes). Drechsel *et al.* (1991) have recently shown that *Rhizopus microsporus* variety *rhizopodiformis* produces a citric acid-containing siderophore which has been named rhizoferrin (Figure 6a). Thieken and Winkelmann (1992) showed also that this novel siderophore was not confined to the genus *Rhizopus*, but was found in different families of the Mucorales and

Table 6. Siderophores produced by fungi.

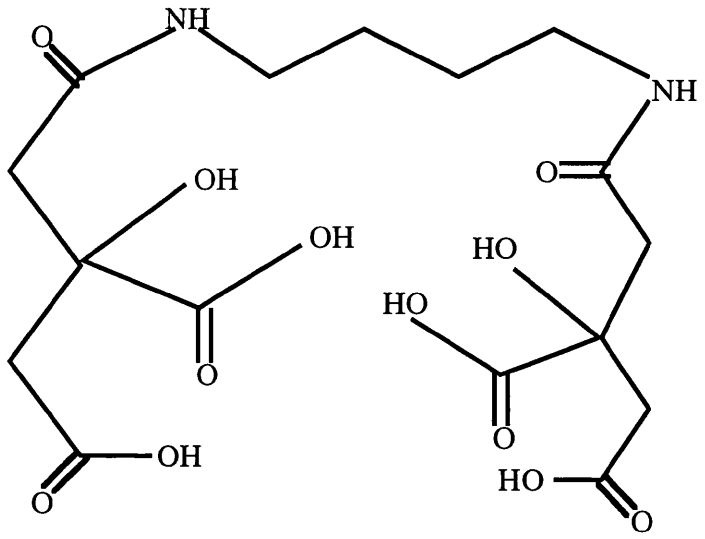
Name of siderophore	Organism
Ferrichrome	*Aspergillus niger*
	Penicillium parvum
	Ustilago sphaerogena
	Cintractia sorghi-vulgaris
	Farysia olivacea
	Moesziomyces penicillaris
	Schizonella melanogramma
	Sorosporium cenchri
	Spacelotheca andropogonis
	Graphiola phoenicis
	Protomyces macrosporus
	Tilletiaria anomala
Tetraglycyl-ferrichrome	*Neovossia indica*
Ferrichrome A	*Ustilago sphaerogena*
	Schizonella melanogramma
	Sphacelotheca andropogonis
Malonichrome	*Fusarium roseum*
Ferrichrome C	*Cryptococcus melibiosum*
	Neurospora crassa
	Aspergillus duricaulis
Ferricrocin	*Aspergillus aureolus*
	Microsporum gypseum
	Neurospora crassa
Ferrichrysin	*Aspergillus melleus*
Ferrirubin	*Aspergillus ochraceus*
	Penicillium rugulosum
	Paecilomyces varioti
	Spicaria sp.
Ferrirhodin	*Aspergillus nidulans*
Des(diserylglycyl)-ferrirhodin	*Aspergillus ochraceus*

Table 6 (cont.)

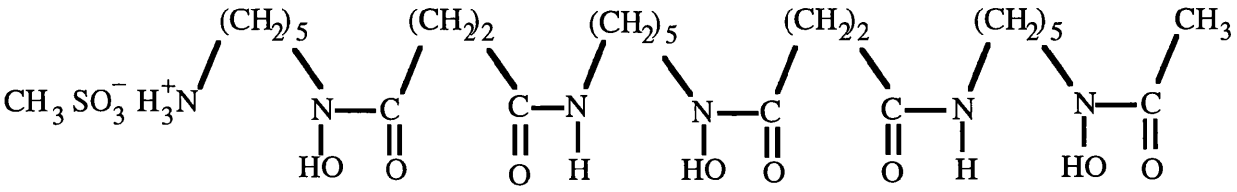
Name of siderophore	Organism
Asperchromes (A, B1, B2, B3, C, D1, D2, D3 and E)	*Aspergillus ochraceus*
Fusarinins (A,B)	*Fusarium roseum*
Fusigen (= Fusarinin C)	*Fusarium cubense*
	Aspergillus fumigatus
	Giberella fujikuroi
	Penicillium chrysogenum
	Paecilomyces varioti
Triacetylfusigen (= Triacetyl-fusarinin)	*Aspergillus fumigatus*
	Penicillium purpurogenum
Dimerium acid	*Fusarium dimerum*
Coprogen	*Neurospora crassa*
	Penicillium camemberti
	Microsporum gypseum
Triornicin	*Epicoccum purpurascens*
Isotriornicin	*Epicoccum purpurascens*
Neocoprogen I	*Curvularia lunata*
Neocoprogen II	*Curvularia lunata*
Neurosporin	*Neurospora crassa*
Coprogen B	*Fusarium dimerium*
	Myrothecium roridum
	Nectria cinnabarina
	Neurospora crassa
	Histoplasma capsulatum
Rhodotorulic acid	*Rhodotorula pilimanae*
	Leucosporidium scottii
	Rhodosporidium toruloids
	Sporidiobolus johnsonii
	Sporobolomyces hispanicus
	Ustilago violacea

(From Winkelmann and Huschka , 1987)

Figure 6. Structures of two siderophore types (***a***) Rhizoferrin, a novel polycarboxylate siderophore produced by *Rhizopus microsporus* (Thieken and Winkelmann, 1992); (***b***), Deferriferrioxamine B mesylate salt, from *Streptomyces* spp., available commercially as Desferal (Ciba-Geigy).



(*a*) **Rhizoferrin**



(*b*) **Deferriferrioxamine B**

Entomophthorales, indicating that rhizoferrin represents a typical siderophore of the Zygomycetes. Charlang *et al.* (1981) showed that *Aspergillus nidulans* and *Penicillium chrysogenum* produce extracellular siderophores and specific cellular siderophores which have a role in conidial germination and function even under conditions of adequate iron nutrition.

Siderophores may function as iron storage forms in fungal systems. Matzanke *et al.* (1987, 1994) showed that the spores of *Neurospora crassa* 74A are lacking in ferritin-like iron pools, as demonstrated by Mossbauer spectroscopic analysis. The cyclic hexapeptide siderophore ferricrocin constituted 47% of the total iron content in spores . After germination and growth, the ferricrocin iron pool disappeared, indicating the metal was utilized. In spores of *Aspergillus ochraceus*, 74% of the total iron content was bound by ferrichrome-type siderophores. Ferricrocin also occurs in the yeast *Rhodotorula minuta* and in *Ustilago sphaerogena* (Matzanke *et al.*, 1990).

8.5 Possible mechanisms for microbial siderophore utilisation

Three possible separate mechanisms for microbial siderophore utilisation have been described in the following analogies by Bergeron (1986). The mechanisms are assigned on the basis of the point of iron release and the final fate of the ligand.

8.5.1 The taxi cab mechanism

The iron siderophore complex delivers the metal to the cell surface where it is released to a secondary transport apparatus. The ligand, which remains in the extracellular environment, can then be reused by the microorganism.

8.5.2 The American mechanism

The iron siderophore complex is transported across the cell wall and the iron released by a process that results in ligand destruction.

8.5.3 The European mechanism

The complex is transported across the cell wall and the iron released by a process which does not destroy the ligand. The ligand is then excreted by the microorganism for further use.

8.6 Genetics of iron transport by microorganisms

There are at least three high-affinity and one low-affinity Fe transport systems in *E.coli* (Fig. 7). The two different siderophores synthesized by *E.coli* are enterobactin (phenolate) and aerobactin (hydroxamate). Transport of these siderophores involves two separate systems, one for enterobactin, and a second for aerobactin and some other hydroxamate siderophores produced by other bacteria and fungi such as coprogen, rhodotorulic acid, and certain ferrichromes and ferrioxamines. Lastly, two Fe-transport systems, not mediated by siderophores, include one for ferric-dicitrate which is inducible under low Fe conditions in the presence of 0.1 mM citrate, and a second system for transport of Fe^{2+} (Crowley *et al.*, 1991).

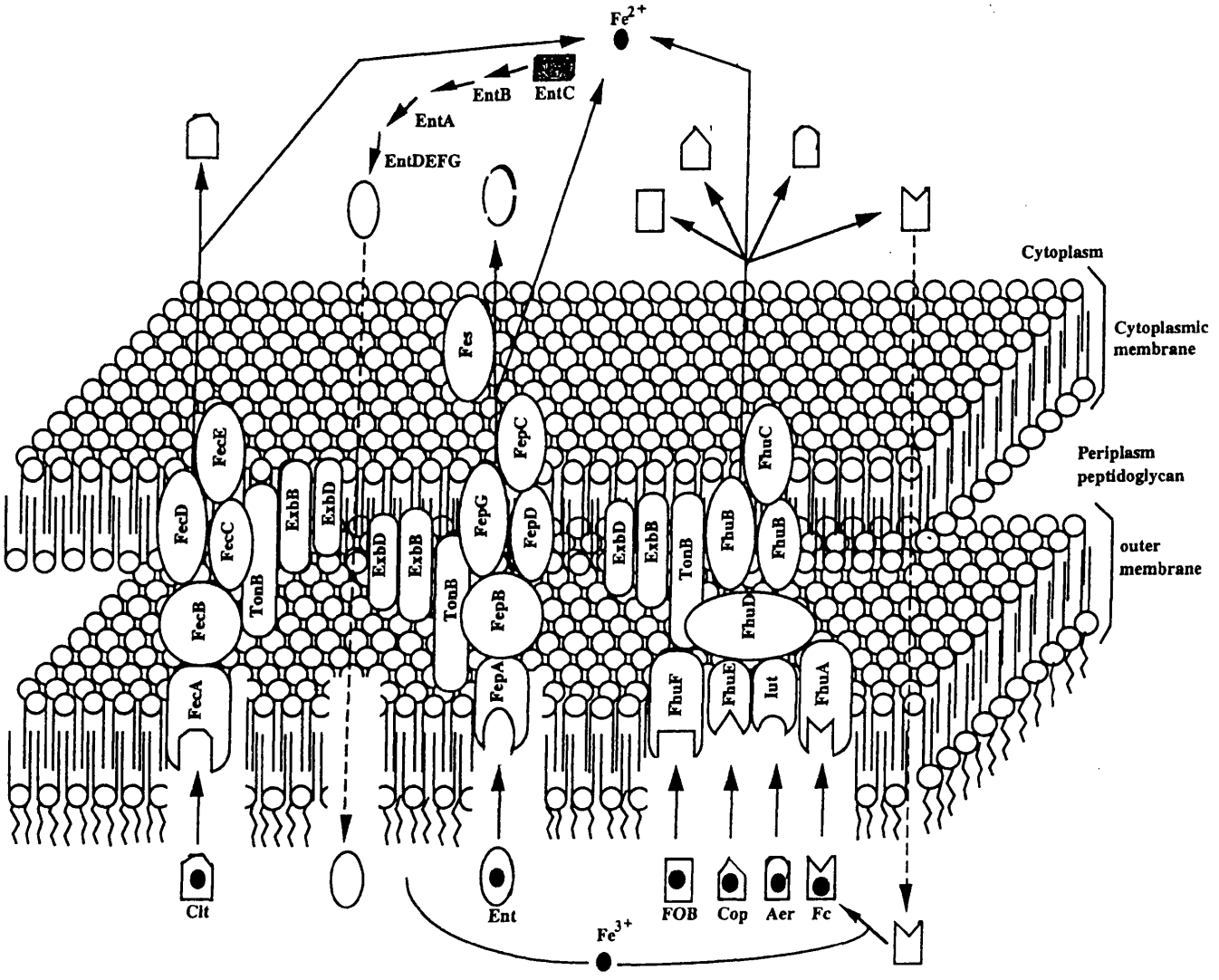
Siderophore transport systems in *E.coli* require several gene products, all of which are negatively regulated by an Fe-binding protein designated, Fur, for ferric uptake regulation. The enterobactin synthesis and transport system alone requires at least sixteen genes; seven for production of the siderophore, eight for ferrienterobactin transport proteins in the outer envelope, periplasm, and cytoplasmic membrane, and one for a protein that removes Fe from the siderophore in the cell cytoplasm. Transport of ferric-hydroxamate siderophore by *E coli* requires genes for two cytoplasmic membrane proteins, FhuB and FhuC, a periplasmic protein FhuD, and individual genes for receptor proteins for each ferric-hydroxamate siderophore type used by a particular *E.coli* strain. Lastly, three gene products designated TonB, ExbB, and ExbD are required for the ferrienterobactin, ferric-hydroxamate, ferric-dicitrate transport systems. The TonB protein appears to be involved in transfer of energy from the cytoplasm to the outer membrane (Postle

and Skare, 1988). The ExbB and ExbD proteins have been located in the cytoplasmic membrane and may be involved in physically stabilizing TonB (Fischer *et al.*, 1989). Outer-membrane receptor proteins are highly specific for individual siderophore type and for ferric-dicitrate. These include, FepA for ferrienterobactin, Iut for ferric-aerobactin, FhuA, FhuE, and FhuF for the ferric-hydroxamate siderophores, ferrichrome, coprogen, and ferrioxamine, respectively, and FecA for ferric-dicitrate (Braun *et al.*, 1987; Nelson *et al.*, 1988). After transport into the periplasm, subsequent transport of ferric-siderophores or ferric-dicitrate into the cytoplasm involves corresponding sets of cytoplasmic membrane proteins, including FepB, FepC, FepD, and FepG for ferrienterobactin, FecB, FecC, FecD, and FecE for ferric-dicitrate, and three proteins designated FhuB, FhuC, and FhuD for ferric-hydroxamate siderophores (Crowley *et al.*, 1991).

8.7 Non-siderophore mediated iron uptake by bacteria

Meiwes *et al.* (1990) isolated a compound, termed staphyloferrin, from low-iron culture broth of *Staphylococcus hyicus* , which exhibited siderophore activity; they found no evidence that *Staphylococci* synthesize catechol- or hydroxamate- type siderophores. Similarly, *Yersinia enterocolitica* has been shown to produce yersiniophore which was negative in the hydroxamate or phenolate assay (Chambers and Sokol, 1994). *Haemophilus influenzae* is able to acquire iron from human transferrin and it has the ability to use a variety of other iron and haem compounds as iron sources (Holland *et al.*, 1991). Several reports have studied the mechanisms of iron acquisition *in vitro* by *Listeria monocytogenes*. This pathogen does not produce siderophores, but instead releases a soluble, low molecular mass (8-10 KDa) reductant that removes Fe^{3+} from transferrin and produces Fe^{2+}, allowing direct interaction of Fe^{2+} with bacterial surface binding sites (Rouguette *et al.*, 1995).

Figure 7. Iron transport systems of *Escherichia coli* for ferrienterobactin, ferriaerobactin, ferric-dicitrate, and the ferric-hydroxamate siderophores, ferrichrome, ferrioxamine B, coprogen, and rhodotorulic acid. Adapted from Crowley *et al.* (1991).


Fe2+
EntB
EntC
EntA
EntDEFG
Cytoplasm
Cytoplasmic membrane
Periplasm peptidoglycan
outer membrane
Fes
FecE
FecD
FecC
FecB
FecA
TonB
ExbB
ExbD
FepC
FepG
FepD
FepB
FepA
FhuC
FhuB
FhuD
FhuF
FhuE
Iut
FhuA
Cit
Ent
FOB
Cop
Aer
Fc
Fe3+

8.8 Non-siderophore mediated iron uptake by *C. albicans*

Moors *et al.* (1992) proposed a novel mechanism by which *C. albicans* may obtain iron for growth in the human host, involving the use of complement receptor-like (CR-like) molecules. In addition, they showed that *C. albicans* uses CR-like molecules to bind complement-coated RBC, allowing the fungus to obtain RBC-derived iron for growth. They suggested also that *C. albicans* may have two mechanisms for iron uptake. The first, involving siderophores would operate, for example, in the human gut where an abundance of nutrients, including elemental iron, are available as a result of digestive processes; under circumstances such as these, in which iron is not bound by transferrin, Candida-derived siderophores could function in scavenging iron. However, acquisition of iron from RBC by the second mechanism that they describe may be important in disseminated candidiasis, at sites of invasive infection such as the kidney and liver, where elemental iron is not readily available and where there is an abundance of RBC. In addition, *C. albicans* may utilize this mechanism in mucocutaneous lesions in which the organism has penetrated the superficial layers of the skin resulting in an inflammatory response. It is likely that the ability to utilize more than one mechanism for iron acquisition, depending upon the local environment, would contribute to the pathogenic versatility of the organism (Moors *et al.*, 1992). An alternative strategy to obtain biologically active ferrous iron involves the extracellular reduction of ferric iron followed by specific uptake of the ferrous iron. This is a mechanism used by a variety of organisms, including yeasts such as *Saccharomyces cerevisiae* (Lesuisse and Labbe, 1989) and bacteria such as *Legionella pneumophila* (Johnson *et al.*, 1991). Such extracellular reductases could potentially produce ferrous ions from a range of ferric iron sources including ferric-siderophore complexes. Recently, Morrissey *et al.* (1996) demonstrated that *C. albicans* has ferric- and cupric-reductase activities which are both regulated in a manner similar to that of the *Saccharomyces cerevisiae* reductase; they also showed that *C. albicans* cell-associated ferric-reductase activity is dependent on iron

concentration and is induced at low concentration. It was found that the regulation and quantity of *C. albicans* reductase activity was unchanged at a higher temperature (from 30 to 37°C). All experiments were carried out with yeast-form organisms and not with hyphal forms.

8.9 Correlation between siderophores and microbial virulence

Since the levels of free iron *in vivo* are well below microbial requirements, it might be expected that possession of high-affinity iron-scavenging systems would constitute on important element in microbial virulence. *Salmonella typhimurium* mutants blocked in the synthesis of the siderophore enterochelin are less virulent in infections in mice than wild-type bacteria (Yancey *et al.*, 1979).

One simple and efficient method of protecting animals from bacterial and fungal infection is to deprive the invading organisms of iron. The siderophores of *Shewanella putrefaciens* were not sufficiently strong to inhibit growth of other bacteria under iron-restricted conditions. However, siderophore-producing *Pseudomonas* bacteria were always inhibitory to *Shewanella putrefaciens* under iron- limited conditions (Gram, 1994). The ability to grow in iron-restricted conditions, possibly with the assistance of siderophore-mediated iron uptake, may contribute to the increased pathogenicity of *Staphylococcus aureus* when compared with that of coagulase-negative staphylococci, which are considered less virulent than *S. aureus*, mostly infecting patients compromised in some way (Lindsay and Riley, 1994). In clinical haemolytic strains of *Vibrio parahaemolyticus*, the production of siderophore and haemolysin was significantly enhanced in iron-limited culture; cell adherence and lethality for mice were also enhanced. The environmental nonhemolytic strain that was cultured in iron-limited medium exhibited lethal activity for mice, and other factors except hemolysis were also enhanced, like the responses of clinical strains (Dai *et al.*, 1992). The virulence of *Klebsiella pneumoniae* in a mouse peritonitis model is enhanced by possession of the aerobactin determinants (Nassif and Sansonetti, 1986). Disruption of the

vibriobactin uptake system reduces the ability of *V. cholera* to cause disease in an infant mouse model (Henderson and Payne, 1994). These examples demonstrate that siderophores are now firmly established as important determinants of virulence for microbial infection of animals.

9. Factors affecting siderophore synthesis

A number of environmental factors can affect siderophore synthesis by microorganisms. Some of those affecting siderophore synthesis by fungi, particularly, are considered here.

9.1 Effect of temperature on siderophore production

Temperature is an important factor for the activity of membrane- associated transport systems. In an attempt to characterize the siderophore transport systems in fungi, Winkelmann and Huschka (1987) studied the temperature dependence of siderophore uptake. Using *Neurospora crassa* and *Aspergillus melleus*, they measured the uptake of ^{55}Fe-labelled coprogen and ferrichrysin at different temperatures. Uptake of coprogen in *Neurospora crassa* revealed a continuous increase of transport rates from 4°C to 45°C with a maximum at 39°C, followed by a complete loss of transport activity above 45°C. A similar temperature profile was observed with ferrichrysin. Uptake of ferrichrysin in *Aspergillus melleus* revealed a rather symmetrical temperature profile with a maximum at 29°C (Winkelmann and Huschka, 1987). Grieger and Kluger (1977) showed also that *Aeromonas hydrophila* grows equally well at 38°C and 41°C. When iron levels in the medium are reduced, or the iron chelated with desferal, the growth rate at 41°C is inhibited more than that at 38°C.

9.2 Effect of pH on siderophore production

It is well known that yeasts and fungi prefer a slightly acidic environment for optimal growth. Moreover, most fungi rapidly acidify their growth medium by excreting organic acids. Winkelmann and Huschka (1987) measured the acidification of a chemically defined medium by *Neurospora crassa* and found that after germination the pH generally decreased to a value of 4-5 whether the medium was iron-deficient or not. Siderophore transport in *N. crassa* has been found to be strictly dependent on the pH of the medium. Thus when ^{14}C-Coprogen transport into cells of *N. crassa* was tested, maximum uptake was observed at pH 5 which corresponds well with the pH optimum for growth. Uptake at pH 4 and 6 was only 50% and transport above pH 7 was nearly zero (Winkelmann and Huschka, 1987).

9.3 Other factors affecting siderophore production

Oxygen may affect siderophore synthesis by some microbial strains. Microaerophiles such as *Spirillum volutans* and *Campylobacter fetus* grew well in 17% oxygen if provided with dihydroxyphenolate, and in 21 % oxygen if given both the phenolate and additional iron, whereas a supply of neither was required for growth at 6% oxygen (Bowdre *et al.*, 1976). In other organisms, such as *Mycobacterium smegmatis*, the quantity of siderophore product may be altered by such trace metals as manganese , cobalt and zinc (Lankford, 1973; Ratledge and Hall, 1971). Glucose, while generally the preferred energy substrate, may not be optimum for siderophore production. This is well illustrated by aerobactin formation in *Aerobacter aerogenes* (Warner *et al.*, 1981). Growth of this organism on glucose affords very low yields of aerobactin. Growth on succinate, on the other hand, gives high yields. Deferration of the medium by different methods also affects siderophore production (Waring and Werkman, 1942; Neilands, 1984a; Simpson and Oliver, 1983).

10. Possible Uses of Siderophores in Medicine

Different siderophores have been used for chelation therapy. An iron chelator is administered which can form a complex with iron and then be excreted from the body in urine and faeces. Chelation therapy is a widely used treatment for iron poisoning, and for haemochromatosis and related disorders. Haemochromatosis involves a progressive increase in iron body stores causing iron deposits in cells of organs. The most effective siderophore for chelation therapy is desferrioxamine B from *Streptomyces pilosus* (Muller and Raymoned, 1984), which is produced commercially as the methane sulphonate salt, "Desferal" (Fig. 6b) (Jacobs, 1977, 1980; Messenger and Ratledge, 1986). Calver *et al.* (1979) have suggested that Desferal may have a chemotherapeutic role in severe neisserial disease , even in individuals with a normal iron load.

The properties required of a siderophore for successful use in chelation therapy are as follows. It must bind the metal strongly enough to compete with biological ligands such as transferrin or ferritin without inhibiting haem synthesis. The siderophore should be able to discriminate against the relatively abundant calcium and zinc cations *in vivo.* It should have a low toxicity and should not support microbial growth. The siderophore should have access to a chelatable iron pool and reach the site of heavy metal deposition. It must also not interfere with normal intracellular iron metabolism. The siderophore must be produced in large quantities by the producing microorganism and be easily and cheaply extracted and purified (Hoffbrand, 1980; Messenger and Ratledge , 1986).

Siderophores also have antibiotic activity. The sideromycins are iron-chelating antibiotics produced by *Streptomyces* species. Albomycin and ferrimycin are two members of this group, and are possibly the best understood examples (Messenger and Ratledge, 1986). *In vivo* experiments with mice show ferrimycin to be more effective than penicillin against some microorganisms (Hider, 1984). Other siderophores which have been isolated show some weak antibiotic activity. For instance, Desferritriacetylfusarinine C, produced by *Aspergillus deflectus*,

inhibits the growth of bacterial species whereas yeasts and fungi are only weakly affected, if at all. Since the potency of this activity is inhibited by addition of iron to the siderophore it would appear that its mode of action is to sequester iron and make it unavailable to bacterial species (Anke, 1977).

A hydroxamate siderophore produced by *Pseudomonas alcaligenes* and siderochelin produced by *Nocardia* spp. SC11 340 also have antibiotic activity against bacteria and fungi (Barker *et al.*, 1979; Itoh *et al.*, 1979; Liu *et al.*, 1981). Experiments *in vitro* have shown that desferrioxamine inhibits the growth of a number of bacteria by depriving them of iron (Calver *et al.*, 1979; Finkelstein and Yancey, 1981; Van Asbeck *et al.*, 1983). Rhodotorulic acid, synthesised by *Rhodotorula* sp.(Atkin and Neilands, 1968) and which can be produced in large quantities, has also been considered for clinical use. However, it induces zinc secretion and local inflammatory reactions (Grady *et al.*, 1979; Jacobs, 1979).

11. Applications for Siderophores outside Medicine

The siderophore, pseudobactin, is produced by *Pseudomonas putida* following inoculation into soil , and increases the growth and yield of various plants. *Pseudomonas putida* colonizes the roots of potato, sugar beet and radish and may act by depriving deleterious fungi and bacteria of iron or by supplying the plant roots with iron via pseudobactin (Kloepper *et al.*, 1980). Another pseudomonad, *Pseudomonas* spp. strain 346, suppresses wilt of cucumber, radish and pea caused by *Fusarium* species; addition of iron (III) to the soil significantly reduced the ability of strain 346 to limit chlamydospore germination of *Fusarium* species (Elad and Baker, 1985; Sneh *et al.*, 1984 ; Leong, 1986). Siderophores are probably of importance in keeping the mixed microbial population in soil in balance. Burton *et al.* (1954) have shown that some microbes synthesize siderophores whilst others use them without synthesizing any (Messenger and Ratledge, 1986). Siderophores may also be valuable in the growth of plants on

hydroponic solutions. Ferrichrome and ferrichrome A are effective in supplying tomato plants grown in this way with iron (Messenger and Ratledge, 1986).

12. Production of a Haemolytic Factor by *C. albicans*

Manns *et al.* (1994) found that *C. albicans* has the ability to utilize iron derived from haemoglobin and exhibits haemolytic activity when grown on glucose-enriched blood agar at 37°C in 5% CO_2. Hemoglobin released from lysed erythrocytes could restore the transferrin-inhibited growth of *C. albicans*. The investigators concluded that *C. albicans* expresses a haemolytic factor which allows it to acquire iron from host erythrocytes. However, no lysis occurred on plates containing sucrose, lactose, or galactose instead of glucose in the medium or when cultures were grown at temperatures other than 37°C. Hyphae showed greater haemolytic activity than yeast cells (Manns *et al.*, 1994).

13. Pigment Production by *Candida albicans*

Pigment production can be an important aid in both identification and classification of microorganisms. Several workers have reported that some *Candida* spp. produce pigments when grown under certain conditions (Table 7). Synthesis of green pigment by *C. albicans* was first described in 1940 by Jones and Peck, who used it as an additional means of differentiating *Candida stellatoidea* from *C. albicans* . They observed a greenish zone around colonies on blood agar. *C. stellatoidea* produced more than ten times the amount of pigment produced by strains of *C. albicans* . The pigment was found to be soluble in acetone, ethyl acetate and acetic acid; it appeared to be practically insoluble in ether and in methyl, ethyl and amyl alcohols. It appeared also to be unstable, solutions gradually assuming an irreversible yellow colour on standing for a few days (Jones and Peck, 1940). A later study revealed that green pigment was secreted by *C. albicans* during prolonged incubation in liquid medium containing 500 mM galactose (McCourtie and Douglas, 1985). *C. albicans* grown in a high concentration of galactose where

green pigment is produced showed ten times greater adherence to denture acrylic than *C. albicans* grown in low-glucose medium where no green pigment is produced.

Pigment formation associated with tryptophan metabolism has been noted in several organisms. *Candida pulcherrima* produces the red pigment pulcherrimin (Cook and Slater, 1956). Some Phycomycetes, Ascomycetes, Basidiomycetes and Schizomycetes produce a reddish-brown pigment when grown on media containing tryptophan (Swack *et al.*, 1960; Benjamin and Tamhane, 1966). Schindler and Zahner in 1971 reported that *Candida lipolytica* also synthesizes a reddish-brown pigment when growing on tryptophan medium (Chaskes and Phillips, 1974). However, *C. albicans*, *C. tropicalis* and *C. parapsilosis* produce a pink pigment on glucose-salts-biotin medium containing tryptophan as the major nitrogen source (Chaskes and Phillips, 1974). *Candida krusei*, *C. pseudotropicalis* and *C. gulliermondii* on the other hand, form brown pigments whereas pigmentation of *C. stellatoidea* is variable, some strains producing a brown pigment and others a pink pigment. The pink pigment is sensitive to alkali and on addition of sodium hydroxide it is converted to a light yellow-brown, returning to pink with the addition of acid (Chaskes and Phillips, 1974).

Chaskes and Phillips (1974) suggested that the pink pigments synthesized by *Candida* species might represent indole or tryptophan derivatives while Schindler and Zahner (1971) proposed that the reddish-brown pigment of *C. lipolytica* was a derivative of indole pyruvic acid. Indole derivatives are sensitive to physical agents such as light. Pink pigment production by *Candida* species was strongly catalyzed by light; cultures incubated in the dark produced only trace amounts of pink pigment (Chaskes and Phillips, 1974). Furthermore, although exposure of sterile pink supernatants to ordinary laboratory light (6 months) caused no colour change, direct sunlight converted the pink pigment to an orange-brown pigment. Riboflavin, a yellow-orange vitamin produced by some *Candida* species is decomposed by ultraviolet radiation and visible light (Prescott and Dunn, 1959).

Table 7. Pigment production by *Candida* species.

Pigment	**Colour**	***Candida* species**	**Reference**
Pulcherrimin	red	*Candida pulcherrima*	*a*
Green pigment	Green	*C. albicans*	*b, c*
		C. stellatoidea	
Tryptophan derivatives	Pink	*C. albicans*	*d*
		C. tropicalis	
		C. parapsilosis	
Tryptophan derivatives	Brown	*C. krusei*	*d*
		C. pseudotropicalis	
		C. gulliermondii	
Tryptophan derivatives	Brown or pink	*C. stellatoidea*	*d*
Derivatives of indole pyruvic acid	Reddish-brown	*C. lipolytica*	*d*
Riboflavin	Yellow-orange	*Candida spp.*	*e*

Ref. (*a*), Cook and Slater, 1956; (*b*), Jones and Peck, 1940; (*c*), Sweet and Douglas, 1991a; (*d*), Chaskes and Phillips, 1974; (*e*), Prescott and Dunn, 1959.

However, light is not required for red pigment production by *Candida pulcherrima* (Roberts, 1946).

13.1 Influence of iron on pigmentation of *Candida* species

Chaskes and Phillips (1974) showed that iron enhanced pigment production by *Candida* species, especially production of brown pigment. Moreover, they eliminated the possibility that iron was responsible for the brown colour, by control experiments. Pigment formation did not occur in media containing $FeSO_4$ that lacked tryptophan as the major nitrogen source. In addition, Sweet and Douglas (1991a) have demonstrated that green pigment production by *C. albicans* is regulated by the availability of iron and that maximal synthesis is attained at 0.026 μM (growth-limiting) concentration of the element. An iron concentration of 0.001% enhanced formation of the red pigment, pulcherrimin, by *C. pulcherrima* (Roberts, 1946). Finally, the production of riboflavin, a yellow-orange vitamin, by *Candida* species (Prescott and Dunn, 1959) was also shown to be linked to the iron concentration. Optimum iron concentrations were 10 μg/litre whereas levels of 100 μg/litre were inhibitory.

OBJECT OF RESEARCH

Siderophore production by *Candida* species has not been extensively investigated. The earliest report in 1983 suggested that *C. albicans* synthesises a siderophore of the hydroxamate type while subsequent studies indicated that certain isolates are capable of simultaneous secretion of both hydroxamate and phenolate chelators. More recent work in this Division showed that twelve strains of *C. albicans*, when grown in the yeast form in a defined medium, secreted hydroxamate-type siderophores; phenolate siderophores were not detected. Siderophore synthesis by *C. albicans* was maximal during growth in 0.026-0.2μM iron. These low concentrations of iron also induced the synthesis of a green pigment, with maximal production at 0.026μM. Synthesis of a green pigment by *C. albicans* was first reported in 1940 but has not been studied in detail; the role of the pigment in fungal metabolism and pathogenicity is unknown.

The aim of this project was to compare the production of siderophores by yeast and hyphal forms of *C. albicans* under different cultural conditions. The properties and possible functions of the iron-regulated green pigment were also explored, and contrasted with those of a pink pigment synthesised by *C. albicans*.

Specific objectives included the following:

(i) Development of protocols (media, growth temperature) for the production of pure yeast and pure hyphal cultures of *C. albicans*.

(ii) Measurement of siderophore production by non-specific and specific assays, and correlation with fungal dry weight for yeasts and hyphae.

(iii) Measurement of green pigment production by *C. albicans* grown in the yeast and hyphal forms.

(iv) Determination of the effect of various environmental conditions on green pigment production.

(v) Characterisation of the green pigment by thin layer chromatography and spectroscopy, and comparison of its properties with those of a pink pigment also produced by *C. albicans*.

MATERIALS AND METHODS

1. Origin and maintenance of organisms

Twelve strains of *Candida albicans* were used in this study. Strains GRI 681 and GRI 682 were obtained from cervical smears taken from asymptomatic women at Glasgow Royal infirmary; strains GDH 2023 and GDH 2346 were isolated from patients with denture stomatitis at Glasgow Dental Hospital. *C. albicans* 'Outbreak' strain, which was isolated as the causative agent of an outbreak of systemic candidosis at The London Hospital, was kindly supplied by Dr J. P. Burnie. *C. albicans* 1001-wild type and two mutants derived from it unable to form yeasts (strain 1001-FR) or hyphae (strain 1001-92') were kindly supplied by Dr. C. Gil, Universidad Complutense, Madrid, Spain. These mutants were obtained after treatment of the wild type by UV irradiation (90μJ mm^{-2}) (Pomes *et al.*, 1985). *C. albicans* ATTC 10261 and its mutant hOG301 were kindly supplied by Prof. M. G. Shepherd, University of Otago, Dunedin, New Zealand. Isolation of strain hOG301 followed mutagenesis of strain ATCC 10261 with N-methyl-N-nitro-N-nitrosoguanidine (Hubbard *et al.*, 1986). *C. albicans* MEN and its mutant MM2002 were kindly supplied by Dr. R.D. Cannon, University of Otago, Dunedin, New Zealand. Mutant MM2002 was isolated by a physical separation technique (Cannon, 1986).

The organisms were maintained on slopes of Sabouraud dextrose agar (Difco) at 4°C and subcultured monthly. Every two months the cultures were replaced by new ones freshly grown from freeze-dried stocks. Freeze-dried yeasts were maintained in a dried state in small evacuated glass ampoules kept at -20°C.

2. Growth media

2.1 Growth media for the production of hyphae

Seven media were tested for their ability to stimulate hyphal growth.

2.1.1 Lee's amino acid synthetic medium (Lee's medium)

The formulation was as given by Lee *et al.* (1975). Amino acid stock solution (100 ml) was combined with salts and glucose (see appendix), and made up to 1,000 ml with distilled water. A separate solution of biotin was prepared, filter sterilised, and added aseptically to the cooled, autoclaved medium.

2.1.2 Lee's amino acid synthetic medium + arginine, pH 4.5 and 6.7 (Lee's medium+arginine)

The method described by Staebell and Soll (1985) was used. Lee's synthetic medium was supplemented with 70µg arginine per ml, and the pH was adjusted to a value of 4.5 or 6.7 before autoclaving.

2.1.3 Glucose-glycine broth (GGB)

Glucose-glycine broth was composed of 1% glucose, 1% glycine and 0.1% yeast extract (Oxoid). The glycine-yeast extract was adjusted to pH 7.5 with solid $NaHCO_3$ before the glucose solution was added (Muerkoester *et al.*, 1979).

2.1.4 Neopeptone starch broth (NSB)

Neopeptone starch broth was prepared with 2.5% neopeptone , 0.1% yeast extract, 0.3% NaCl and 0.2% soluble starch. Before autoclaving, the neopeptone starch broth was adjusted to pH 7.5 with 10M NaOH (Muerkoester *et al.*, 1979).

2.1.5 Casein-yeast extract medium (CY)

Casein-yeast extract medium contained 5g casein hydrolysate and 5g yeast extract (Oxoid) per litre (Marichal *et al.*, 1986).

2.1.6 N-Acetylglucosamine-yeast nitrogen base-proline medium (NYP)

NYP medium contained 0.22g N-acetylglucosamine (Sigma), 0.12g proline (Sigma), 4.5g NaCl and 3.35g yeast nitrogen base (Difco) per litre (Marichal *et al.*, 1986).

2.1.7 Yeast nitrogen base medium (YNB)

One litre of 50 mM glucose solution or 500 mM galactose solution (as carbon source) was supplemented with 0.67g yeast nitrogen base (McCourtie and Douglas, 1981). These media had a final pH value of 5.4 and were autoclaved at 10 lb for 10 min. They were also used for studies on the production of green pigment under different growth conditions.

2.2 Growth media for the detection of siderophores

2.2.1 Blue agar for the detection of siderophores

Siderophores produced by different *C. albicans* strains and mutants were detected using the blue agar plate method of Schwyn and Neilands (1987). Glucose, as carbon source (0.45g), was dissolved in 90 ml distilled deionized water, and 0.6g yeast nitrogen base plus 1.08g agar (Agar technical No. 3, Oxoid) were added and the mixture was autoclaved. Chrome azurol S (2mM; 5ml) was mixed with 1 ml iron (III) solution (1 mM $FeCl_3.6H_2O$ and 10 mM HCl). This blue solution was slowly added to 4 ml of 5 mM hexadecyltrimethylammonium bromide (HDTMA). The resulting dark blue liquid was autoclaved. The dye solution was finally added to YNB agar along the glass wall of the flask with enough agitation to achieve mixing without generation of foam. Each plate received 30 ml of blue agar. Plates were inoculated from fresh slopes and incubated at 37°C for 24h. Production of siderophores was indicated by a pink halo around the colonies. Wet films were

prepared from the colonies after incubation for 24h to determine the percentage of yeasts or hyphae.

2.3 Growth media for pigment production

2.3.1 Blood agar for detection of green pigment

Sheep blood (5ml) was added to 100 ml of autoclaved cooled Sabouraud Dextrose Agar (Difco) for the preparation of blood agar plates. The plates were inoculated with a loopful of freshly grown yeast from SDA slopes and incubated at 37°C for 24h. Production of green pigment was noted by a dark green zone around colonies (Jones and Peck, 1940).

2.3.2 Tryptophan medium for production of pink pigment

This chemically defined medium contained 20µg biotin, 4g KH_2PO_4, and 2.5g $MgSO_4.7H_2O$ per litre of distilled water; the final glucose concentration was 1% and the nitrogen source consisted of proline (0.5g/litre) and tryptophan (1g/litre). The pH of the medium was adjusted to 5.5 with K_2HPO_4. It was sterilised by filtration through 0.45µm filter units (Sterifil-D, Millipore) and transferred to sterilised Erlenmeyer flasks. In some experiments iron (0.2g $FeSO_4$/litre) was added; the $FeSO_4$ was dissolved in deionized sterile water and was sterilised separately by filtration through 0.45µm membranes. After addition of this solution, the medium was used immediately (Chaskes and Phillips, 1974).

2.4 Growth media for the detection of haemolysis

2.4.1 Sabouraud dextrose blood agar

Blood agar plates were prepared by adding 3 ml of sheep red blood cells (SRBCs), washed with phosphate-buffered saline (pH 7.2) and suspended in sterile PBS to 100 ml of Sabouraud dextrose agar which had been enriched with

glucose (to a final concentartion of 7% wt/vol.). The plates were incubated at 37°C in 5% CO_2 for 24 to 48 h (Manns *et al.*, 1994).

2.4.2 Trypticase soy blood agar

Blood agar plates were prepared by adding 3 ml of washed SRBCs suspended in sterile PBS (pH 7.2) to 100 ml of Trypticase Soy agar which had been enriched with 3% glucose (wt/vol.). The plates were incubated at 37°C in 5% CO_2 for 24 to 48 h (Manns *et al.*, 1994).

2.4.3 Brain heart infusion agar (BHIA)

BHIA (Oxoid; 47g) was suspended in 1 litre of distilled water, boiled to dissolve the medium completely, and sterilized by autoclaving at 121°C for 15 min. After cooling to 45°C, 5% SRBCs washed in sterile PBS (pH 7.2) were added and plates were poured immediately.

2.5 Low-iron growth media

2.5.1 Treatment of glassware

To minimize iron contamination, disposable plasticware was used wherever possible; glassware was washed with Extran (BDH), soaked in 1% (v/v) HCl for at least 48h and rinsed three times with distilled water and another three times with distilled deionized water. Glassware was sterilized by dry heat treatment at 120°C overnight.

2.5.2 Deferration of growth medium using Chelex-100 ion exchange resin (batch method)

Chelex 100 ion exchange resin (Bio-Rad) can be used for analysis, removal or recovery of trace metals. It is an analytical grade resin which shows unusually

high preference for copper, iron and other heavy metals over such cations as sodium, potassium and calcium. The quantity of cations absorbed by this weakly acidic resin is a function of pH: it is very low below pH 2, increases sharply from pH 2 to pH 4, and reaches a maximum above pH 4. Any metal removed from solution is replaced by an equivalent amount of the ions originally on the resin. An alkaline form of resin usually gives the best results and so a sodium form was used in this study .

The batch method consists of adding resin (Chelex 100 sodium-form resin; Bio-Rad) directly into the sample, followed by stirring. About 5g of resin were weighed out for every 100 ml of sample, the resin was added to the sample and the mixture was stirred or shaken (gently) for 1 h. The sample was filtered or decanted from the resin.

The most effective agents to elute metals from the resin are acids. Regeneration of the resin to a salt form is a two-step process. The resin is first converted to the hydrogen form using acid, then converted to the desired ionic form using the hydroxide of the cation desired.

$$\text{Resin-Fe} + 2\,\text{HCl} \longrightarrow \text{Resin-H} + \text{FeCl}_2$$

$$\text{Resin-H} + \text{NaOH} \longrightarrow \text{Resin-Na} + \text{H}_2\text{O}$$

The following sequence was used: 2 bed volumes of 1 M HCl; 5 bed volumes of water rinse; 2 bed volumes of 1 M NaOH; 5 bed volumes of water rinse. Single-step conversions are adequate when going from weakly-held to strongly-held ions. Thus the calcium form or magnesium form is prepared from the sodium form using 2 bed volumes of 1 M calcium chloride and 1 M magnesium chloride.

$$\text{Resin-Na} + \text{CaCl}_2 \longrightarrow \text{Resin-Ca} + 2\text{NaCl}$$

$$\text{Resin-Na} + \text{MgCl}_2 \longrightarrow \text{Resin-Mg} + 2\text{NaCl}$$

The following sequence was used: 2 bed volumes of 1 M $CaCl_2$ or 1 M $MgCl_2$ added to 1 bed volume of sodium-form resin; 5 bed volumes of water rinse.

2.5.3 Preparation of deferrated yeast nitrogen base (dYNB) agar

Purified agar (Oxoid; 1.2g) was added to 87.2 ml Chelex water (distilled deionized water after mixing with resin) , 10 ml of deferrated yeast nitrogen base stock solution (a 10-fold concentrate) and 2.8 ml 1 M glucose to give 100 ml of deferrated YNB agar. Autoclaving was carried out in a pressure cooker at a pressure of 10 lbs p.s.i. for 10 min. *C. albicans* strains grown on this agar were used to inoculate deferrated yeast nitrogen base media after washing and suspending in 0.03 M citrate- buffered saline, pH 5.

2.5.4 Preparation of dYNB medium containing different concentrations of iron

The medium used was a modification of yeast nitrogen base (Difco), prepared from individual constituents (see appendix) and containing either 50 mM glucose or 500 mM galactose as the carbon source. Prior analysis of the medium components by graphite furnace atomic absorption spectrometry revealed that the main source of contaminating iron was monobasic potassium phosphate; this was therefore replaced by the dibasic salt which contained approximately 40-fold less iron. Deferration of the medium was accomplished by treatment with of Chelex 100 ion-exchange resin (Bio-Rad) using the batch procedure recommended by the manufacturer (see section 2.5.2). A stock solution containing most of the medium components (except glucose, $MgSO_4$, $CaCl_2$, $CuSO_4$, $MnSO_4$, $ZnSO_4$ and $FeCl_3$), made up in distilled deionized water, was treated with sodium -form resin. Glucose, as a 1 M solution, and the distilled deionized water to be used for diluting the concentrated medium, were also mixed with sodium-form resin. Stock solutions of $CaCl_2$ and $MgSO_4$ were treated with calcium-form and magnesium-form resin, respectively. Solutions of zinc, copper and manganese salts were not treated with

Chelex 100 because they were of low concentration and contained insignificant amounts of iron. The various stock solutions were combined to give a 10-fold concentrate of medium which was sterilised by filtration through 0.45 μm Sterifil-D filter units (Millipore) and stored at 4°C until use.

When diluted, the medium had an iron content of 0.026 μM as determined by graphite furnace atomic absorption spectrometry. This was identical to the value obtained previously (Sweet and Douglas, 1991a). It was supplemented, as indicated, by adding solutions of $FeCl_3$ freshly prepared from a concentrated stock; storage of dilute solutions was avoided to minimize adsorption of iron to the plastic containers (Sweet and Douglas, 1991a).

2.6 Growth media containing different concentrations of salts and trace elements

YNB medium containing different low concentrations of phosphate, magnesium, manganese, zinc and copper were prepared for studies on the production of the green pigment.

2.6.1 YNB containing low phosphate concentrations

YNB was made up from individual constituents as outlined in the Difco manual (see appendix). The stock solution of 10 mM KH_2PO_4 was diluted to give the following concentrations: 0, 0.05, 0.1, 0.2, and 0.5 mM phosphate. Glucose (50 mM) was used as the carbon source. Control flasks contained medium with 500 mM galactose or 50 mM glucose and 7 mM phosphate (the concentration of phosphate in YNB powder).

2.6.2 YNB containing low magnesium concentrations

YNB was made up from individual constituents as outlined in the Difco manual. The stock solution of 15 mM $MgSO_4$ was diluted to give the following concentrations: 0, 0.05, 0.1, 0.5, and 1 mM magnesium. Glucose (50 mM) was used as the carbon source. Control flasks contained medium with 500 mM galactose or 50 mM glucose and 2 mM magnesium (the concentration of magnesium in YNB powder).

2.6.3 YNB containing low manganese concentrations

YNB was made up from individual constituents as outlined in the Difco manual. The stock solution of 20 μM manganese sulphate was diluted to give the following concentrations: 0, 0.1, 0.5, and 1 μM manganese. Glucose (50 mM) was used as the carbon source. Control flasks contained medium with 500 mM galactose or 50 mM glucose and 1.79 μM manganese (the concentration of manganese in YNB powder).

2.6.4 YNB containing low zinc concentrations

YNB was made up from individual constituents as outlined in the Difco manual. The stock solution of 20 μM zinc sulphate was diluted to give the following concentrations: 0, 0.1, 0.5, and 1 μM zinc. Glucose (50 mM) was used as the carbon source. Control flasks contained medium with 500 mM galactose or 50 mM glucose and 1.39 μM zinc (the concentration of zinc in YNB powder).

2.6.5 YNB containing low copper concentrations

YNB was made up from individual constituents as outlined in the Difco manual. The stock solution of 10 μM copper sulphate was diluted to give the concentrations: 0, 0.025, 0.05, and 0.1 μM copper. Glucose (50 mM) was used as

the carbon source. Control flasks contained medium with 500 mM galactose or 50 mM glucose and 0.16 μM copper (the concentration of copper in YNB powder).

2.7 Sterilisation of growth media

Media not containing glucose or galactose were autoclaved at 121°C for 15 min. Media which did contain these sugars were sterilised in a pressure cooker at 10 lbs p.s.i. for 10 min. In some experiments, e.g.. iron-limitation experiments, sterilisation was accomplished by filtration through disposable 0.45μm Sterifil-D filter units (Millipore). Small volumes were sterilised using disposable syringe filters (0.45 μm; Minisart, Sartorius).

3. Preparation of inocula and growth conditions

Preparation of inocula and growth conditions varied according to the type of experiment.

3.1 Growth conditions for freeze-drying

For freeze-drying, yeasts were grown in yeast nitrogen base (Difco) containing 500 mM sucrose. Batches of medium (50 ml, in 250 ml Erlenmeyer flasks) were inoculated with overnight yeast cultures (5ml) and incubated at 37°C on an oribital shaker operating at 150 r.p.m./min. After 24h, cells were harvested (MSE bench centrifuge: 5 min, 1200 x g) and washed in sterile 0.15M phosphate-buffered saline (pH 7.2; PBS). Yeast cells were resuspended in a small volume of 2% (w/v) skimmed milk and a drop of suspension was added to a sterile glass ampoule. Ampoules were dried in a centrifugal freeze-drier (Edwards High Vacuum Ltd., Sussex).

3.2 Studies on the production of yeasts and hyphae by clinical isolates

For *Candida albicans* strains GRI 682, GRI 682, GDH 2023 and GDH 2346, blastospores were harvested from Sabouraud dextrose agar (Oxoid) slopes which had been incubated for 48h at room temperature. The growth was washed off with sterile distilled water, and centrifuged at 5000 r.p.m (MSE bench centrifuge) for 10 min at room temperature, and the cells were resuspended in sterile distilled water. To standardise the inoculum, the spectrophotometric absorbance of the cell suspensions at 550 nm was measured; the standardised cell suspension used to inoculate media had an absorbance of 0.3 against a blank of sterile distilled water, corresponding to a cell concentration of 10^8 cells/ml. One ml of the adjusted cell suspension was added to each 100 ml of medium in 250 ml Erlenmeyer flasks. Half of the flasks were incubated at 25°C (for the production of yeasts) and the other half were incubated at 37°C (for the production of hyphae), all flasks being in orbital shakers operating at 100 r.p.m.

3.3 Studies on the production of yeasts and hyphae by mutant strains

Candida albicans strain 1001 (wild type) and the mutant strains 1001-92' (HY^-) and 1001-FR (Y^-) were inoculated into 100 ml medium in 250-ml Erlenmeyer flasks with a loopful of culture from SDA slopes which had been incubated at 37°C for 24h. All flasks were incubated at 37°C in an orbital shaker at 100 r.p.m.

3.4 Studies on green pigment production

In some experiments, e.g.. effect of carbon source, incubation time, temperature and shaking on green pigment production, four *C. albicans* strains were grown in YNB medium containing 50 mM glucose or 500 mM galactose as

carbon source. A loopful of 48h culture grown on SDA at 37°C was inoculated into 50 ml of YNB in Erlenmeyer flasks (250 ml), and incubated at 25°C, 30°C and 37°C either as a static culture or in an orbital shaker (150 r.p.m) for 15 days. Pigment production and growth were measured after 2, 5, 10 and 15 days.

Growth conditions for cultures in YNB containing low concentrations of phosphate, magnesium, manganese, zinc and copper were as follows : 48h old *C. albicans* strains grown on SDA at 37°C were resuspended in sterile deionized water. Portions (10 µl) of the suspension containing $2x10^6$ cells/ml (adjusted using a haemocytometer) were inoculated into 20 ml of YNB in Erlenmeyer flasks (100 ml) to give a concentration of 10^3 cell/ml, and incubated at 37°C with shaking at 100 r.p.m. for 2 and 5 days. Glucose (50 mM) was used as the carbon source. Control cultures were grown in medium containing 500 mM galactose or 50 mM glucose and normal trace element or salts concentrations. Growth in all media was measured in a spectrophotometer at 550 nm.

3.5 Studies on pink pigment production

C. albicans 'Outbreak' strain grown on SDA agar for 24h at 37°C was inoculated, using a heavy inoculum (washed cells, resuspended in sterile deionized water to $4x10^6$ cells/ml), into tryptophan medium with or without $FeSO_4$ (0.2 g/litre). Cultures were incubated at 30°C in an orbital shaker at 100 r.p.m. for 1-3 days (Chaskes and Phillips, 1974).

3.6 Growth in low-iron media

Yeasts were grown in deferrated yeast nitrogen base medium, containing 0.026, 0.1, 0.2, 0.4 and 0.8 µM iron. Batches of medium (50 ml, in 250 ml acid-washed Erlenmeyer flasks) were inoculated with 25 µl of yeast suspension ($2x10^6$ cells ml^{-1}) to give a concentration of 10^3 cells ml^{-1}, and incubated at 37°C in an

orbital shaker operating at 100 r.p.m. Inocula were prepared by washing and suspending organisms freshly grown on plates of deferrated yeast nitrogen base agar in 0.03 M-citrate- buffered saline, pH 5.

3.7 Cell counts of yeasts and hyphae

Wet films were made by placing one drop of culture on a slide and applying a coverslip. Ten fields of 100 cells were counted under X400 magnification. Buds less than half the diameter of the mother cells were not scored; longer structures (twice normal bud length) were scored as hyphae. Hyphae and blastospores were scored individually, and the hyphae were expressed as a percentage of the total (Fig.1). Counts were not made from heavily clumping preparations.

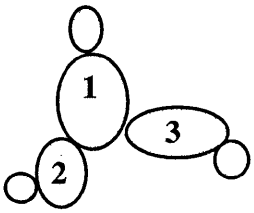
3.8 Preparation of culture supernates for siderophore, hydroxamic acid and phenolate assays

After incubation for 24 or 48 h at 25°C (for the production of yeasts) or 37°C (for the production of hyphae), samples of cultures (20 ml) were centrifuged at 5000 r.p.m for 10 min. The resulting supernates were tested for the presence of siderophores, hydroxamates and phenolates.

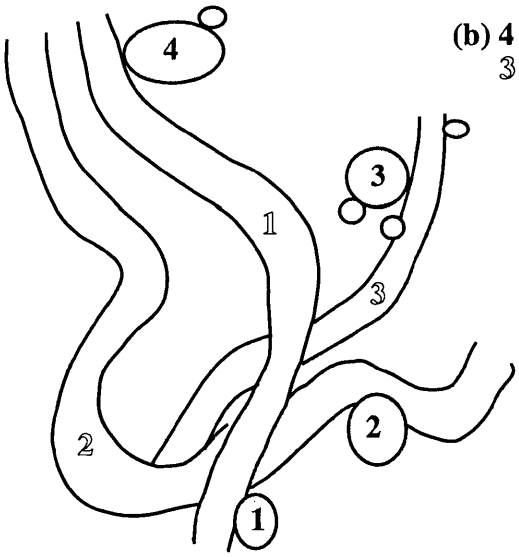
3.9 Determination of cell dry weight

Cell dry weights were determined using the method of Sweet and Douglas (1991a,b). Organisms from portions (10 ml) of 48 h cultures were collected on pre-weighed cellulose nitrate filters (0.45 μm pore size; 25 mm diameter; Whatman) and given three washes with distilled water (5 ml). The filters were dried to constant weight at 80°C and the cell dry weight per ml of culture calculated.

Figure 8. Counting method for yeasts and hyphae of *C. albicans*

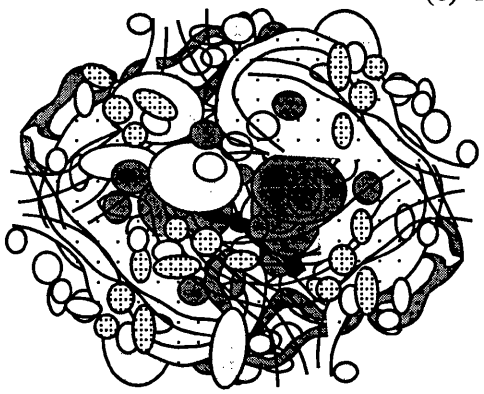


(a) 3 yeasts



(b) 4 yeast
3 hyphae

(c) heavy clumps of hyphae



4. Chemical assays

4.1 Non-specific Assay for Siderophores

Siderophore production was determined by the universal chemical assay for siderophores described by Schwyn and Neilands (1987). This assay is based on the removal of iron by siderophores from a dye complex of Chrome azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA). When a strong chelator removes the iron from the dye, its colour turns from blue to orange. Siderophores in culture supernates were assayed by mixing 0.5 ml of supernate with 0.5 ml of CAS (Aldrich) solution prepared as described by Schwyn and Neilands (1987). A 6 ml volume of 10 mM HDTMA (Sigma) solution was placed in a 100 ml volumetric flask and diluted with water. A mixture of 1.5 ml iron (III) solution (1 mM $FeCl_3.6H_2O$, 10 mM HCl) and 7.5 ml 2mM aqueous CAS solution was slowly added under stirring. A 4.307g quantity of anhydrous piperazine (Sigma) was dissolved in water and 6.25 ml of 12M hydrochloric acid was carefully added. This buffer solution (pH 5.6) was rinsed into the volumetric flask which was then filled with water to afford 100 ml of CAS assay solution. Siderophores were detected after 1 hour by a decrease in absorbance at 630 nm. A 0.5 mM solution of desferal (Ciba) was used as a positive control.

4.2 Detection of Hydroxamic acids

Hydroxamate-type siderophores were detected by the method of Holzberg and Artis (1983); this was based on the procedure described by Emery and Neilands (1960, 1962), who demonstrated that the oxidation of certain hydroxamic acids yields a material with a very strong absorption at 264 nm. To 0.4 ml of culture supernatant were added, in order, 1.0 ml of 2.19 mM periodic acid, 2.6 ml of distilled water, and 2 drops of glycerol. The final volume was mixed, and the

absorbance at 264 nm was determined. In this assay, 0.5 mM desferal (Ciba) was used as a positive control. Catechol (Sigma) was used as a negative control.

4 .3 Detection of Catechol

Phenolate-type siderophores were detected by a method was based on that described by Arnow (1937), in which catechol gives a yellow colour when reacted with nitrous acid and changes to an intense orange-red when made strongly basic (~pH 10). To 1.0 ml of supernatant were added, in order with mixing, 1.0 ml of 0.5 M HCl, 1.0 ml of nitrite-molybdate reagent (10g of sodium nitrite and 10g of sodium molybdate in 100 ml of deionized water), 1.0 ml of 1.0 M NaOH, and 1.0 ml of deionized water. In this assay, 174μM catechol (Sigma) was used as a positive control. Desferal (Ciba) was used as a negative control (Holzberg and Artis, 1983).

5. Pigment Production

5.1 Green pigment production

Pigment production was determined by measuring the absorbance of culture supernates at 444 nm, the absorption maximum of the pigment (Sweet and Douglas, 1991a). Blanks consisted of uninoculated medium. Although largely negligible, cells or cell debris not removed from the supernates occasionally gave a measurable absorbance at 444 nm. Therefore, A_{520} values were subtracted from the values measured at 444 nm since cells and cell debris gave similar absorbances at 520 and 444 nm, whereas the green pigment showed no absorbance at 520 nm.

5.2 Pink pigment production

C. albicans 'Outbreak' strain was grown in a chemically defined medium (tryptophan medium) containing tryptophan as nitrogen source and 0.2g $FeSO_4$ per

litre. After incubation at 30°C for 1-3 days, pink pigment production was determined by measuring the absorbance of culture supernates at 535-540 nm (Chaskes and Phillips, 1974).

6. Extraction of pigments

6.1 Extraction of green pigment using different solvents

Five-day cultures of *C. albicans* in dYNB containing 0.026μM iron, and giving high A_{444} readings for pigment production, were centrifuged. The supernates and cell pellets were freeze-dried. Portions (100 mg) of each were transferred to a conical flask (50 ml) together with 30 ml of different solvents and stirred for 6 h or overnight. After centrifugation, absorbance measurements were made at A_{444} and 520 nm to determine the most suitable solvent for pigment extraction. The solvents tested were acetone, acetic acid, ethyl acetate, water, ethanol and amyl alcohol.

6.2 Extraction of green pigment with acetone

Freeze-dried supernate (500 mg) from 5-d cultures in dYNB with 0.026 μM iron, which had given high readings at A_{444} , was transferred to a flask with 100 ml acetone, and stirred for 1h. The acetone was recovered by centrifugation, concentrated using a rotary evaporator and subjected to wavelength scanning in a silica cuvette.

6.3 Extraction of pink pigment with ethanol

Freeze-dried supernates (100 mg) from 3-d cultures in tryptophan medium (with 0.2g/litre $FeSO_4$) showing pink pigment production were placed in conical flasks with 30 ml of ethanol and stirred for 30 min. After centrifugation, the

extracted pigment was removed and wavelength scanning carried out using ethanol as a blank.

7. Thin-Layer Chromatography (TLC)

7.1 TLC of green pigment

Different samples of freeze-dried or solvent extract were subjected to TLC. Different plates and solvent systems were used. The TLC plates used were: (i) pre-coated TLC glass plates (sizes 5x10, 10x20 and 20x20cm; DC-Fertigplatten SIL G-25 UV_{254}), containing 0.25 mm silica gel 60 with fluorescent indicator (Camlab); (ii) TLC aluminium sheets containing silica gel 60, without fluorescent indicator (20x20 cm ; layer thickness 0.2 mm; Merck).

Ascending chromatography was done at room temperature for 2-4h using a Shandon tank with different solvents (Table 1). The TLC plates were air dried before analysis.

7.1.1 TLC using different sample volumes

Freeze-dried supernates of dYNB cultures containing 0.026 µM iron and showing obvious green pigment production were dissolved in deionized water at a concentration of 1 mg/ml. Different volumes of the resulting concentrated solution of green pigment were applied to TLC plates to determine the volume giving the best separation. The sample volumes used were 20, 30, 40, 50, 60, 80, 120, and 150 µl.

7.1.2 TLC using different culture supernates

Five supernates of dYNB cultures containing different iron concentrations (0.026, 0.1, 0.2, 0.4, and 0.8 µM) were freeze-dried and subjected to TLC using

Table 8. Solvent systems used for TLC

Solvent system	Used for separation of
Butanol : ethanol : water (4:1:1)	Pink pigment[a]
Butanol : acetic acid : water (65:10:25)	Pink pigment[a]
n-Butanol : pyridine : water (1:1:1)	Amino acid and peptides[b]
Methanol : water : pyridine (20:5:1)	Amines[b]
n-Propanol : ethyl acetate : water (7:1:2)	Carbohydrates[b]
Ethyl acetate : pyridine : water (12:5:4)	Carbohydrates[b]
Acetic acid : conc. HCl : water (30:3:10)	Phenolic derivatives[b]
Ethanol : water : NH_3 (18:1:1)	Simple phenols[b]
Phenol saturated with water	Amino acid and peptides[b]
Phenol sat. H_2O: ethanol : water (15:4:1)	Amino acid and peptides[b]
Chloroform : formic acid : water (125:73:3)	Simple phenols[b]
Chloroform : acetic acid : water (125:73:3)	Simple phenols[b]
5% Ammonium formate plus 0.5% formic acid	Catechols[c]
Petroleum ether :diethyl ether : acetic acid (70:30:1)	Cholesteryl esters and free fatty acids[b]
Chloroform : methanol : water : acetic acid (75:25:3:8)	Phospholipids

[a] Chaskes and Phillips (1974)
[b] Dawson *et al.* (1986)
[c] Rogers (1973)

butanol: ethanol: water (4:1:1) as solvent. Solutions of glucose and YNB powder were run in parallel for comparison with the green pigment.

7.2 TLC of pink pigment

Ethanol-extracted pink pigment and freeze-dried pigment-containing culture supernates (tryptophan medium) were subjected to TLC. The solvent system of butanol: ethanol: water (4:1:1) was used.

7.3 Analysis of TLC

7.3.1 Ultraviolet radiation

The dried TLC plates were examined under ultraviolet light using a Chromato- VUE Cabinet (UV Transilluminator, Model TM-40) to detect fluorescent spots.

7.3.2 Staining

Siderophore compounds were detected by spraying TLC plates with CAS solution (Schwyn and Neilands, 1987) and air-drying, or spraying with 0.1 M $FeCl_3$ in 0.1 M HCl (for the detection of hydroxamates). Desferal (Ciba) was used as a positive control for hydroxamates.

For visualisation of lipids, developed plates were sprayed with 50% sulphuric acid, then heated in an oven at 100°C for 15-30 min. All lipids char dark brown or black on a cream background as a result of this treatment.

For components which appeared on TLC plates, Rf values were calculated as follows:

$$R_f = \frac{\text{Distance travelled by component (cm)}}{\text{Distance travelled by solvent (cm)}}$$

7.4 Purification of green pigment by TLC

A concentrated solution of green pigment (2 mg dissolved in 2 ml water) was applied as a baseline streak to a series of TLC plates (20x20cm; DC-Fertigplatten SIL G-25 UV_{254}; 0.25 mm layer of silica gel 60 with fluorescent indicator). Butanol: ethanol: water (4:1:1) was used as the solvent. Separated, fluorescent components were marked under UV light , scraped from plates, dissolved from the silica in deionized distilled water and freeze-dried.

8. Absorption Wavelength Scanning

8.1 Wavelength scanning of green pigment

Fluorescent components of the green pigment were prepared by TLC as described in Section 7.4 above and dissolved in distilled water. Automatic wavelength scanning was carried out in a Shimadzu UV-3101 PC spectrophotometer using quartz cuvettes and a water blank. Culture supernates containing green pigment were subjected to automatic wavelength scanning using a Shimadzu UV-240 spectrophotometer.

8.2 Wavelength scanning of pink pigment

Ethanol-extracted pink pigment and pigment- containing culture supernates (tryptophan medium) were subjected to automatic wavelength scanning using a Shimadzu UV-240 spectrophotometer and quartz cuvettes. Ethanol or distilled water was used as a blank.

9. Fluorescence Wavelength Scanning

Yellow and blue fluorescent components scraped from TLC plates after separation in butanol: ethanol: water (4:1:1) and butanol: acetic acid: water (65:10:25) were scanned using a Hitachi F2000 fluorescence spectrophotometer.

Pink pigment solutions and different stock chemical solutions used in preparing dYNB were also scanned for fluorescence.

10. Statistical Analyses

The technique of analysis of variance was used to evaluate differences in experiments on siderophore and green pigment production. This technique examines the variation within the whole group of sample means, such as four strains , two morphologies and two incubation times (Wardlaw, 1992). Linear regression was used to treat the results from some experiments, e.g. determinations of cell dry weight. Standard errors of the mean (SEM) are included for most of the data presented graphically here.

Minitab analysis using the boxplot technique was also used (Fig. 8a).

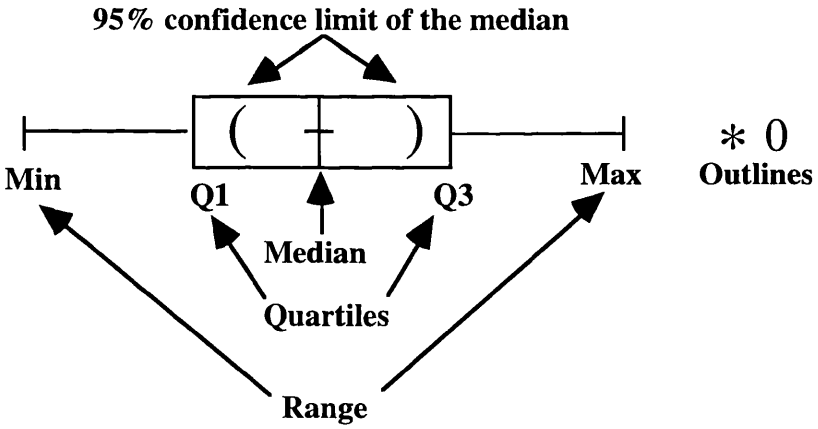


Figure 8a. Statistical analyses using the boxplot technique

This graphically displays the main features of data from a single variable. The middle half of each variable is represented by a box and the median is marked with a "+". The extent of the data (minimum and maximum) and the location of possible extraordinary values (* 0) are indicated on either side of the box with special symbols. Curved brackets indicate 95% confidence limits. Results are given in detail in the Appendix.

RESULTS

1. Comparison of several liquid media for the production of pure yeast and hyphal cultures of *C. albicans*

Strains of *C. albicans* from different sources were tested for their ability to grow as pure yeast or pure hyphal forms in several liquid media. Each strain was grown in 100 ml shake flask cultures at 25°C for pure yeast forms and at 37°C for pure hyphal forms. The percentages of yeasts and hyphae were determined using wet film preparations, and culture supernates were stored at -20°C for further studies on siderophore production.

1.1. Growth of *C. albicans* in amino acid synthetic medium (Lee's medium)

Pure yeast forms were obtained with Lee's medium at 25°C for all strains tested after 24h and 48h. The percentage of hyphal forms produced at 37°C differed from strain to strain (Fig. 9), with a higher percentage after 24h (50-85%) than after 48h (48-68%). Large clumps of hyphae were observed sticking to the walls of the flasks.

C. albicans 1001 (wild-type strain) and its mutant, strain 1001-92'(HY^-) both showed pure yeast forms following incubation in Lee's medium at either 25°C or 37°C. A high percentage of hyphal forms (72% - 86%) was noted with *C. albicans* mutant strain 1001-FR(Y^-) grown on this medium at 37°C for 24h, 48h, or 7h (Fig. 13). Growth curves of the wild type and its mutants in Lee's medium (Fig. 15) showed that the wild-type strain and yeast-form mutant give optical density readings higher than 1.5 at 550 nm whereas the hyphal form grows poorly ($O.D._{550} = 0.5$).

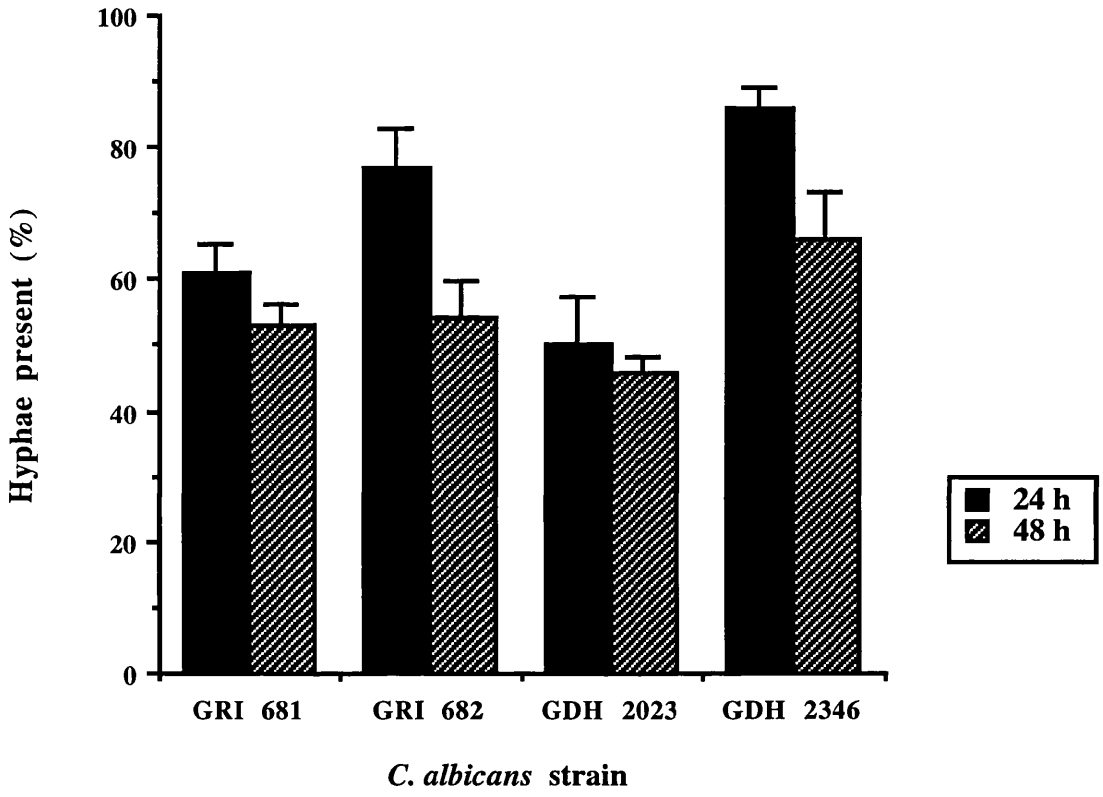
1.2. Growth of *C. albicans* in Lee's medium + arginine

This medium is identical to Lee's medium except for the addition of 70µg/L arginine; the medium is adjusted to pH 4.5 or pH 6.7 by adding drops of HCl. Two strains of *C. albicans* were used at 25°C and 37°C. At both pH values,

Figure 9.

Production of hyphae by *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 after incubation in Lee's medium at 37°C for 24 and 48 h.

Data represent the mean results of four experiments done in duplicate ± SEM


100
80
60
40
20
0
Hyphae present (%)
GRI 681
GRI 682
GDH 2023
GDH 2346
C. albicans strain
24 h
48 h

pure yeast forms were obtained at 25°C after 24h or 48h. However, at 37°C (Fig. 10) a higher proportion of hyphae (68-85%) was present in medium at pH 6.7 than in medium at pH 4.5 (5-30%).

1.3. Growth of *C. albicans* in neopeptone starch broth (NSB)

Four strains of *C. albicans* were inoculated into NSB medium which contains neopeptone, yeast extract, NaCl and soluble starch. This medium was incubated at 40°C for 48h. A low percentage (1- 26%) of hyphal forms were noted (Table 9) . Pure yeast cultures were obtained when the organisms were grown at 25°C.

1.4. Growth of *C. albicans* in casein-yeast extract (CY) medium

Standardised inocula (1 ml or 5 ml) of *C. albicans* strain GDH 2346 were added to 100 ml batches of CY medium and incubated at 25°C and 37°C. Inocula were prepared by resuspending the yeast cells in sterile CY medium and adjusting to an optical density of 0.3 at 550 nm in a spectrophotometer. Pure yeast cultures were obtained at 25°C, and low numbers of hyphae (9-13%) at 37°C (Table 10).

1.5. Growth of *C. albicans* in N-acetylglucosamine-yeast nitrogen base-proline (NYP) medium

Four strains of *C. albicans* were tested with NYP medium. They showed good growth and good hyphal formation (60-80%) at 37°C after 24h incubation (Fig. 11). They grew as pure yeast forms at 25°C.

C. albicans 1001(wild-type) and its mutant 1001-92'(HY^-) both grew solely in the yeast form at 25°C and 37°C. Excellent hyphal production (98% - 100%) was noted with the mutant strain 1001-FR(Y^-) when grown on this medium at 37°C for 24h, 48h, or 72h (Fig. 13). Growth curves of the wild type and its mutants in this medium (Fig. 14) showed that the wild type strain and yeast-form mutant (1001-92') gave optical density readings of 0.5 at 550 nm.

Figure 10.

Production of hyphae by *C. albicans* strains GRI 681 and GDH 2346 after incubation in Lee's medium + arginine pH 4.5 or pH 6.7 at 37°C for 24 and 48 h.

Data represent the mean results of two experiments done in duplicate ± SEM

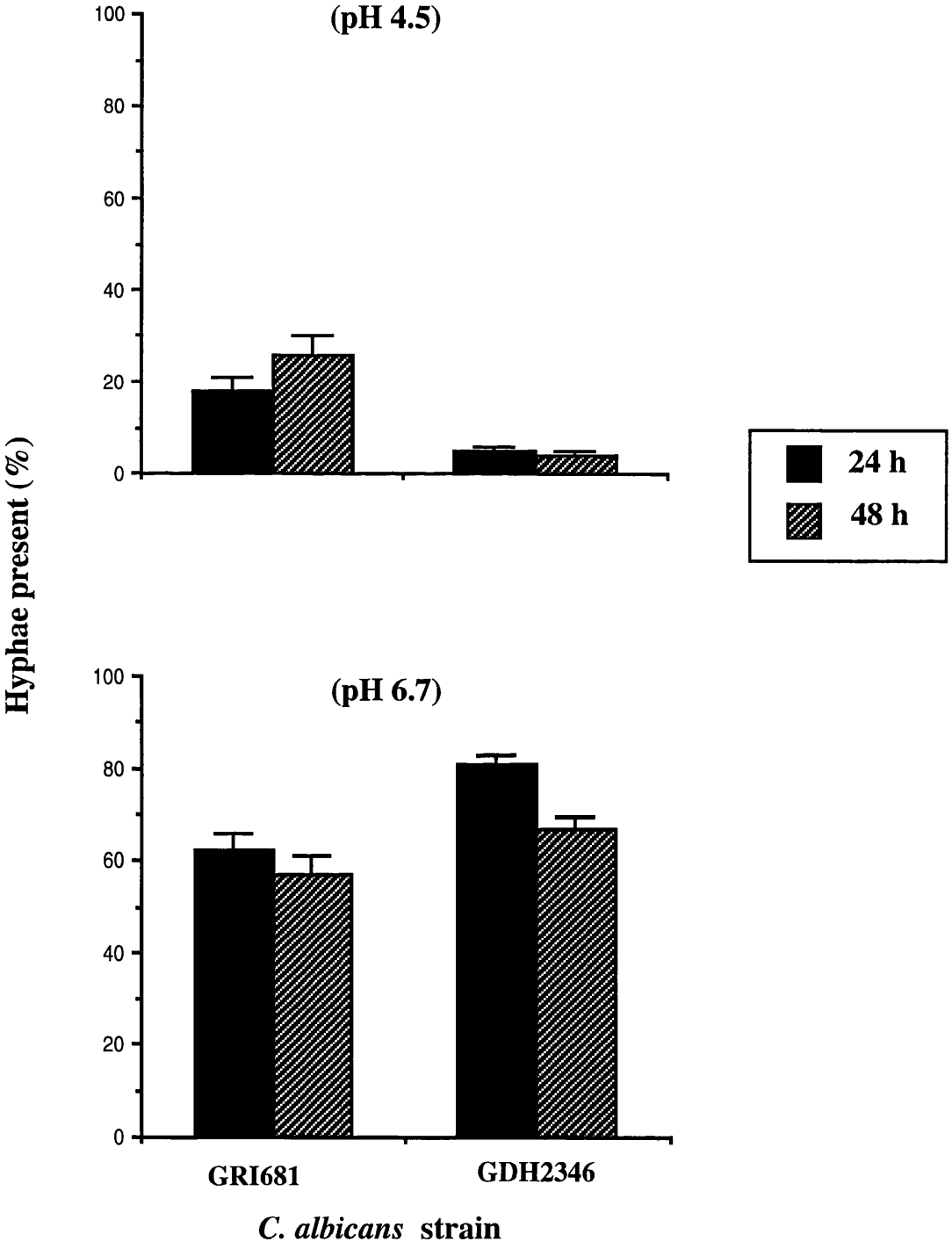

(pH 4.5)
100
80
60
40
20
0
24 h
48 h
Hyphae present (%)
(pH 6.7)
100
80
60
40
20
0
GRI681
GDH2346
C. albicans strain

Table 9.

Production of hyphae by *C. albicans* strains after incubation in neopeptone starch broth for 48 h at 40°C.

C. albians strain	Hyphae present (%)
GRI 681	1
GRI 682	2
GDH 2023	3
GDH 2346	26

Table 10.

Production of hyphae by *C. albicans* strain GDH 2346 after incubation in casein-yeast extract medium for 24 h at 37°C.

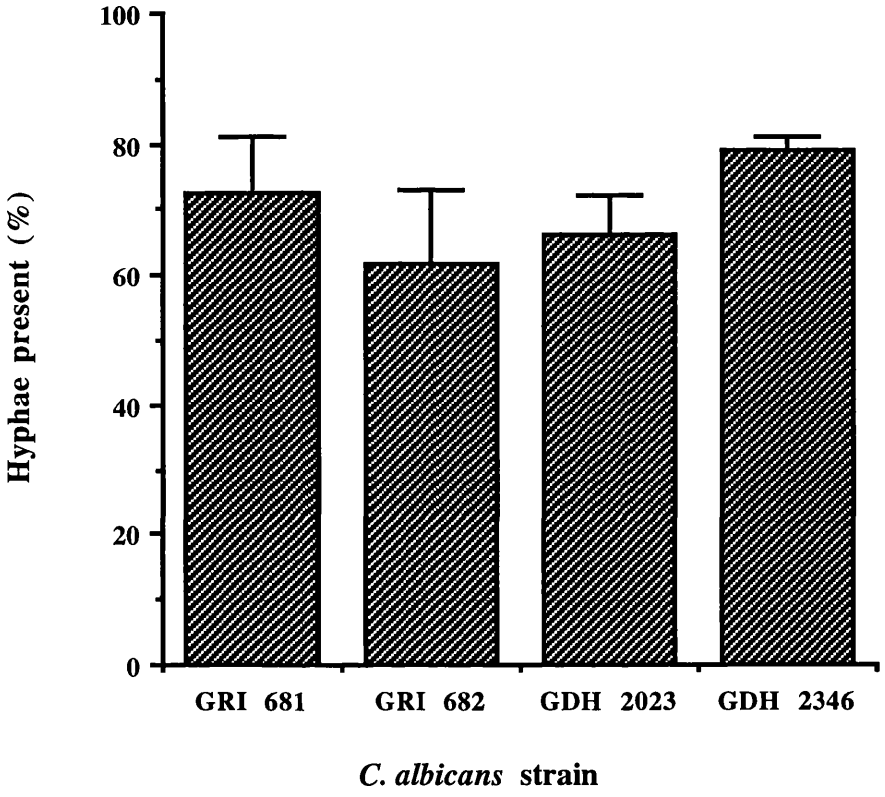
Inoculum size (ml)*	Hyphae present (%)
1	13
5	9

* Inocula were prepared by resuspending the yeast cells in sterile CY medium to an optical density of 0.3 at 550nm. 1ml or 5ml was added to 100ml of medium.

Figure 11.

Production of hyphae by *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 after incubation in N-acetylglucosamine-yeast nitrogen base-proline (NYP) medium at 37°C for 24 h.

Data represent the mean results of three experiments done in duplicate ± SEM


100
80
60
40
20
0
Hyphae present (%)
GRI 681
GRI 682
GDH 2023
GDH 2346
C. albicans strain

Poorer growth was observed with the hyphal form mutant (1001-FR) and the optical density readings decreased with increasing incubation times.

1.6. Growth of *C. albicans* in glucose glycine broth

The highest percentage of hyphae was obtained with this medium (which contains 1% glucose, 1% glycine and 0.1% yeast extract) compared to all other media tested. Strain GRI 681 gave 53-71% hyphae; strain GRI 682 gave 50-70%; strain GDH 2023 gave 60-78%; strain GDH 2346 gave 88-97% (Fig. 12). All strains tested grew exclusively in the yeast form at 25°C.

C. albicans 1001(wild-type) and its mutant 1001-92'(HY^-) both grew only in the yeast form at 25°C and 37°C. The wild type showed the same behaviour even when grown on solid media such as Sabouraud dextrose agar. Pure hyphal cultures were obtained with *C. albicans* 1001-FR(Y^-) in glucose glycine broth at 37°C following incubation for 24h, 48h, and 72h (Fig. 13). Growth curves of the wild type and its mutants in this medium (Fig. 14) demonstrated that cultures of both the yeast-form mutant and hyphal-form mutant showed increasing optical density with increasing incubation time.

Another wild-type strain and its mutant were tested for their ability to grow on glucose glycine broth as pure yeasts or hyphae at 37°C for 24h. *C. albicans* ATTC 10261 (wild-type) gave 87- 94% yeast-form cultures with an optical density of 0.54-0.58. Its mutant, HOG 301, showed a low percentage of hyphae (20-43%) with an optical density of 0.34.

1.7. Growth of *C. albicans* 1001 and its mutants in yeast nitrogen base (YNB) medium

C. albicans 1001(wild-type) and its mutants (1001-92'(HY^-) for pure yeast forms; 1001-FR(Y^-) for pure hyphal forms) were incubated for different times at 25°C and 37°C in YNB medium. The wild type and mutant 1001-92' (HY^-) both grew exclusively as yeasts at 25°C and 37°C. Almost pure hyphal

Figure 12.

Production of hyphae by *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 after incubation in glucose glycine broth at 37˚C for 24 and 48 h.

Data represent the mean results of five experiments done in duplicate ± SEM

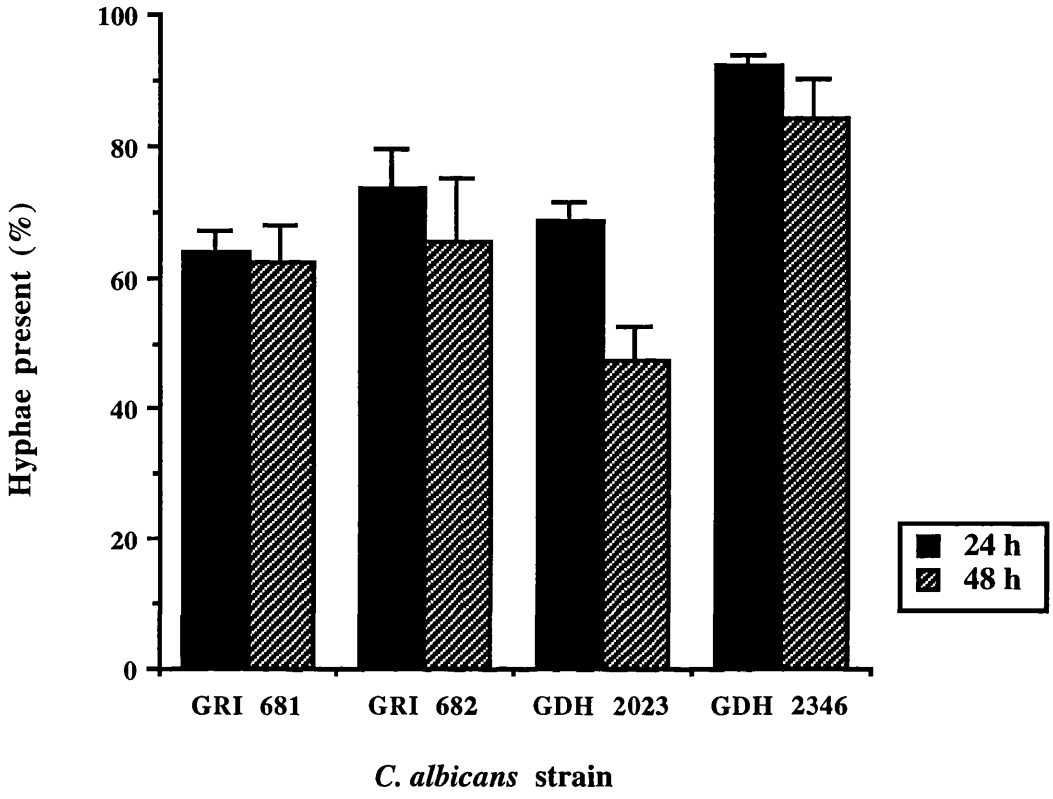

100
80
60
40
20
0
Hyphae present (%)
GRI 681
GRI 682
GDH 2023
GDH 2346
C. albicans strain
24 h
48 h

Figure 13.

Production of hyphae by *C. albicans* mutant strain 1001-FR (Y^-) after incubation in various media at 37°C for 24, 48 or 72 h.

Data represent the mean results of two experiments done in duplicate ± SEM

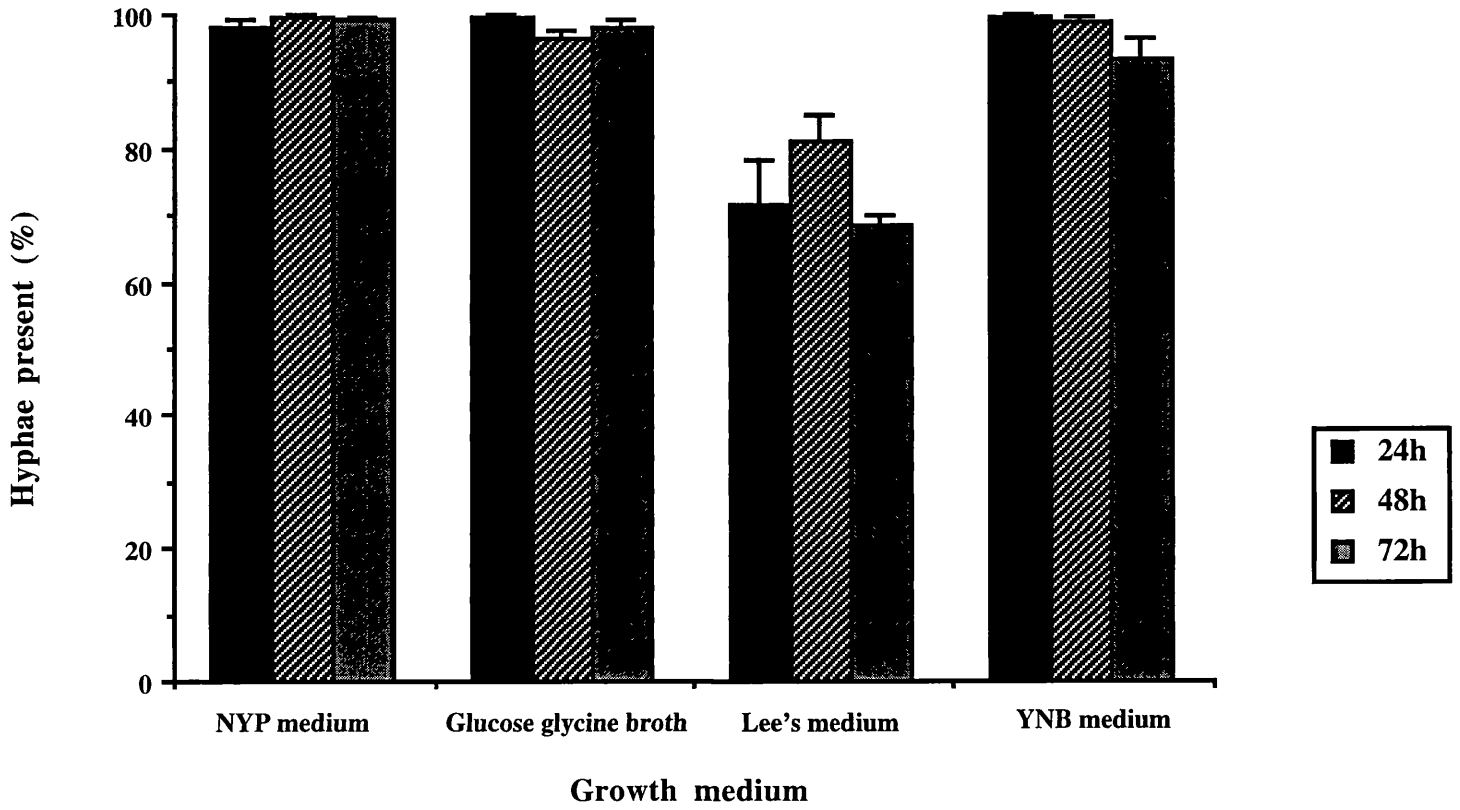
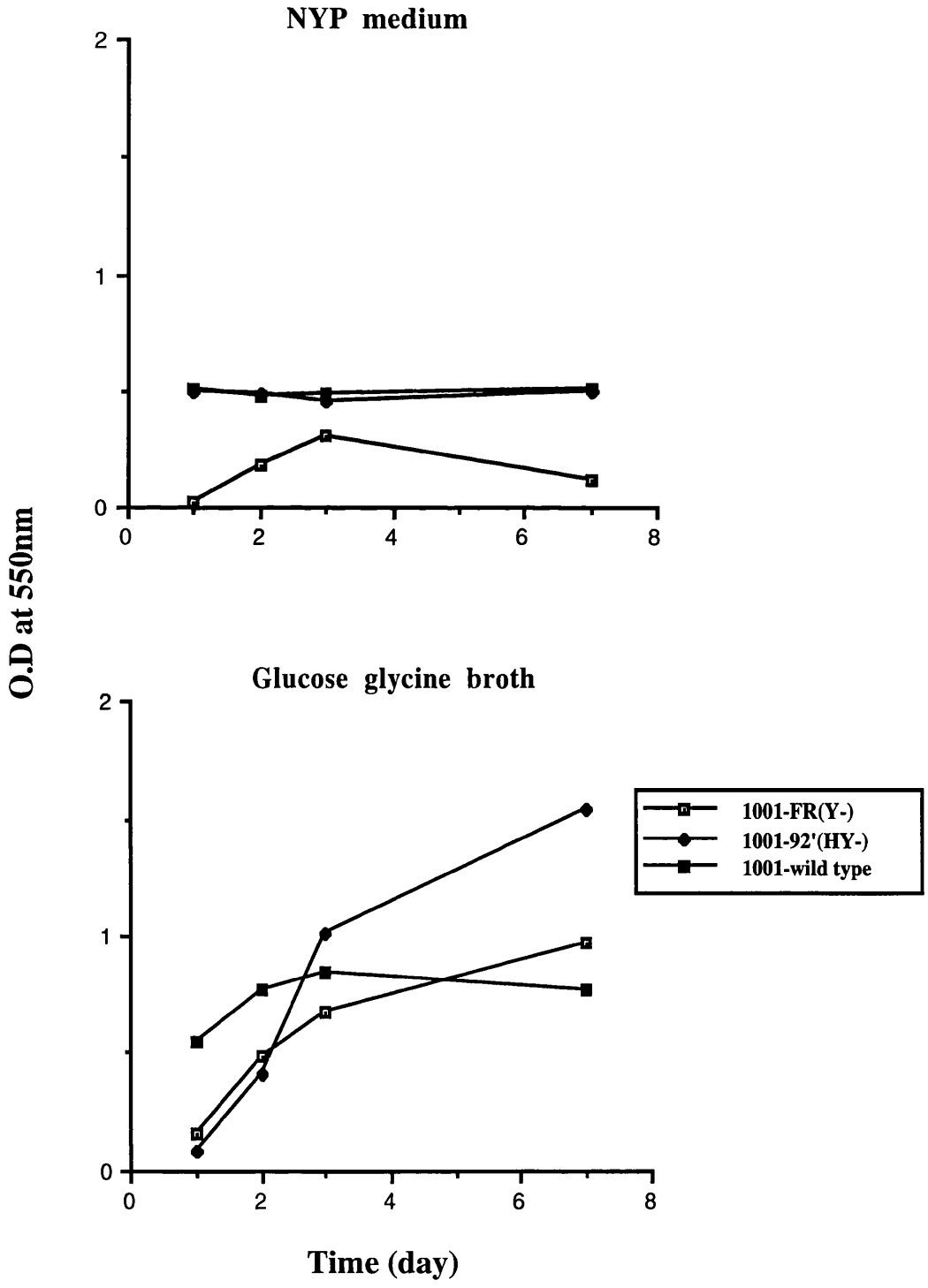

Hyphae present (%)
100
80
60
40
20
0
NYP medium
Glucose glycine broth
Lee's medium
YNB medium
Growth medium
24h
48h
72h

Figure 14.

Growth of *C. albicans* strain 1001 and its mutants 1001-FR(Y^-) and 1001-92'(HY^-) in NYP medium and glucose glycine broth at 37°C.


NYP medium
Glucose glycine broth
O.D at 550nm
Time (day)
1001-FR(Y-)
1001-92'(HY-)
1001-wild type
0
1
2
0
2
4
6
8

cultures (94-100%) were obtained with mutant 1001-FR(Y^-) grown in YNB medium at 37°C for 24h, 48h, and 72h (Fig. 13). A lower proportion of hyphae (60-85%) was observed when the same strain was grown on YNB medium at 25°C. Growth curves of *C. albicans* 1001 and mutant 1001-92'(HY^-) revealed optical density readings of 1.5 in YNB medium. Mutant 1001-FR (Y^-) grew very slowly, reaching an optical density of 1.0 only after 7 days (Fig. 15).

2. Detection of siderophores by non-specific assays

Culture supernates of *C. albicans* strains grown in the yeast form at 25°C or hyphal form at 37°C in several media were subjected to chemical assays to determine which morphological form produces greater amounts of siderophore. All media contained normal iron concentrations since growth of *C. albicans* under iron-limitation might affect the formation of hyphae.

Siderophore production in liquid media was determined by the universal chemical assay described by Schwyn and Neilands (1987). This assay utilises a dye complex of chrome azural S (CAS) and hexadecyltrimethyl ammonium bromide (HDTMA) that has a high affinity for iron . The iron dye complex is blue with an absorption maximum at 630 nm. When a strong chelator removes the iron from the dye, its colour turns from blue to red.

Siderophore synthesis during growth on solid medium was also investigated by incorporating the dye into deferrated yeast nitrogen base agar. The formation of a pink zone around *Candida* colonies after 48h was indicative of siderophore production.

2.1. Siderophore production by *C. albicans* in glucose glycine broth

Culture supernates of *C. albicans* strains grown in glucose glycine broth at 25°C to produce yeast forms and at 37°C to produce hyphal forms were examined for the presence of siderophores. Figure 16 shows that there were highly significant differences between the two *C. albicans* morphologies (using

Figure 15.

Growth of *C. albicans* strain 1001 and its mutants 1001-FR(Y^-) and 1001-92'(HY^-) in Lee's medium and YNB medium at 37°C.

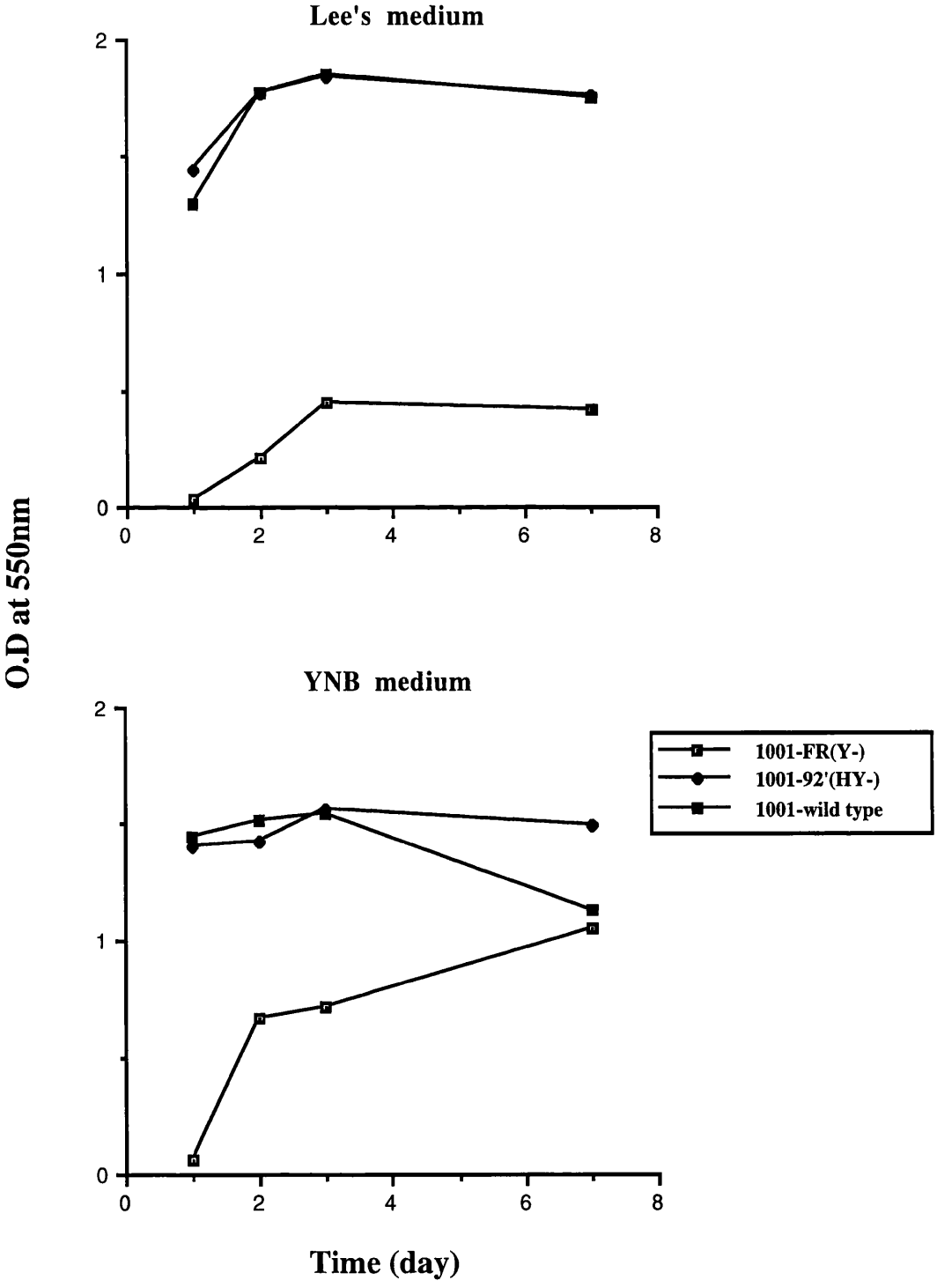
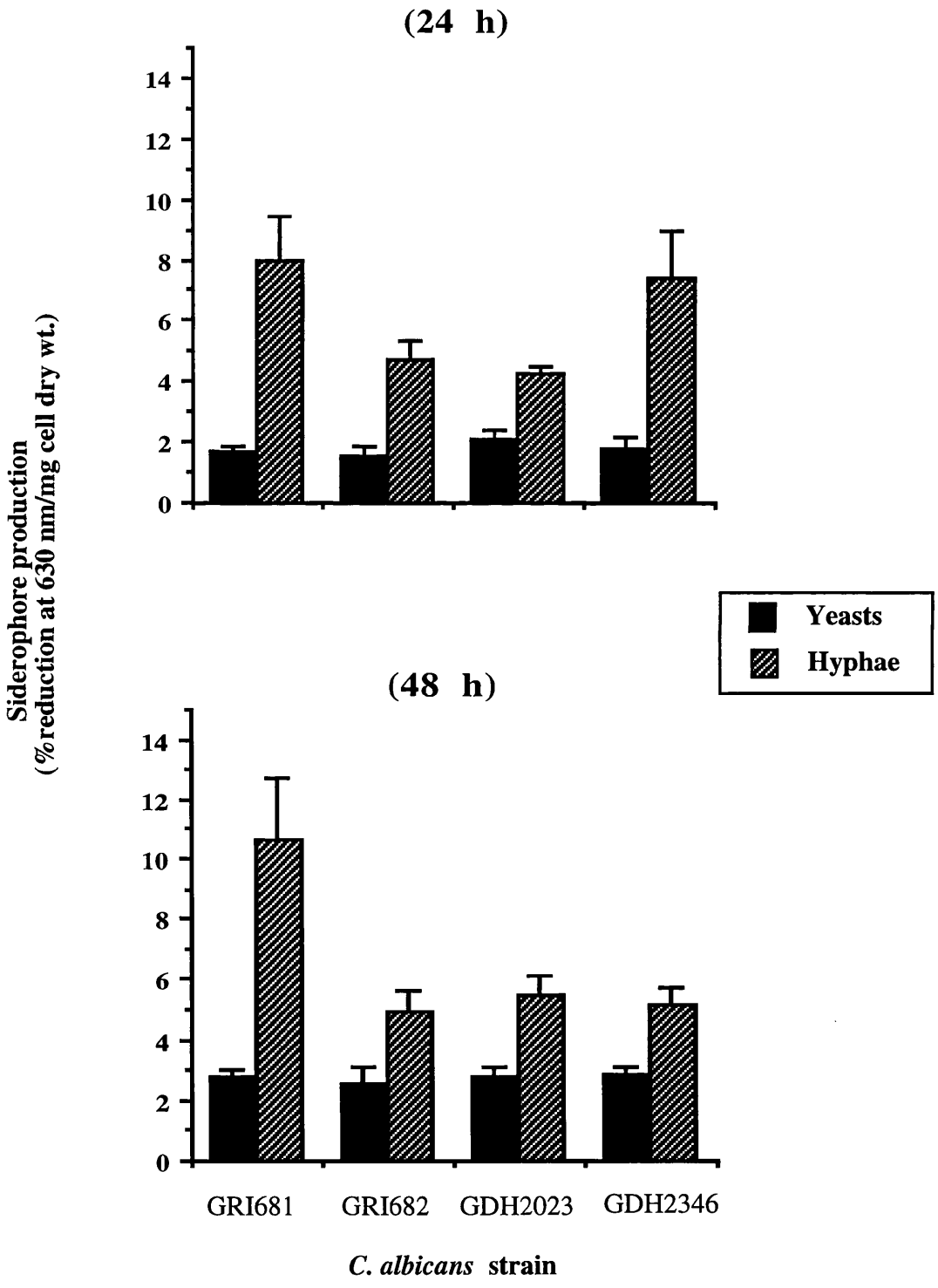

Lee's medium
YNB medium
O.D at 550nm
Time (day)
1001-FR(Y-)
1001-92'(HY-)
1001-wild type
0
1
2
0
2
4
6
8

Figure 16.

Siderophore production by yeast and hyphal forms of *C. albicans* strains after incubation in glucose glycine broth at 25°C (yeasts) or 37°C (hyphae) for 24 and 48 h.

Data represent the mean results of two experiments done in quadreplicate ± SEM


(24 h)
14
12
10
8
6
4
2
0
Siderophore production
(%reduction at 630 nm/mg cell dry wt.)
Yeasts
Hyphae
(48 h)
14
12
10
8
6
4
2
0
GRI681
GRI682
GDH2023
GDH2346
C. albicans strain

analysis of variance; see Appendix) when siderophore production was related to fungal dry weight. Hyphal forms synthesised substantially greater amounts of siderophores than did yeast forms after incubation for either 24h or 48h. Siderophore production varied quantitatively from strain to strain, but generally, *C. albicans* GRI 681 seemed produce more than the other three strains.

2.2. Siderophore production by *C. albicans* in NYP medium

Assay of siderophores in culture supernates of NYP medium after growth of *C. albicans* strains at 25°C for yeast forms and 37°C for hyphal forms showed no difference between the two forms (Fig. 17). The amounts of siderophore detected varied from strain to strain. Strains GRI 682 and GDH 2023 showed greater siderophore production by hyphae but the other two strains (GRI 681 and GDH 2346) did not. No siderophore production was detected with the yeast form of strain GRI 682 in this medium.

2.3. Siderophore production by *C. albicans* in Lee's medium

Lee's medium was also selected for comparison of siderophore production by yeasts and hyphae since it was relatively successful in generating cultures of either morphological form. When culture supernates were examined for siderophore production , the results showed greater production by hyphae than by yeast forms when related to the cell dry weight (Fig. 17). However, there appeared to be some interference by constituents of Lee's medium in the universal chemical assay (see section 5.2.1).

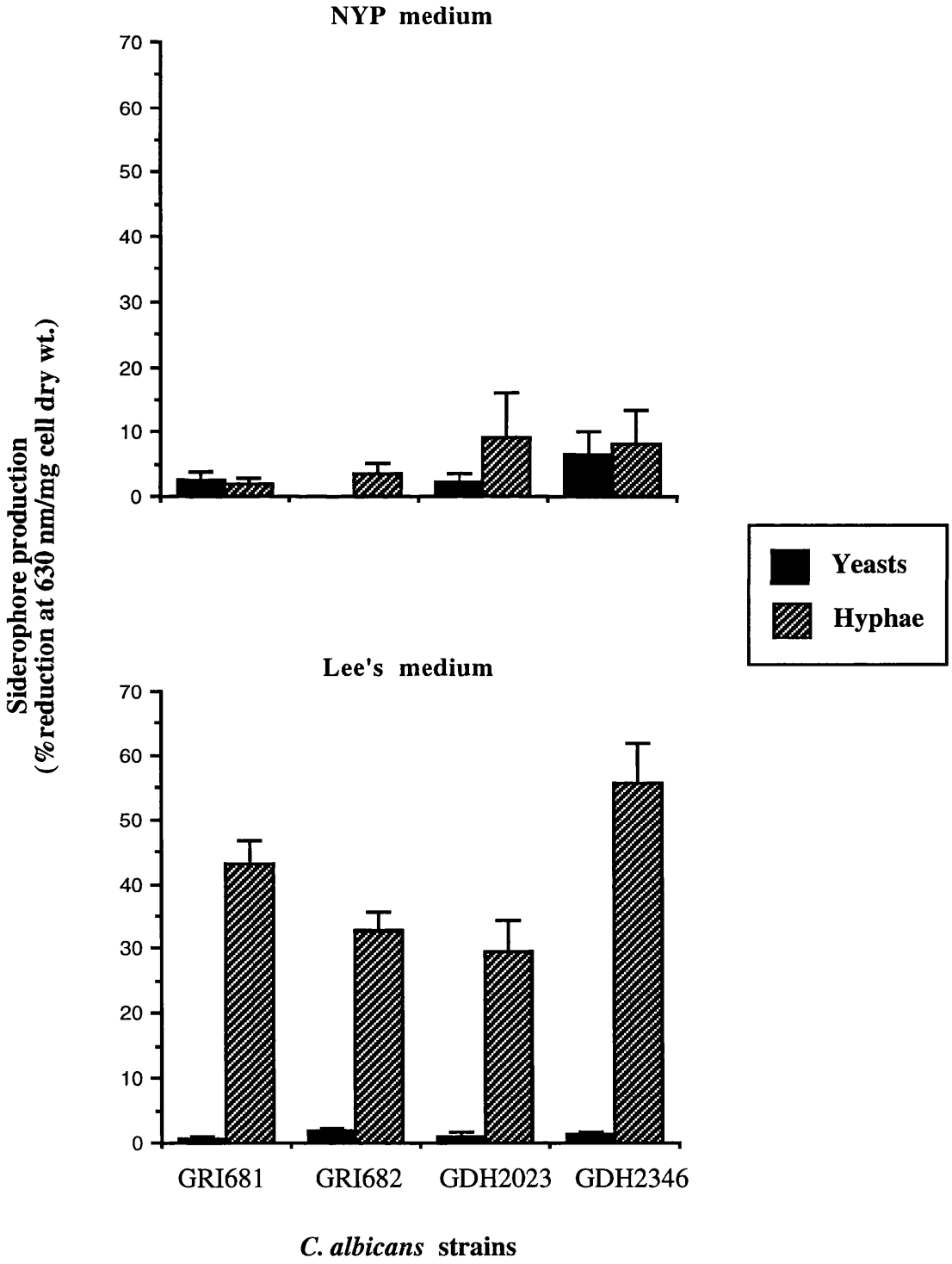
2.4. Comparison of siderophore production by *C. albicans* wild-type strains and morphological mutants

C. albicans wild-type strain 1001 and its mutants 1001-92' (HY^-; yeast form growth) and 1001-FR(Y^-; hyphal form growth) were cultured in four liquid media: NYP medium, glucose glycine broth, Lee's medium and YNB medium.

Figure 17.

Siderophore production by *C. albicans* strains after growth in NYP medium and Lee's medium at 25°C (yeasts) or 37°C (hyphae) for 24 h.

Data represent the mean results from three single experiments with NYP medium and from two experiments done in duplicate with Lee's medium ± SEM


NYP medium
70
60
50
40
30
20
10
0
Siderophore production
(% reduction at 630 nm/mg cell dry wt.)
Yeasts
Hyphae
Lee's medium
70
60
50
40
30
20
10
0
GRI681
GRI682
GDH2023
GDH2346
C. albicans strains

Culture supernates were tested for the presence of siderophores. *C. albicans* ATCC 10261 and its mutant HOG 301 (hyphal form growth) were treated similarly. Figure 18 indicates that both wild-type strains and their mutants produce siderophores after incubation for 24h in glucose glycine broth. In each case, however, siderophore production by the wild type was greater than that of the mutant (s). The three other media tested also failed to reveal any clear difference in siderophore production by mutant strains, even when incubation was continued for up to 7 days (Tables 11, 12, and 13). Again, possible interference (in the form of very high readings) was noted in some of the assays involving Lee's medium.

2.5. Detection of siderophores by the universal chemical assay with "blue agar" plates.

Siderophores produced by *C. albicans* strains and morphological mutants were also detected using the agar plate method of Schwyn and Neilands (1987) in which the blue dye complex was incorporated into deferrated yeast nitrogen base agar. Nine strains of *C. albicans* grew well on the blue agar and pink halos were seen around the colonies after 24h of growth, indicating siderophore production (Table 14). All strains tested, including the mutants, produced clearly visible pink halos (Figs. 19, 20 and 21). These results suggest that all of the *C. albicans* strains express a high-affinity iron uptake system involving the secretion of a siderophore. Removal of iron from the blue dye complex by the siderophore allows growth in the deferrated medium and results in the production of pink halos around the *C. albicans* colonies.

Wet films prepared from the colonies grown on blue agar plates after 24h at 37°C showed that 83-97% hyphae were present with mutant strains 1001-FR (Y^-) and HOG 301. 100% yeast forms were observed with other strains (Table 14). The pink halos around the colonies varied from 0.4-1.4 cm in diameter, with no obvious differences between yeast-form and hyphal-form colonies. These agar

Figure 18.

Siderophore production by *C. albicans* strain 1001 and its mutants (1001-FR and 1001-92') and strain ATTC 10261 and its mutant HOG 301 (hyphal form growth) after growth in glucose glycine broth at 37°C for 24h.

Data represent the mean results of three experiments done in duplicate ± SEM

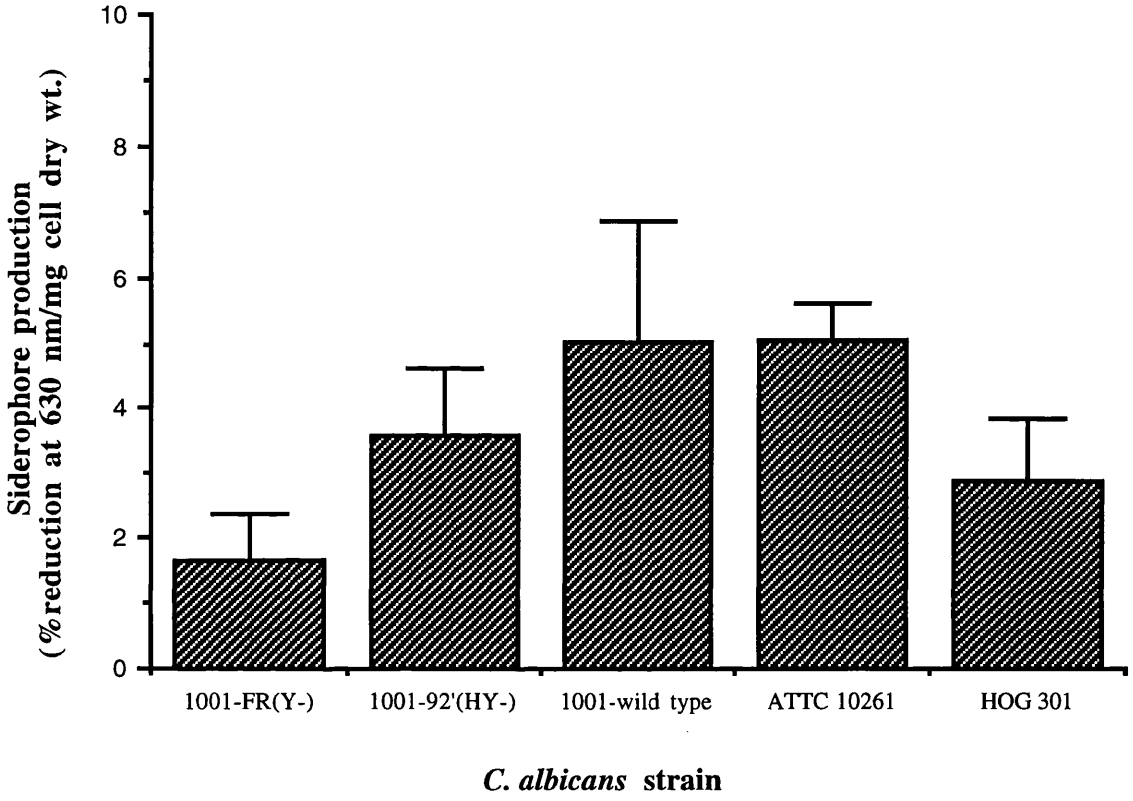

Siderophore production
(%reduction at 630 nm/mg cell dry wt.)
10
8
6
4
2
0
1001-FR(Y-)
1001-92'(HY-)
1001-wild type
ATTC 10261
HOG 301
C. albicans strain

Table 11. Siderophore production by *C. albicans* wild type and mutant strains in NYP medium at 37°C after different incubation periods.

	% reduction at 630 nm after incubation for			
***C. albicans* strain**	**24 h**	**48 h**	**72 h**	**7 d**
1001-wild type	1.4	2.8	11.1	1.6
1001-FR(Y^-)	2.3	2.7	2.3	1.1
1001-92'(HY^-)	1.1	0.5	2.1	1.7

(Data represent mean results from two experiments)

Table 12. Siderophore production by *C. albicans* wild type and mutant strains in Lee's medium at 37°C after different incubation periods.

	% reduction at 630 nm after incubation for			
***C. albicans* strain**	**24 h**	**48 h**	**72 h**	**7 d**
1001-wild type	0	0	0.9	0.9
1001-FR(Y^-)	37.8	30.9	26.3	4.2
1001-92'(HY^-)	40.3	28.8	27.1	4.8

(Data represent mean results from two experiments)

Table 13. Siderophore production by *C. albicans* wild type and mutant strains in YNB medium at 37°C after different incubation periods.

	% reduction at 630 nm after incubation for			
***C. albicans* strain**	**24 h**	**48 h**	**72 h**	**7 d**
1001-wild type	4.2	0.1	5.3	2.5
1001-FR(Y^-)	0	0	0	0.3
1001-92'(HY^-)	1.7	1.6	0.6	0

(Data represent mean results from two experiments)

Table 14.

Detection of siderophores by the Schwyn and Neilands assay iusing YNB blue agar plates.

C. albicans strain	Growth	Sid. production	Morphological form (%)	Diameter of pink halos (cm)
GRI 681	++	+	100 Y	0.5-1
GRI 682	++	+	100 Y	0.46-0.6
GDH 2023	++	+	100 Y	0.4-0.7
GDH 2346	++	+	100 Y	0.44-1
1001-Wild Type	++	+	93 Y, 7 Ps	0.8-1.4
1001-FR(Y^-)	++	+	83 HY, 17 Y	0.4-1
1001-92'(HY^-)	++	+	100 Y	0.6-1.4
ATTC 10261	++	+	100 Y	0.4-1
HOG 301	+++	+	97 HY, 3Y	0.4-1.2

Sid. production = Siderophore production demonstrated by colour change (blue to pink) around the colony; HY= hyphal form; Y= yeast form; Ps = Pseudohyphal form.

Figure 19.

Siderophore production by *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 on YNB blue agar after incubation at 37˚C for 24h.

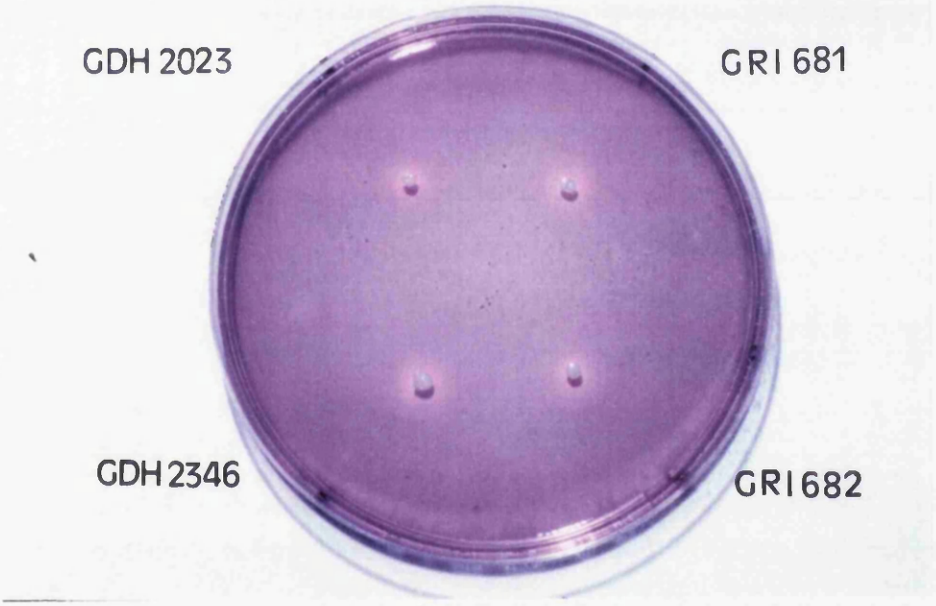

GDH 2023
GRI 681
GDH 2346
GRI 682

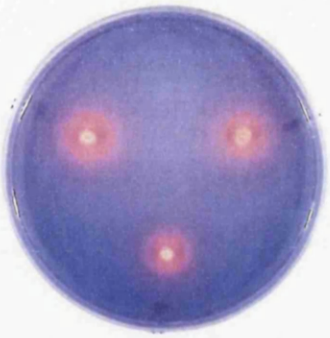
Figure 20.

Siderophore production by *C. albicans* strain 1001 and its mutants 1001-FR(Y^-) and 1001-92'(YH^-) on YNB blue agar after incubation at 37°C for 24h.

Figure 21.

Siderophore production by *C. albicans* strain ATTC 10261 and its mutant HOG 301 (hyphal form) on YNB blue agar after incubation at 37°C for 24h.

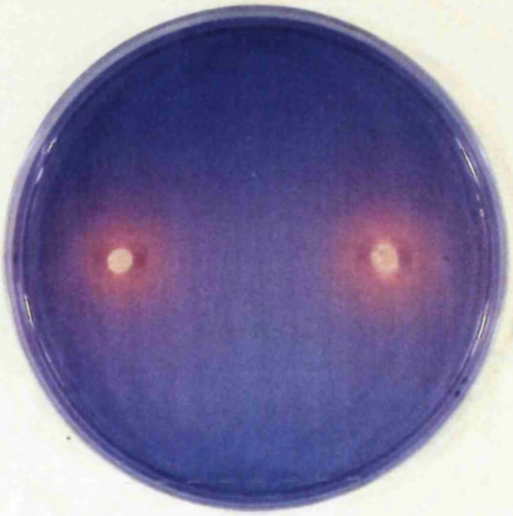
1001-WILD TYPE



1001-FR[Y⁻]

1001-92'[HY⁻]

ATTC 10261



HOG 301

plate assays clearly demonstrate that either morphological form of *C. albicans* is capable of producing significant amounts of siderophores.

2.6. The effect of iron concentration on siderophore production

The medium used was a modification of yeast nitrogen base, prepared from individual constituents and containing 50 mM glucose as the carbon source. *C. albicans* grows entirely in the yeast form in this medium. Deferration of the medium was accomplished using Chelex 100 ion-exchange resin (Bio-Rad) via the batch procedure recommended by the manufacturer. The various stock solutions were combined to give a 10-fold concentrate of medium which was sterilised by filtration through 0.45μm Sterifil-D filter units (Millipore) and stored at 4°C until use. When diluted, the medium had an iron content of 0.026 μM as determined by graphite furnace atomic absorption spectrometry. It was supplemented, as indicated, by adding solutions of $FeCl_3$ freshly prepared from a concentrated stock; storage of dilute solutions was avoided to minimize adsorption of iron to the plastic containers (Sweet and Douglas, 1991).

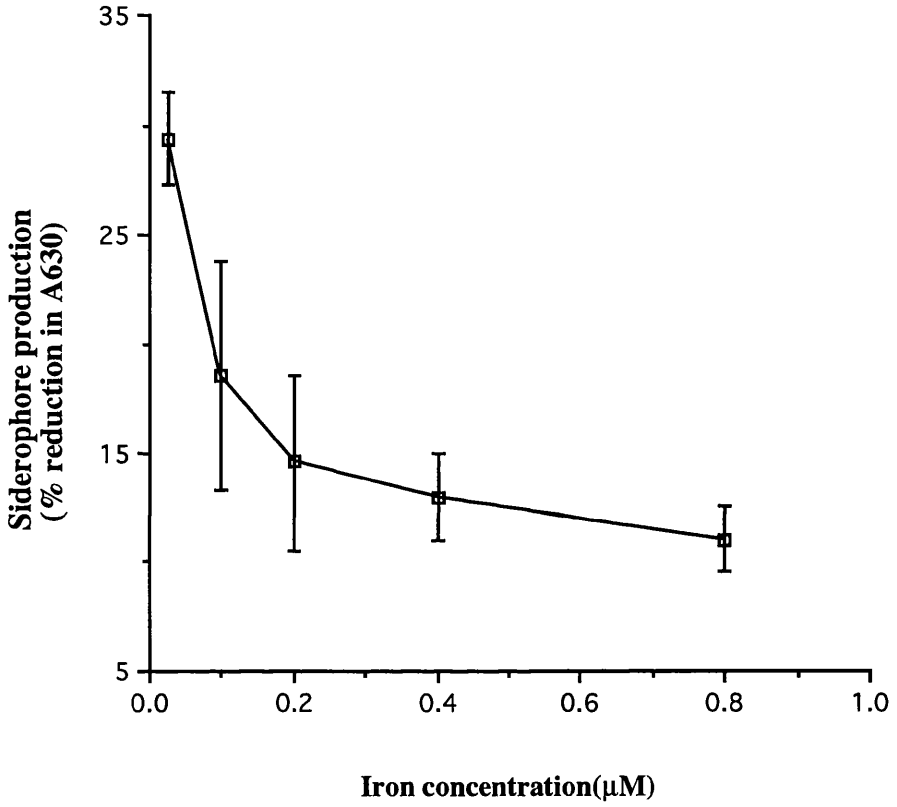
C. albicans 'Outbreak' strain was grown in deferrated yeast nitrogen base medium, containing 0.026, 0.1, 0.2, 0.4 and 0.8 μM iron. Batches of medium (50 ml, in 250 ml acid-washed Erlenmeyer flasks) were inoculated with 25 μl of yeast suspension ($2x10^6$ cells ml^{-1}) to give a concentration of 10^3 cells ml^{-1}, and incubated at 37°C in an orbital shaker operating at 100 r.p.m. Inocula were prepared by washing and suspending organisms freshly grown on plates of deferrated yeast nitrogen base agar in 0.03 M-citrate- buffered saline, pH 5.

Siderophore production was maximal at the lowest iron concentration (0.026 μM) and decreased when iron concentration was increased from 0.026 to 0.8 μM (Fig. 22). A concentration of 0.8 μM $FeCl_3$ provided excess iron, since adding more $FeCl_3$ did not increase growth (Sweet and Douglas, 1991).

Figure 22.

Siderophore production by *C. albicans* 'Outbreak' strain grown in YNB medium containing different concentrations of iron.

Culture supernates were assayed for siderophores using the universal chemical assay of Schwyn and Neilands (1987). Data represent the mean results of two experiments done in duplicate ± SEM (except data for 0.026 μM iron, where results are means of four experiments done in duplicate)


Siderophore production
(% reduction in A630)
35
25
15
5
0.0
0.2
0.4
0.6
0.8
1.0
Iron concentration(μM)

3. Detection of hydroxamate-type siderophores in culture supernates

Siderophores can be classified chemically into two major categories: the hydroxamic acids and phenolates. Under controlled conditions *in vitro*, growth in an iron-deficient medium leads to an increased synthesis of hydroxamates by hydroxamate-producing microorganisms and phenolates by phenolate-producing microorganisms (Holzberg and Artis, 1983).

The method used to detect hydroxamate-type siderophores was based on that described by Holzberg and Artis (1983), and depends on the fact that oxidation of certain hydroxamic acids yields a material with a very strong absorption at 264 nm. Culture supernates of several *C. albicans* strains grown in the yeast form at 25°C and the hyphal form at 37°C in various liquid media (under normal iron concentrations) were examined for the presence of hydroxamate-type siderophores. Desferal (Ciba Laboratories) was used as positive control and catechol as a negative control.

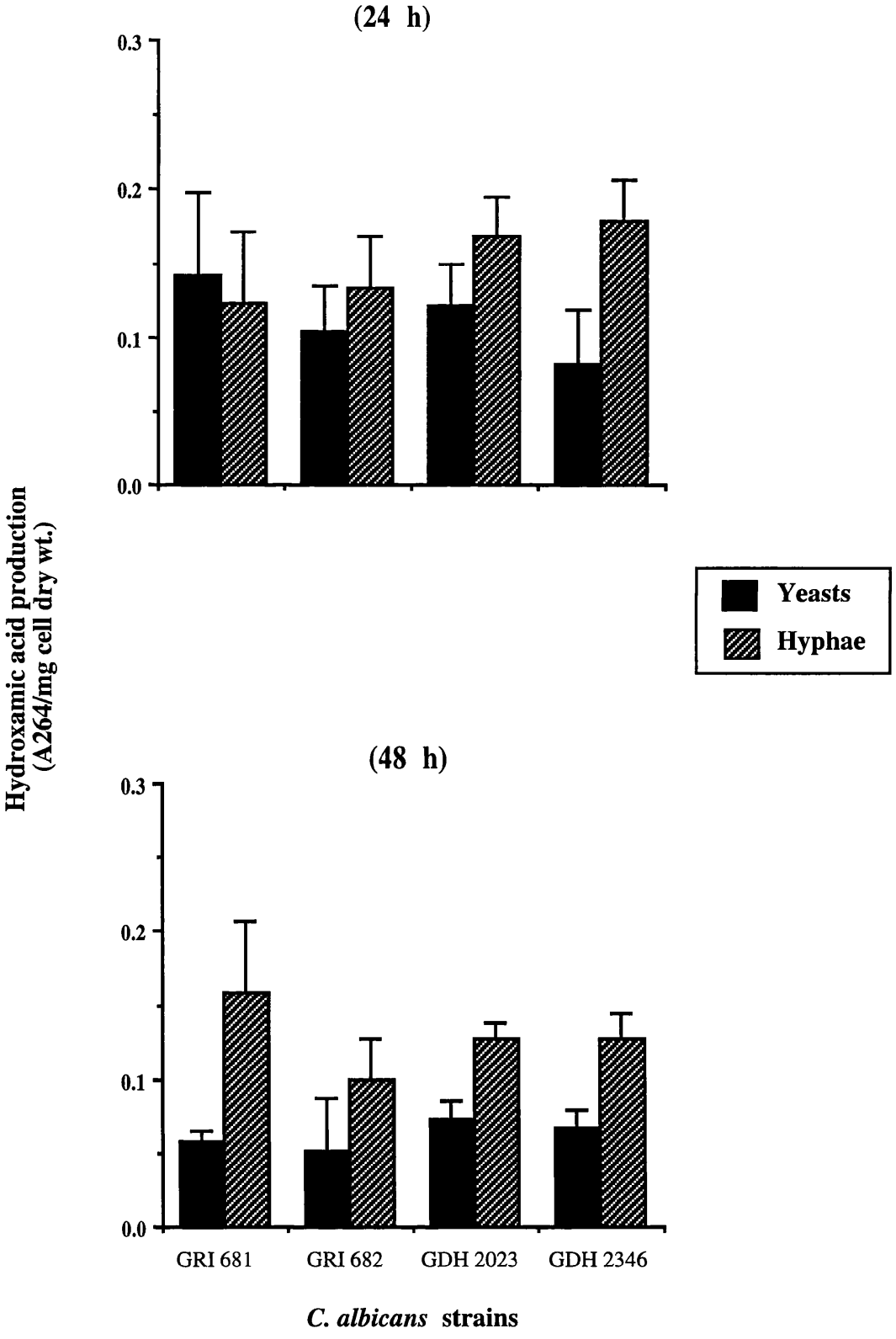
3.1. Hydroxamic acid production by *C. albicans* in glucose glycine broth

Culture supernates of four *C. albicans* strains (GRI 681, GRI 682, GDH 2023 and GDH 2346), grown in glucose glycine broth at 25°C (for yeast forms) and at 37°C (for hyphal forms) showed strong absorption at 264 nm in the assay for hydroxamates. When absorption readings were related to cell dry weight, hydroxamate production by hyphae appeared to be greater than that by yeast forms (Fig. 23). The technique of analysis of variance (see Appendix) was used to evaluate differences in hydroxamate production between the two morphological forms and also between different strains, incubation times and experiments. There was no significant difference between the four strains of *C. albicans* , 24 or 48 h incubation times and four replicate experiments. However, there was a highly significance difference between the results obtained with the two morphological forms, with a clear demonstration that the hyphal forms produce more

Figure 23.

Hydroxamic acid production by yeast and hyphal forms of *C.albicans* strains after incubation in glucose glycine broth at 25°C (yeasts) or 37°C (hyphae) for 24 and 48 h.

Data represent the mean results of two experiments done with five replicates (for 24h) or three replicates (for 48h) ± SEM


(24 h)
0.3
0.2
0.1
0.0
Hydroxamic acid production
(A264/mg cell dry wt.)
Yeasts
Hyphae
(48 h)
0.3
0.2
0.1
0.0
GRI 681
GRI 682
GDH 2023
GDH 2346
C. albicans strains

siderophores than yeast forms. The positive control, desferal showed a strong absorption of 0.529 at 264 nm and there was a weak absorption reading of 0.052 by the negative control, catechol.

3.2. Hydroxamic acid production by *C. albicans* in NYP medium and Lee's medium

The production of hydroxamic acids in other growth media was also investigated. *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 were grown at 25°C and 37°C in Lee's medium and NYP medium; both media provide cultures with a high percentage of hyphae (at 37°C) or pure yeast forms (at 25°C). Culture supernates were examined as before using the hydroxamate assay. The results (Table 15) confirmed that all four *C. albicans* strains tested do produce hydroxamate-type siderophores even in these different media. All the readings except one (strain GRI 682 in NYP medium at 25C°) were above 0.104 (2.0 x absorbance of negative control), which is considered positive. However, hydroxamate secretion appeared to be greater in Lee's medium than in NYP medium. No significant difference in hydroxamate production was noted between the two morphological forms of *C. albicans*.

3.3. Comparison of hydroxamic acid production by *C. albicans* wild-type strains and morphological mutants

C. albicans wild type strain 1001 and its mutants 1001-92' (HY^-) for yeast forms and 1001-FR(Y^-) for hyphal forms were used, together with *C. albicans* wild type strain ATTC 10261 and its mutant HOG 301 for hyphal forms. All of these strains were grown on four liquid media (NYP medium, glucose glycine broth, Lee's medium and YNB medium) and culture supernates were tested for presence of hydroxamates. Figure 24 shows hydroxamic acid production in glucose glycine broth relative to cell dry weight. The results indicate that all of these wild-type strains and mutants produce hydroxamate-type siderophores; there

Table 15.

Hydroxamate-type siderophore production by *C. albicans* grown in Lee's medium and NYP medium at 25°C (yeasts) and 37°C (hyphae).

C. albicans strain	Morphological form	Hydroxamate production (A_{264}) after growth in Lee's medium	Hydroxamate production (A_{264}) after growth in NYP medium
GRI 681	Yeasts	0.470	0.111
	Hyphae	0.376	0.133
GRI 682	Yeasts	0.453	0.099
	Hyphae	0.377	0.170
GDH 2023	Yeasts	0.266	0.114
	Hyphae	0.388	0.210
GDH 2346	Yeasts	0.382	0.172
	Hyphae	0.491	0.123

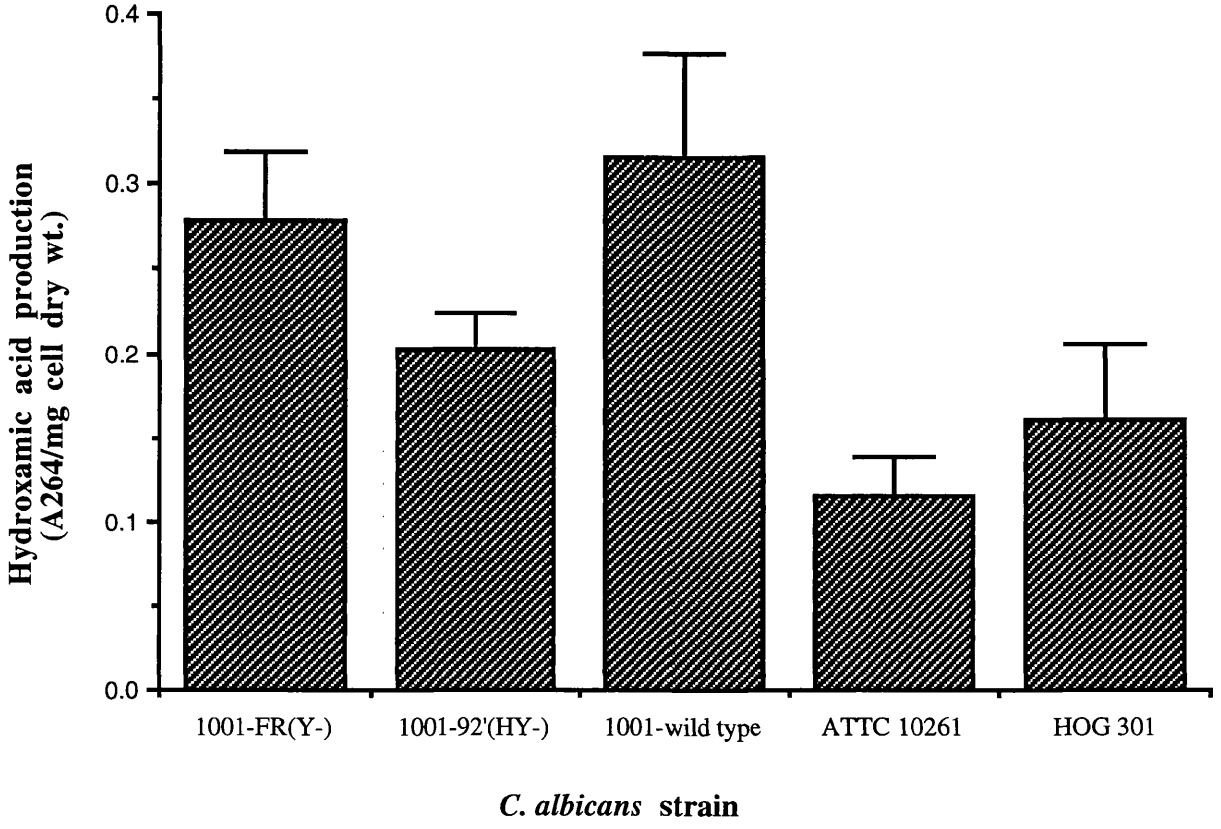
Positive control, Desferal (0.5 mM)	0.529
Negative control, Catechol (174 μM)	0.052

Hydroxamates absorb maximally at 264 nm. An absorbance of less than 0.104 (2.0 x absorbance of negative control, 174 μM catechol) was considered negative. Data represent means result of two experiments (single readings at 264 nm).

Figure 24.

Hydroxamic acid production by *C. albicans* wild-type strains and morphological mutants after growth in glucose glycine broth at 37°C for 24h.

Results shown are mean results of three experiments done in duplicate ± SEM


Hydroxamic acid production
(A264/mg cell dry wt.)
0.4
0.3
0.2
0.1
0.0
1001-FR(Y-)
1001-92'(HY-)
1001-wild type
ATTC 10261
HOG 301
C. albicans strain

was no clear correlation between hydroxamate production and morphological form.

Measurement of hydroxamate-type siderophores in three other media (Tables 16, 17 and 18) after incubation of cultures for up to 7 days also indicated that these wild-type strains and mutants of *C. albicans* produce the chelators. However, siderophore production was very low in these experiments.

4. Detection of phenolate-type siderophores in culture supernates

The second type of siderophore which is produced by some micro-organisms under iron-limitation is a phenolate. Phenolate-type siderophores were detected by a method based on that described by Arnow (1937), in which catechol gives a yellow colour when reacted with nitrous acid; this changes to an intense orange-red when the solution is made strongly basic (~pH 10). Phenolate derivatives absorb maximally at 515 nm. Catechol (174 μM) was used as a positive control and 0.5 mM desferal was used as a negative control.

4.1. Phenolate-type siderophore production by *C. albicans* in glucose glycine broth

Culture supernates of nine *C. albicans* strains grown in glucose glycine broth in the yeast and hyphal form were tested for presence of phenolate-type siderophores (Table 19). The positive control (174 μM catechol) showed an absorption of 0.374 at 515 nm and the negative control (0.5 mM desferal) gave a reading of 0.025. All nine strains produced readings of less than 0.050 (2.0 x absorbance of negative control), which was considered negative. These results indicate that the *C. albicans* strains tested produce only hydroxamate-type siderophores and not phenolate-type compounds.

Table 16. Hydroxamate-type siderophore production by *C. albicans* 1001 and its mutants in NYP medium at 37°C after different incubation periods.

	Hydroxamate production (A_{264}) after incubation for			
***C. albicans* strain**	**24 h**	**48 h**	**72 h**	**7 d**
1001-FR(Y^-)	0.016 ± 0.011	0.028 ± 0.011	0.019± 0.008	0.027
1001-92'(HY^-)	0.012 ± 0.005	0.025 ± 0.009	0.022 ± 0.011	0.024
1001-wild type	0.032 ± 0.010	0.031 ± 0.005	0.002 ± 0.0005	0.024

Table 17. Hydroxamate-type siderophore production by *C. albicans* 1001 and its mutants in Lee's medium at 37°C after different incubation periods.

	Hydroxamate production (A_{264}) after incubation for			
***C. albicans* strain**	**24 h**	**48 h**	**72 h**	**7 d**
1001-FR(Y^-)	0.023 ± 0.012	0.024 ± 0.009	0.035 ± 0.007	0.027
1001-92'(HY^-)	0.023 ± 0.005	0.055 ± 0.035	0.053 ± 0.028	0.048
1001-wild type	0.023 ± 0.013	0.056 ± 0.018	0.031 ± 0.017	0.124

Table 18. Hydroxamate-type siderophore production by *C. albicans* 1001 and its mutants in YNB medium at 37°C after different incubation periods.

	Hydroxamate production (A_{264}) after incubation for			
***C. albicans* strain**	**24 h**	**48 h**	**72 h**	**7 d**
1001-FR(Y^-)	0.044 ± 0.017	0.048 ± 0.004	0.066 ± 0.005	0.032
1001-92'(HY^-)	0.056 ± 0.016	0.047 ± 0.003	0.063 ± 0.007	0.032
1001-wild type	0.036 ± 0.002	0.045 ± 0.004	0.020	0.007

Positive control, Desferal (0.5 mM)	0.568
Negative control, Catechol (174 μM)	0.018

Hydroxamates absorb maximally at 264 nm. An absorbance of less than 0.036 (2.0 x absorbance of negative control, 174 μM catechol) was considered negative. Data represent the mean results of two experiments done in duplicate ± SEM

Table 19.

Phenolate-type siderophore production by *C. albicans* strains grown in glucose glycine broth.

C.albicans strain	Morphological form	Phenolate-type siderophore production (A_{515}) after incubation 24 h	48 h
GRI 681	Yeasts	0.007	0.011
	Hyphae	0.005	0.010
GRI 682	Yeasts	0.008	0.010
	Hyphae	0.007	0.015
GDH 2023	Yeasts	0.010	0.011
	Hyphae	0.005	0.005
GDH 2346	Yeasts	0.011	0.009
	Hyphae	0.015	0.017
1001-FR (Y^-)	Hyphae	0.013	0.021
1001-92' (HY^-)	Yeasts	0.007	0.022
1001-wild type	Yeasts	0.041	0.023
ATTC 10261	Yeasts	0.008	0.007
HOG 301	Hyphae	0.003	0.006

Positive control, Catechol (174 μM)	0.374
Negative control, Desferal (0.5 mM)	0.025

Data represent means of two experiments done in duplicate. An absorbance of less than 0.05 at 515 nm (2.0 x absorbance of negative control) was considered negative.

4.2. Phenolate-type siderophore production by *C. albicans* in NYP medium and Lee's medium

The presence of phenolate derivatives in culture supernates was also investigated using four *C. albicans* strains grown as yeast forms at 25°C and as hyphal forms at 37°C in Lee's medium and NYP medium. Both media permit the growth of cultures with a high percentage of hyphae (at 37°C) and pure yeast forms (at 25°C). Culture supernates were examined using the Arnow method and compared with positive and negative controls. The results (Table 20) confirm that all *C. albicans* strains tested do not produce phenolate-type siderophores.

4.3. Comparison of phenolate production by *C. albicans* 1001 and its morphological mutants

Additional studies on possible phenolate production were carried out with *C. albicans* wild-type strain 1001 and its mutants 1001-92' (HY^-) for yeast forms and 1001-FR(Y^-) for hyphal forms. They were grown in three liquid media (NYP medium, Lee's medium and YNB medium) at 37°C for 24 h, 48 h, 72 h, and 7 days with the normal iron concentration. Culture supernates were collected and examined for the presence of phenolate-type siderophores by the Arnow method. The results (Tables 21, 22 and 23) were all negative; all absorbance values recorded were lower than 0.05 (twice that of the negative control). These experiments further support the conclusion that *C. albicans* produces only one type of siderophore.

Table 20.

Phenolate-type siderophore production by *C. albicans* grown in Lee's medium and NYP medium in the yeast form at 25°C and the hyphal form at 37°C.

C. albicans strains	Morphological form	Phenolate-type siderophore production (A_{515}) after growth in Lee's medium	Phenolate-type siderophore production (A_{515}) after growth in NYP medium
GRI 681	Yeasts	0.018 ± 0.004	0.032 ± 0.015
	Hyphae	0.011 ± 0.002	0.022 ± 0.004
GRI 682	Yeasts	0.015 ± 0.005	0.011 ± 0.005
	Hyphae	0.012 ± 0.002	0.009 ± 0.002
GDH 2023	Yeasts	0.010 ± 0.003	0.028 ± 0.006
	Hyphae	0.012 ± 0.001	0.011 ± 0.004
GDH 2346	Yeasts	0.018 ± 0.004	0.022 ± 0.005
	Hyphae	0.009 ± 0.004	0.022 ± 0.007

Data represent the mean results of two experiments done in duplicate ± SEM. An absorbance of less than 0.05 at 515 nm (2.0 x absorbance of negative control, 0.5 mM desferal) was considered negative.

Table 21. Phenolate-type siderophore production by *C. albicans* 1001 and its mutants in NYP medium at 37°C after different incubation periods.

	Phenolate-type siderophore production (A_{515}) after incubation for			
***C. albicans* strain**	**24 h**	**48 h**	**72 h**	**7 d**
1001-FR(Y^-)	0.006± 0.003	0.002 ±0.002	0.001± 0.001	0
1001-92'(HY^-)	0.002± 0.002	0.003± 0.002	0.001± 0.001	0
1001-wild type	0.004± 0.002	0.006± 0.003	0.003± 0.001	0.002±0.001

Table 22. Phenolate- type siderophore production by *C. albicans* 1001 and its mutants in Lee's medium at 37°C after different incubation periods.

	Phenolate-type siderophore production (A_{515}) after incubation for			
***C. albicans* strain**	**24 h**	**48 h**	**72 h**	**7 d**
1001-FR(Y^-)	0.004± 0.002	0.004± 0.002	0.003 ±0.001	0.004± 0.002
1001-92'(HY^-)	0.017± 0.006	0.016± 0.002	0.009± 0.002	0.007± 0.001
1001-wild type	0.015± 0.005	0.013± 0.002	0.009± 0.002	0.009± 0.002

Table 23. Phenolate- type siderophore production by *C. albicans* 1001 and its mutants in YNB medium at 37°C after different incubation periods.

	Phenolate-type siderophore production (A_{515}) after incubation for			
***C. albicans* strain**	**24 h**	**48 h**	**72 h**	**7 d**
1001-FR(Y^-)	0.015± 0.005	0.017± 0.009	0.017± 0.010	0.010± 0.003
1001-92'(HY^-)	0.009± 0.003	0.010± 0.005	0.004± 0.002	0.005± 0.001
1001-wild type	0.013± 0.006	0.030± 0.008	0.004± 0.001	0.011± 0.002

Positive control, Catechol (174 μM)	0.374
Negative control, Desferal (0.5 mM)	0.025

Data represent means of two experiments done in duplicate ± SEM. An absorbance of less than 0.05 at 515 nm (2.0 x absorbance of negative control) was considered negative.

5. Determination of siderophores by non-specific and specific assays: control experiments

5.1. Standardization of assays using desferal and catechol

5.1.1 Determination of desferal using the universal chemical assay

The objective here was to test the universal chemical assay (Schwyn and Neilands, 1987), which was used for the non-specific detection of siderophores in solution. Desferal solutions (0.1-1.0 mM desferal in deionized distilled water) were prepared in acid-washed universal bottles. A portion (0.5 ml) of each solution was added to 0.5 ml CAS solution. The iron dye complex is blue; when a strong chelator removes the iron from the dye, its colour turns from blue to pink. A strong pink colour was noted with all desferal concentrations; an intense colour appeared with high concentrations. Absorbance readings at 630 nm were taken after 5 minutes and one hour. When the percentage reduction in absorbance was plotted against concentration (Fig. 25), a straight line was obtained. The concentration of desferal (0.5 mM) which was used as a positive control in experiments with culture supernates gave a percentage reduction of 22.7 (Fig. 25).

5.1.2 Determination of desferal using the hydroxamate assay

The purpose of these experiments was to test the specific hydroxamate assay with solutions containing different concentrations of desferal, the positive control. Desferal solutions of 0.1-1.0 mM in deionized, distilled water were used. When absorbance at 264 nm was plotted against desferal concentration, a straight line was obtained (Fig. 26). The concentration of desferal (0.5 mM) which was used a positive control in experiments with culture supernates gave an absorbance of 0.45 (Fig. 26).

Figure 25.

Determination of desferal (desferrioxamine mesylate) using the universal chemical assay (Schwyn and Neilands method).

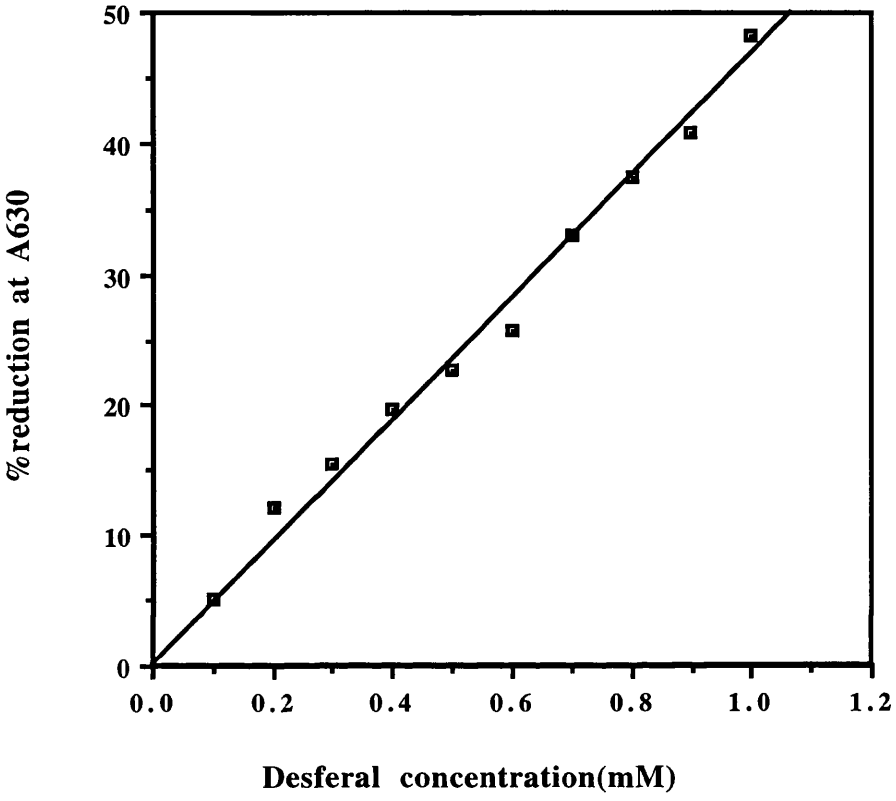
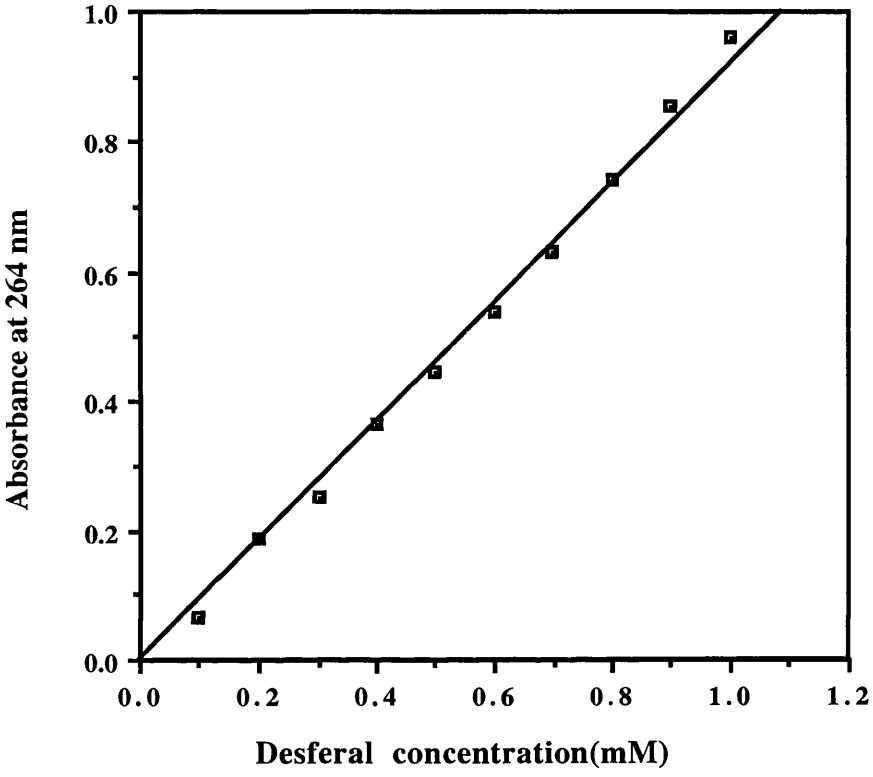

50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
% reduction at A630
Desferal concentration(mM)

Figure 26.

Determination of desferal (desferrioxamine mesylate) using the hydroxamate assay (Holzberg and Artis, 1983).


Absorbance at 264 nm
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Desferal concentration(mM)

5.1.3 Determination of catechol using the Arnow assay for phenolates

In these experiments, the Arnow assay was tested with solutions containing different concentration of catechol. With the Arnow (1937) method, catechol gives a yellow colour when treated with nitrous acid; this changes to an intense orange-red when the solution is made strongly basic (~ pH 10). Phenolate derivatives absorb maximally at 515 nm. All catechol solutions (50-500 μM made up in deionized, distilled water) gave a strong change in colour from yellow to orange-red; a more intense colour appeared at higher concentrations. When absorbance at 515 nm was plotted against concentration, a straight line was obtained. The concentration of catechol (147 μM), which was used as a positive control in experiments with culture supernates gave an absorbance of 0.37 (Fig. 27).

5.2. Interference by medium components in the chemical assays

The aim of these experiments was to establish whether siderophore assays with culture supernates could be affected by interference from medium components.

5.2.1 Effect of Lee's medium, NYP medium and glucose glycine broth on the universal chemical assay

Various desferal concentrations (0.1-1.0 mM) were added to sterile Lee's medium, NYP medium, and glucose glycine broth; 0.5 ml medium was then added to 0.5 ml CAS solution. Absorbance at 630 nm was measured at five minutes and one hour, and the percentage reduction in absorbance was determined as previously (Fig. 28). Figure 28B shows a high level of interference between the chemicals which compose Lee's medium and the universal chemical assay; the linear relationship between percentage reduction in A_{630} and desferal concentration is completely destroyed. With the other two media no interference was noted, both plots (Figs. 28A and 28C) being straight lines. Addition of these

Figure 27.

Determination of catechol using the Arnow assay for phenolates.

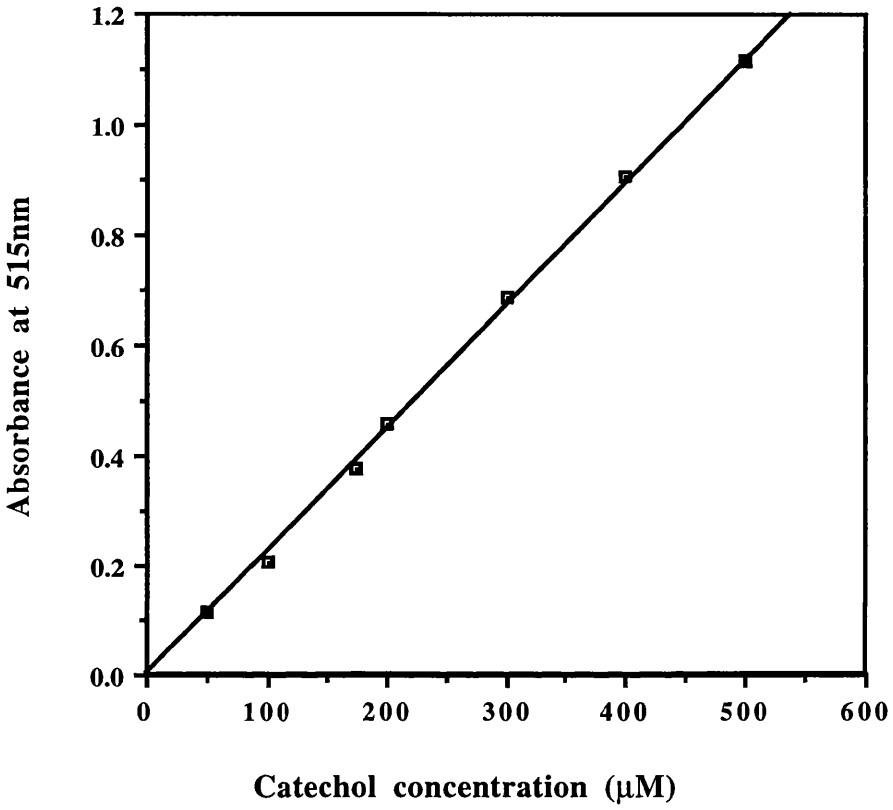

Absorbance at 515nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
600
Catechol concentration (μM)

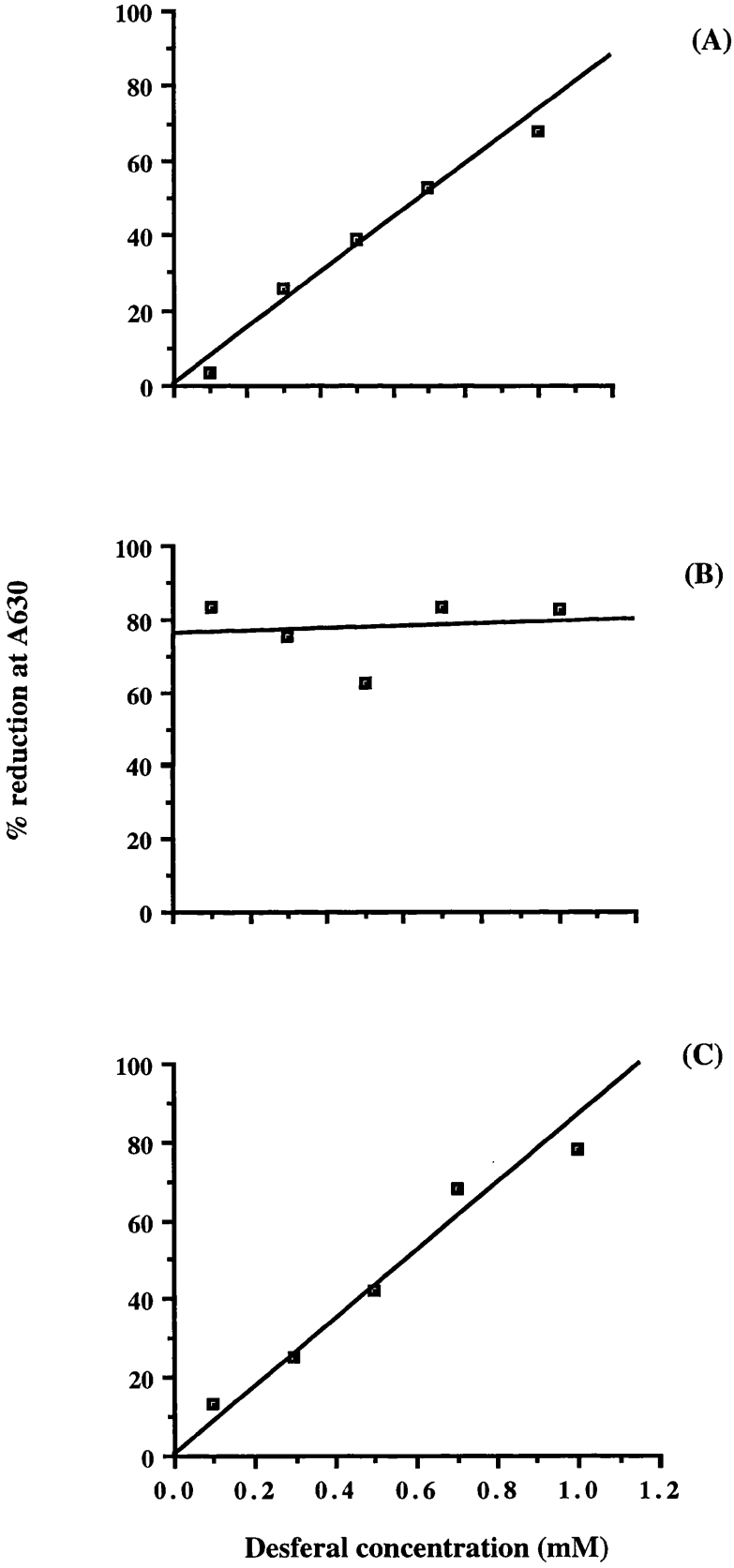
Figure 28.

Effect of three media on the universal chemical assay for siderophores.

(A) NYP medium

(B) Lee's medium

(C) Glucose glycine broth


(A)
(B)
(C)
100
80
60
40
20
0
% reduction at A630
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Desferal concentration (mM)

media (NYP medium and glucose glycine broth) appeared to enhance slightly the colour change from blue to pink (Figs. 25, 28).

5.2.2 Effect of Lee's medium, NYP medium and glucose glycine broth on the hydroxamate assay .

The three media were supplemented with various concentrations of desferal (0.1-1.0 mM), and subjected to the hydroxamate assay. The results for all three media indicated a linear relationship between absorbance at 264 nm and desferal concentration (Fig. 29). These was no evidence of interference in any of these assays.

5.2.3 Effect of Lee's medium, NYP medium and glucose glycine broth on the phenolate assay .

Media were supplemented with various concentration of catechol (100-500 μM) and 1 ml samples were tested by the Arnow method for phenolates. All media tested showed a yellow colour when treated with nitrous acid and this changed to an intense orange-red when the solution was made strongly basic (~pH 10). There was a linear relationship between absorbance at 515 nm and catechol concentration for all three media tested (Fig. 30) indicating that none of them (Lee's medium, NYP medium or glucose glycine broth) interferes with the phenolate assay.

5.3. Determination of cell dry weight of *C. albicans*

In some experiments, siderophore production by *C. albicans* was related to cell dry weight. The method used to determine cell dry weight was that described by Sweet and Douglas (1991a). Organisms from portions (10 ml) of 24 or 48h cultures were collected on pre-weighed cellulose nitrate filters (0.45μm pore size; 25 mm diameter; Whatman) and given three washes with distilled water

Figure 29.

Effect of three media on the hydroxamic acid assay for siderophores.

(A) Lee's medium

(B) Glucose glycine broth

(C) NYP medium

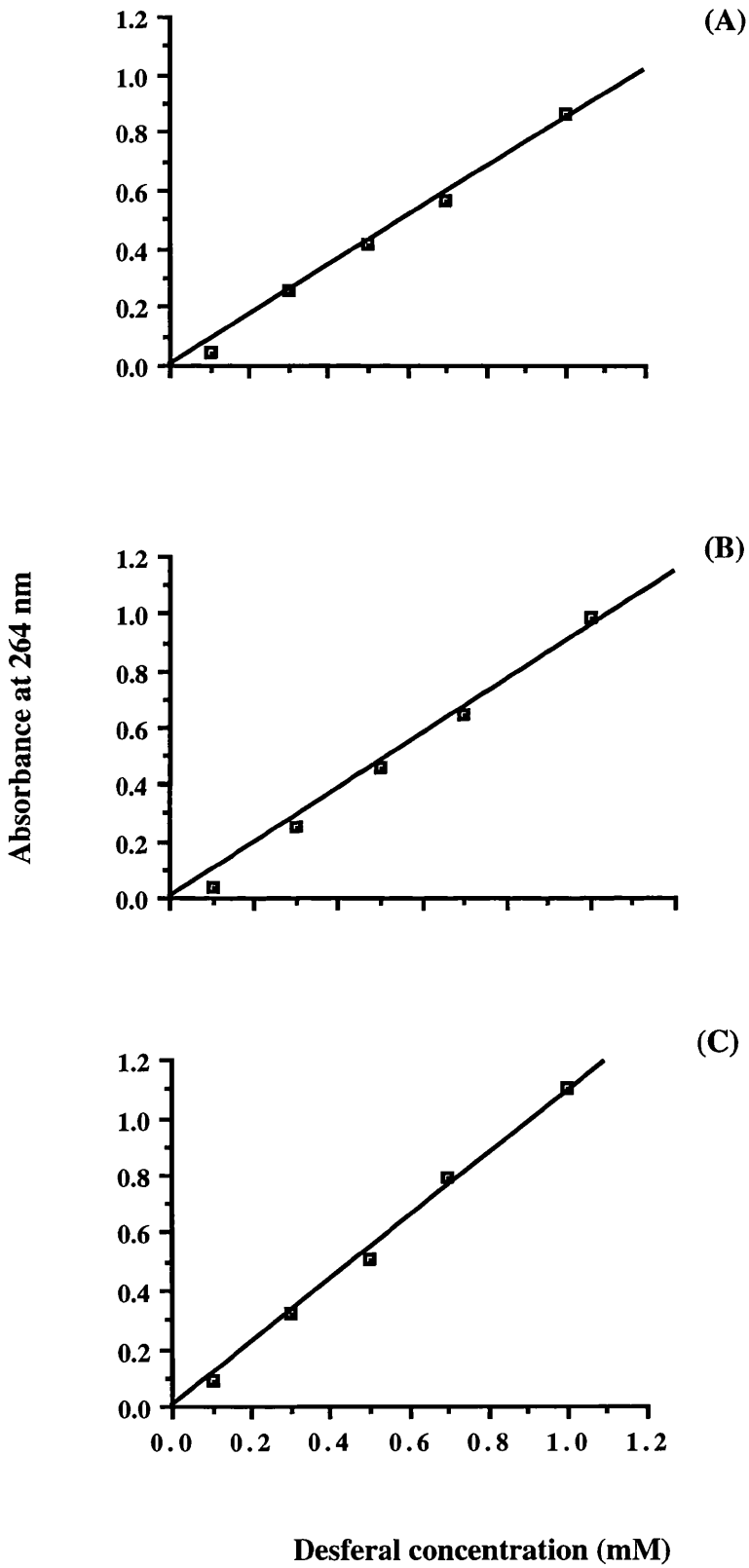

(A)
(B)
(C)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Absorbance at 264 nm
Desferal concentration (mM)

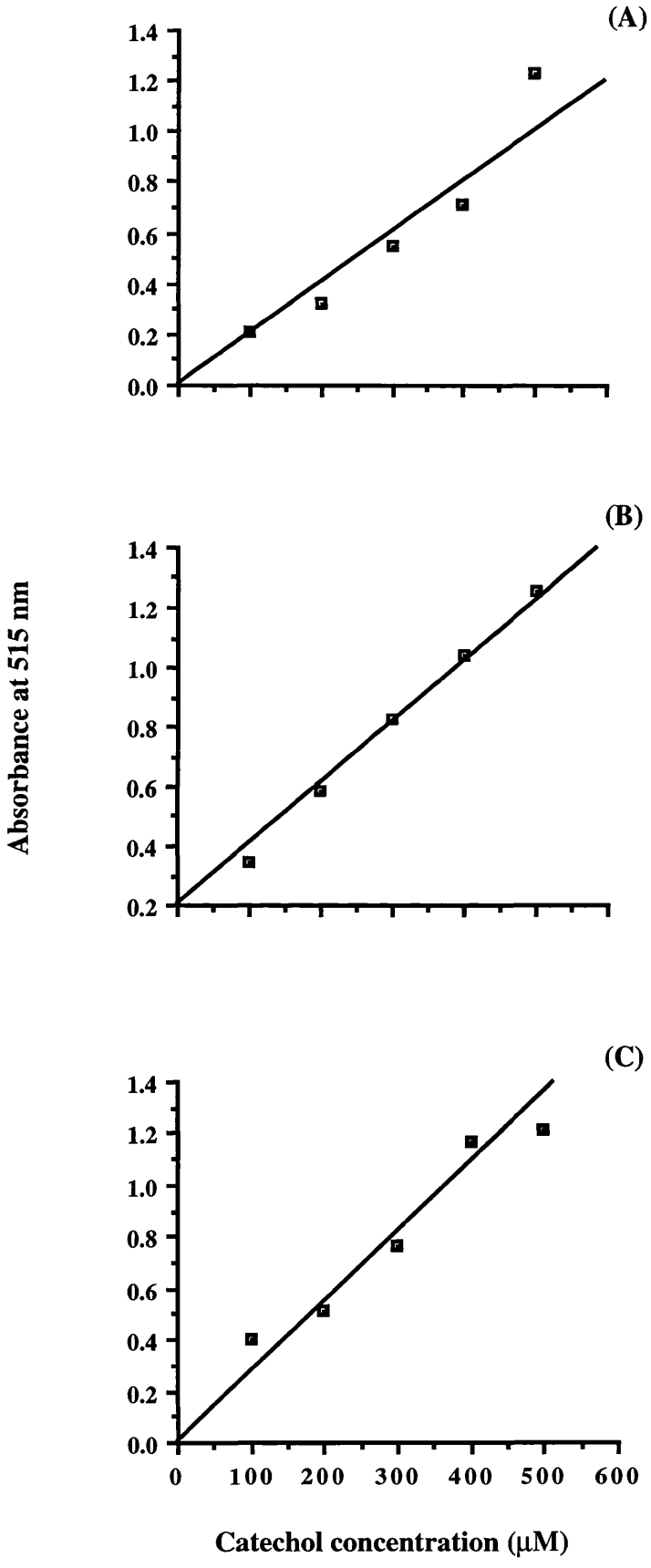
Figure 30.

Effect of three media on the phenolate-type siderophore assay (Arnow method).

(A) NYP medium

(B) Glucose glycine broth

(C) Lee's medium


(A)
(B)
(C)
Absorbance at 515 nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
600
Catechol concentration (μM)

(5ml). The filters were dried to constant weight at 80°C and the cell dry weight per ml of culture calculated.

5.3.1 Determination of cell dry weight of *C. albicans* as hyphae in glucose glycine broth

Four *C. albicans* strains (GRI 681, GRI 682, GDH 2023, and GDH 2346) were grown in glucose glycine broth for 24 and 48 hours at 37°C to produce hyphal cultures. Sample of each (0.5, 1, 1.5, 2, and 2.5 ml volumes) were then collected on filters, and washed, dried and weighed. The results (Fig. 31) show a linear relationship between cell dry weight and culture volume for each strain tested.

5.3.2 Determination of cell dry weight of *C. albicans* as yeasts in glucose glycine broth

C. albicans strains (GRI 681, GRI 682, GDH 2023, and GDH 2346) were grown in glucose glycine broth for 24 and 48 hours at 25°C to produce yeast-form cultures. Samples of each (0.5, 1, 1.5, 2, and 2.5 ml volume) were filtered, washed dried and weighed (Fig. 32). By using analysis of variance on results obtained with both yeasts and hyphae, a highly significant difference was demonstrated between the two morphologies (F -value > P=1%). However, no differences were found between the four strains and two incubation times (see Appendix).

5.3.3 Comparison of cell dry weight determinations of yeast and hyphal forms in Lee's medium, NYP medium and glucose glycine broth

Four *C. albicans* strains (GRI 681, GRI 682, GDH 2023, and GDH 2346) were grown in the three media for 24h at 25°C to produce yeast cultures and 37°C to produce hyphal cultures. Samples (10 ml) were then collected on filters, and washed, dried, weighed and the cell dry weight per ml of culture calculated. The

Figure 31.

Cell dry weight determinations with *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 grown in glucose glycine broth at 37°C as hyphae for 48 h.

Results are means from one experiment done in duplicate

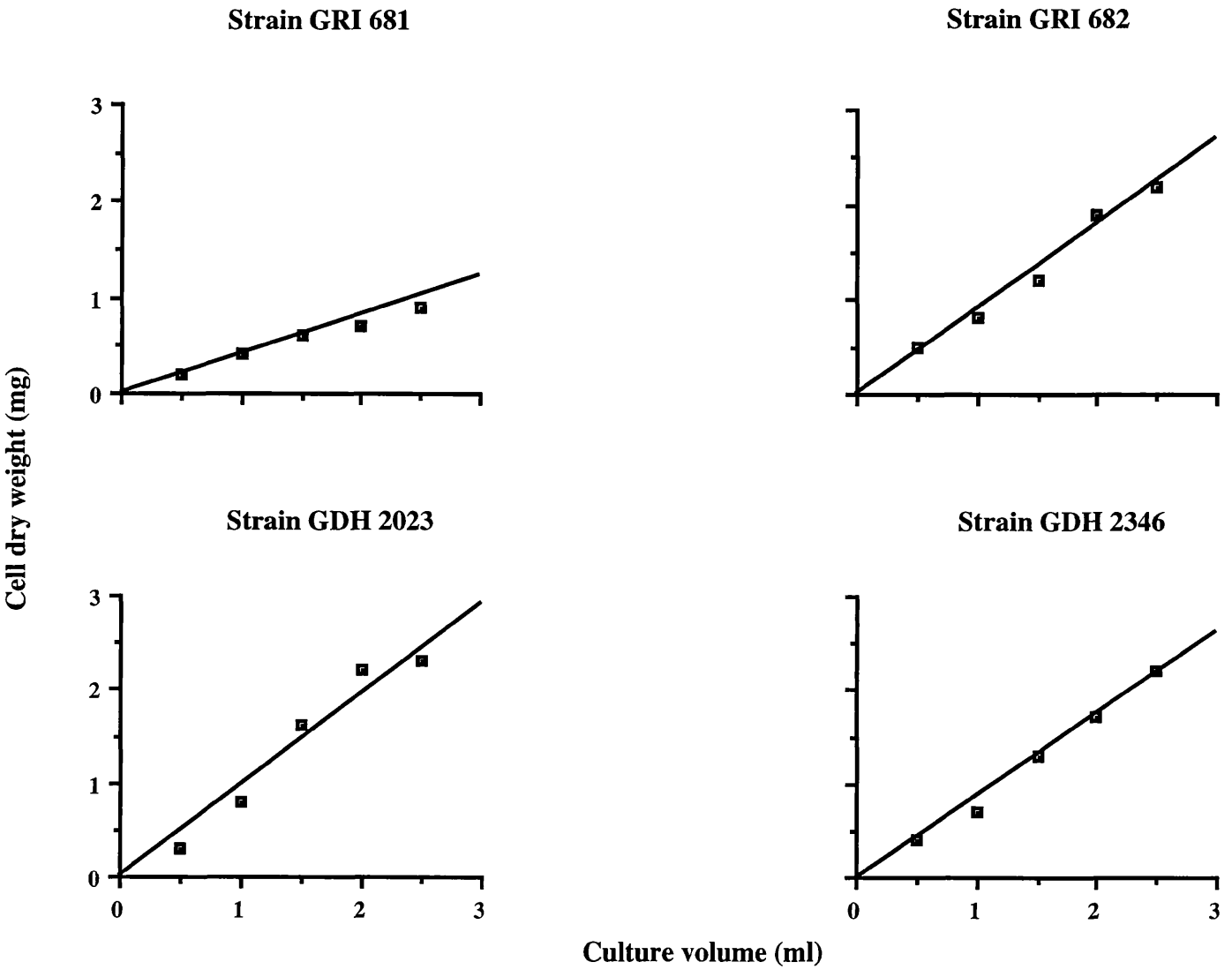
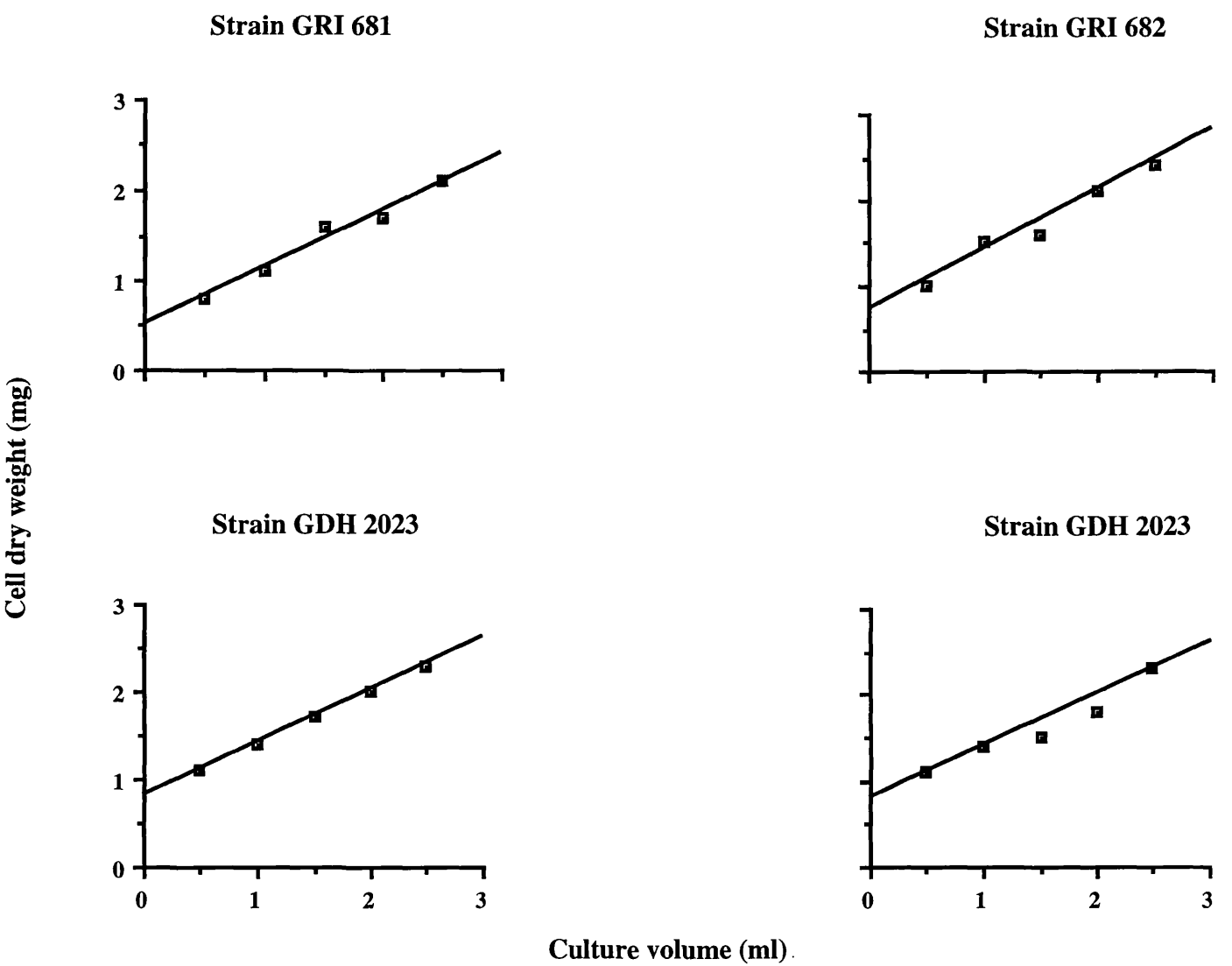

Strain GRI 681
Strain GRI 682
Strain GDH 2023
Strain GDH 2346
Cell dry weight (mg)
Culture volume (ml)
0
1
2
3

Figure 32.

Cell dry weight of *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 grown in glucose glycine broth at 25°C as yeast forms for 48 h.

Results are means from one experiment done in duplicate


Strain GRI 681
Strain GRI 682
Strain GDH 2023
Strain GDH 2023
Cell dry weight (mg)
Culture volume (ml)
0
1
2
3

results (Fig. 33) show high values for cell dry weight in Lee's medium compared with the other two media; NYP medium (Fig. 33d) gave very low readings.

6. Green pigment production by yeast and hyphal forms of *Candida albicans*

Synthesis of a green pigment by *C. albicans* was first described in 1940 by Jones and Peck, who used it as an additional means of differentiating *Candida stellatoidea* from *C. albicans*. They observed a greenish zone around colonies on blood agar. The first report of green pigment production in liquid media was by McCourtie and Douglas (1985) during experiments involving prolonged incubation in medium containing 500 mM galactose. In the present study green pigment synthesis by yeast and hyphal forms of *C. albicans* was investigated; three liquid media were used.

Pigment production was determined by the method of Sweet and Douglas (1991) in which the absorbance of culture supernates at 444nm, the absorption maximum of the pigment, is measured. Blanks consisted of uninoculated medium. Although largely negligible, cells or cell debris not removed from the supernates occasionally gave a measurable absorbance at 444nm. Therefore, A_{520} values were subtracted from the values measured at 444 nm since cells and cell debris gave similar absorbances at 520 and 444 nm, whereas the green pigment showed no absorbance at 520 nm.

6.1. Green pigment production by *C. albicans* in NYP medium

Four strains of *C. albicans* were grown in NYP medium for 24h at 37°C for hyphal forms and at 25°C for yeast forms. When the amount of pigment synthesised by each morphological form was related to the cell dry weight, it was clear that yeast forms produce more pigment than hyphal forms (Fig. 34). No pigment was detected for strain GRI 681 after 24h when grown as hyphae but this strain did produce pigment (A_{444}/mg cell dry wt. = 0.134) when grown as yeasts.

Figure 33.

Cell dry weight determinations with *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 grown in various media at 25°C (yeasts) or 37°C (hyphae).

(A) After incubation in glucose glycine broth for 24h

(B) After incubation in glucose glycine broth for 48h

(C) After incubation in Lee's medium for 24h

(D) After incubation in NYP medium for 24h

Data in A and B represent the mean results of two experiments done in triplicate ± SEM

Data in C and D represent the mean results of two experiments done in duplicate ± SEM

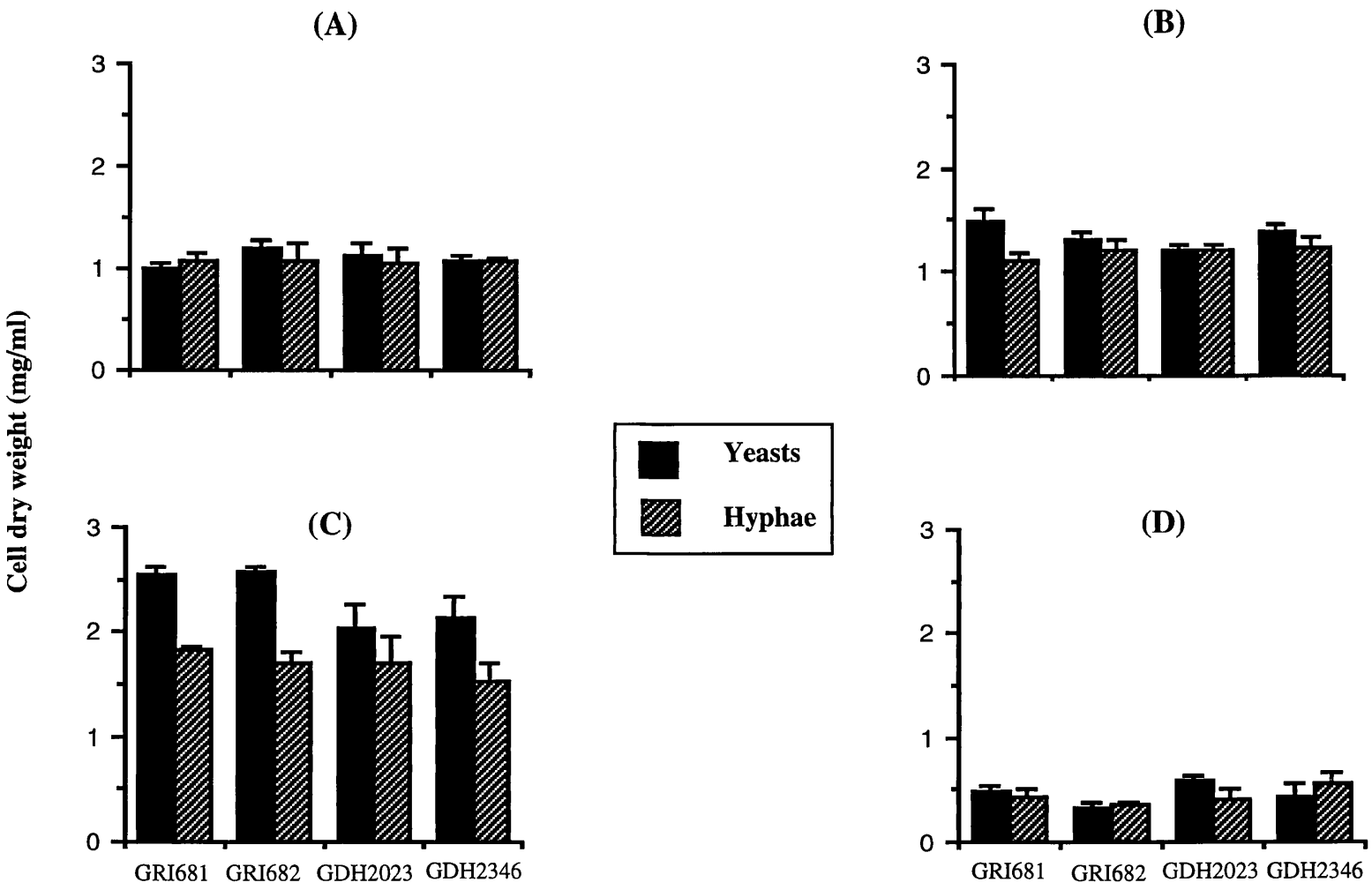
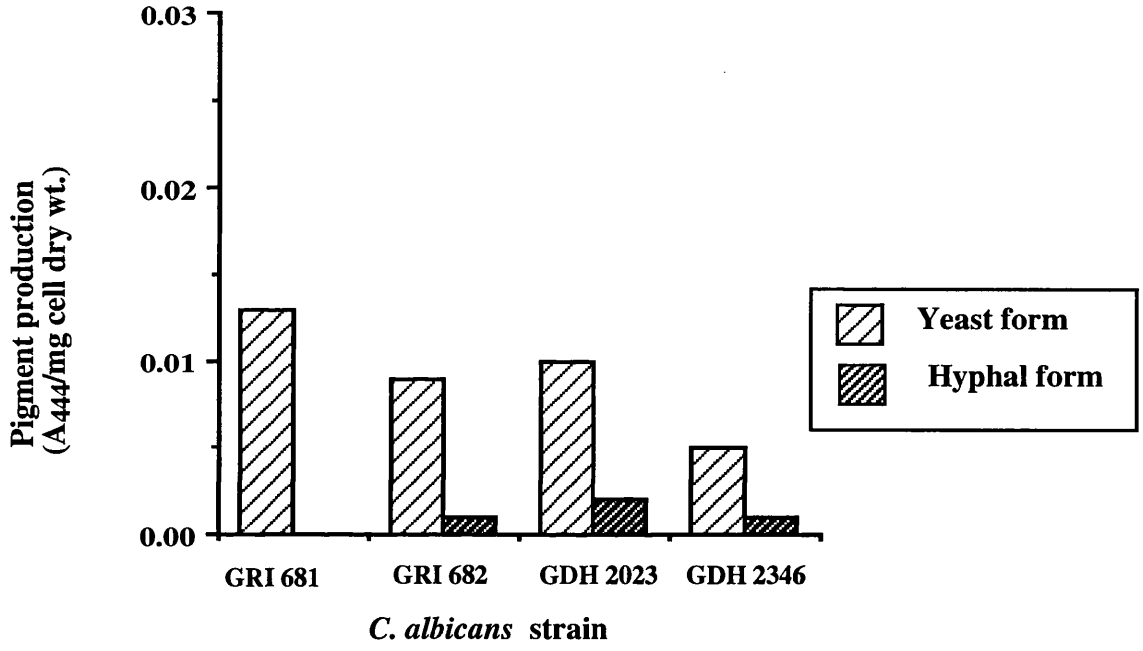

(A)
(B)
(C)
(D)
Cell dry weight (mg/ml)
Yeasts
Hyphae
0
1
2
3
GRI681
GRI682
GDH2023
GDH2346
C. albicans strain

Figure 34.

Green pigment production by *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 after 24h in NYP medium at 25˚C for yeast forms or 37˚C for hyphal forms .


0.03
0.02
0.01
0.00
Pigment production
(A444/mg cell dry wt.)
Yeast form
Hyphal form
GRI 681
GRI 682
GDH 2023
GDH 2346
C. albicans strain

The highest amount of pigment production was observed with strain GDH 2346 (A_{444}/mg cell dry wt. = 0.168).

6.2. Green pigment production by *C. albicans* in Lee's medium

Lee's medium was also used to detect green pigment production by four strains of *C. albicans* grown as yeasts or hyphae. The results, shown in Figure 35, indicate that, as before, yeast forms of *C. albicans* produced more green pigment than hyphal forms although pigment production in the medium was much lower overall. Again, strain GRI 681 produced pigment in the yeast form but not in the hyphal form. The highest amount of pigment production was also noted with this strain (A_{444}/mg cell dry wt. = 0.013).

6.3. Green pigment production by *C. albicans* in glucose glycine broth

Glucose glycine broth, which can be used for the production of pure yeast cultures or for cultures with a very high percentage of hyphae, was also tested in experiments on pigment synthesis by both morphological forms after incubation for 24h. Figure 36 shows that yeasts and hyphae of all strains produced the green pigment in this medium but the amount varied from one strain to another. In contrast to the earlier findings, only strain GDH 2023 showed greater production by yeasts than hyphae; all other strains gave the opposite result. The greatest amount of pigment synthesis was observed with the hyphal form of strain GDH 2346 (A_{444}/mg cell dry wt. = 0.024), and the least was with the hyphal form of strain GDH 2023 (A_{444}/mg cell dry wt. = 0.009).

6.4. Green pigment production by morphological mutants of *C. albicans*

All of the morphological mutants tested, as well as the corresponding wild-type strains, were capable of producing the green pigment. Figure 37 shows green pigment production in glucose glycine broth at 37°C after 24 hours incubation. Wild-type strain ATTC 10261 and its mutant HOG 301 for hyphal

Figure 35.

Green pigment production by *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 after 24h in Lee's medium at 25˚C for yeast forms or 37˚C for hyphal forms.

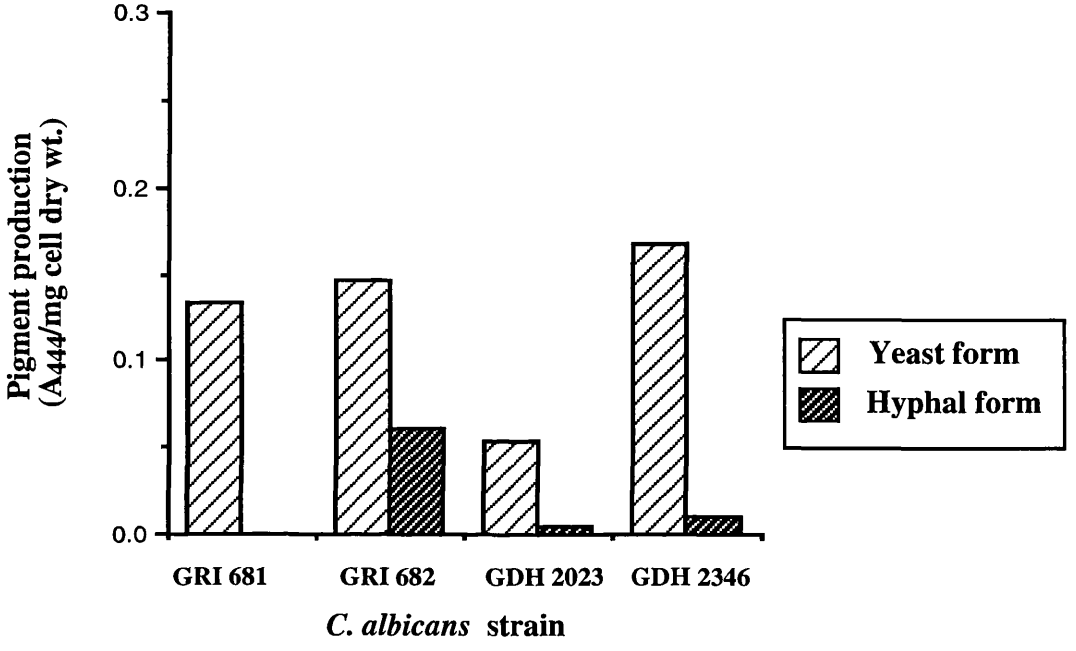

Pigment production
(A444/mg cell dry wt.)
0.3
0.2
0.1
0.0
GRI 681
GRI 682
GDH 2023
GDH 2346
C. albicans strain
Yeast form
Hyphal form

Figure 36.

Green pigment production by *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 after 24h in glucose glycine broth at 25˚C for yeast forms or 37˚C for hyphal forms.

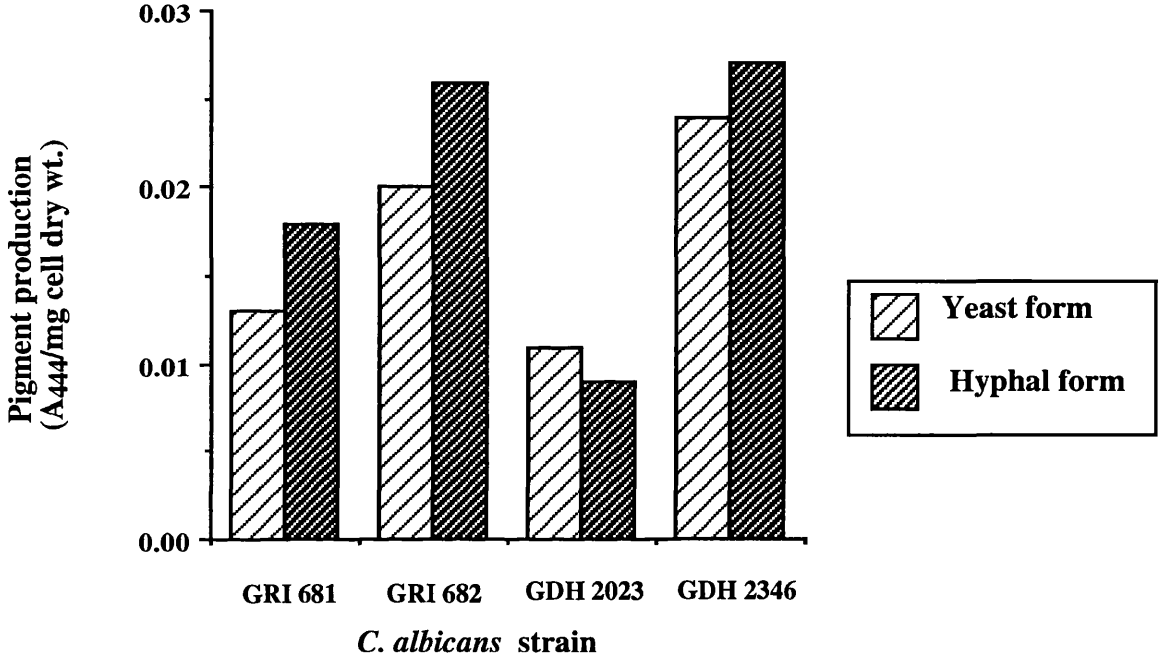
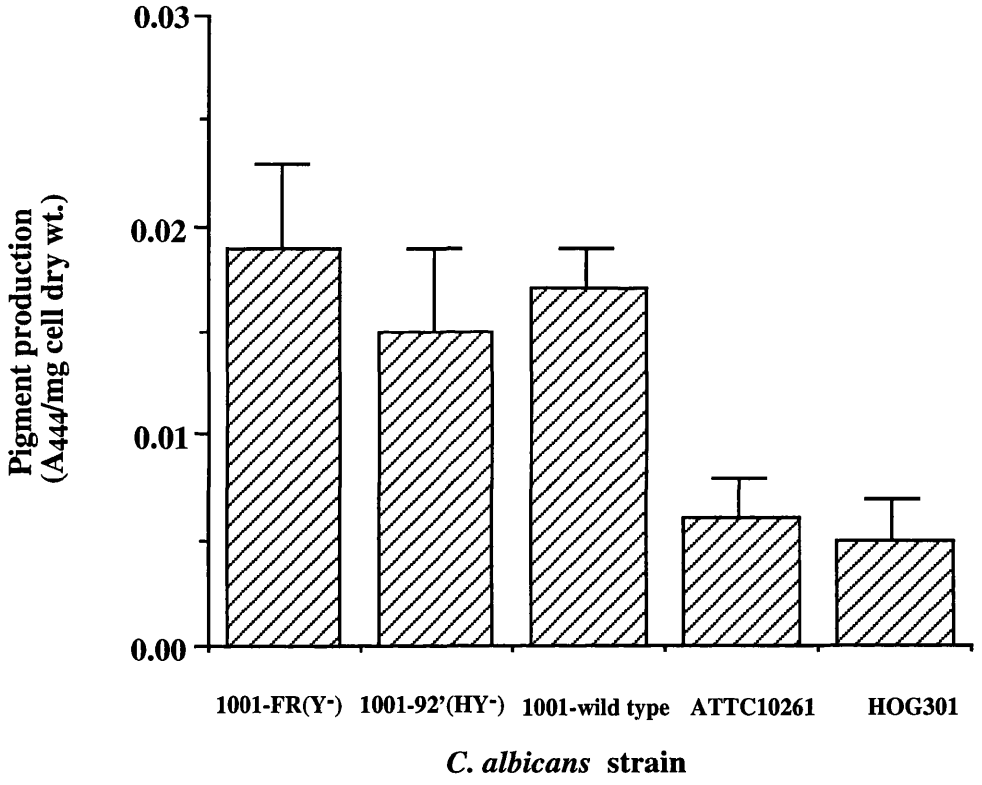

Pigment production
(A444/mg cell dry wt.)
0.03
0.02
0.01
0.00
GRI 681
GRI 682
GDH 2023
GDH 2346
C. albicans strain
Yeast form
Hyphal form

Figure 37.

Green pigment production by mutant and wild-type strains of *C. albicans* after incubation in glucose glycine broth at 37°C for 24 hours.

Data are means of two experiments done in duplicate and are expressed as A444/mg cell dry wt.


0.03
0.02
0.01
0.00
Pigment production
(A444/mg cell dry wt.)
1001-FR(Y-)
1001-92'(HY-)
1001-wild type
ATTC10261
HOG301
C. albicans strain

forms, gave less than half the pigment production of the other strains. With strain 1001, differences in pigment production between the wild type and either the hyphal-form mutant (1001-FR Y^-) or the yeast-form mutant (1001-92' HY^-) were relatively slight.

Pigment production in other media was investigated using only strain 1001 and its mutants, which were grown at 37°C for 24 h, 48 h and 7 days. Tables 24, 25 and 26 indicate that the three strains tested were capable of producing the green pigment in Lee's medium, YNB medium and NYP medium; strains 1001 (wild type) and 1001-92' (HY^-) showed more pigment production after incubation for 48h than after 24 h or 7 days.

Additional studies were carried out on green pigment production in four media by strain 1001 and its mutants. The organisms were grown at 37°C for 72h and the experiment was done three times in duplicate. Figure 38 indicates that the wild-type strain and its mutants produce pigment in all four media. However, the amount of pigment detected in Lee's medium was notably higher than in the other three.

6.5. Green pigment production on blood agar plates.

In 1940, Jones and Peck noted green pigment around colonies of *C. albicans* on blood agar and used this as an additional means of differentiating *Candida stellatoidea* from *C. albicans.* To investigate this phenomenon in the present study, nine *C. albicans* strains were inoculated via a straight wire on to Sabouraud dextrose blood agar and the plates were incubated at 37°C for 24 h. A grey-green zone was noted around the colonies of all nine strains tested, with a thickness of approximately 0.1-0.2 cm (Figs. 39, 40 and 41). Particularly good growth and big colonies were observed with strains 1001-wild type, 1001-92' (HY^-) and HOG 301 on Sabouraud dextrose blood agar (Table 27). Pure yeast-form colonies were obtained with all strains except for the mutants 1001-FR (Y^-) and HOG 301, which produced 48 and 81 % hyphae respectively.

Table 24. Green pigment production by *C. albicans* 1001 and its mutants in NYP medium at 37°C for different incubation periods.

(Data represent the means of A444 values from one experiment done in duplicate).

C. albicans strain	24 h	48 h	7 d
1001-wild type	0.003	0.011	0.003
1001-FR(Y^-)	0.001	0	0.002
1001-92'(HY^-)	0	0.015	0.005

Table 25. Green pigment production by *C. albicans* 1001 and its mutants in Lee's medium at 37°C for different incubation periods.

(Data represent the means of A444 values from one experiment done in duplicate).

C. albicans strain	24 h	48 h	7 d
1001-wild type	0.007	0.019	0.018
1001-FR(Y^-)	0.003	0	0.018
1001-92'(HY^-)	0.008	0.044	0.014

Table 26. Green pigment production by *C. albicans* 1001 and its mutants in YNB medium at 37°C for different incubation periods.

(Data represent the means of A444 values from one experiment done in duplicate).

C. albicans strain	24 h	48 h	7 d
1001-wild type	0.014	0.085	0.007
1001-FR(Y^-)	0.014	0.015	0.020
1001-92'(HY^-)	0.012	0.058	0.009

Figure 38.

Green pigment production by *C. albicans* 1001 and its mutants 1001-FR(Y-) for hyphal forms and 1001-92'(HY-) for yeast forms after incubation in four liquid media at 37°C for 72 h.

Data represent the means of A_{444} values ($\pm$ SEM) from three experiments done in duplicate

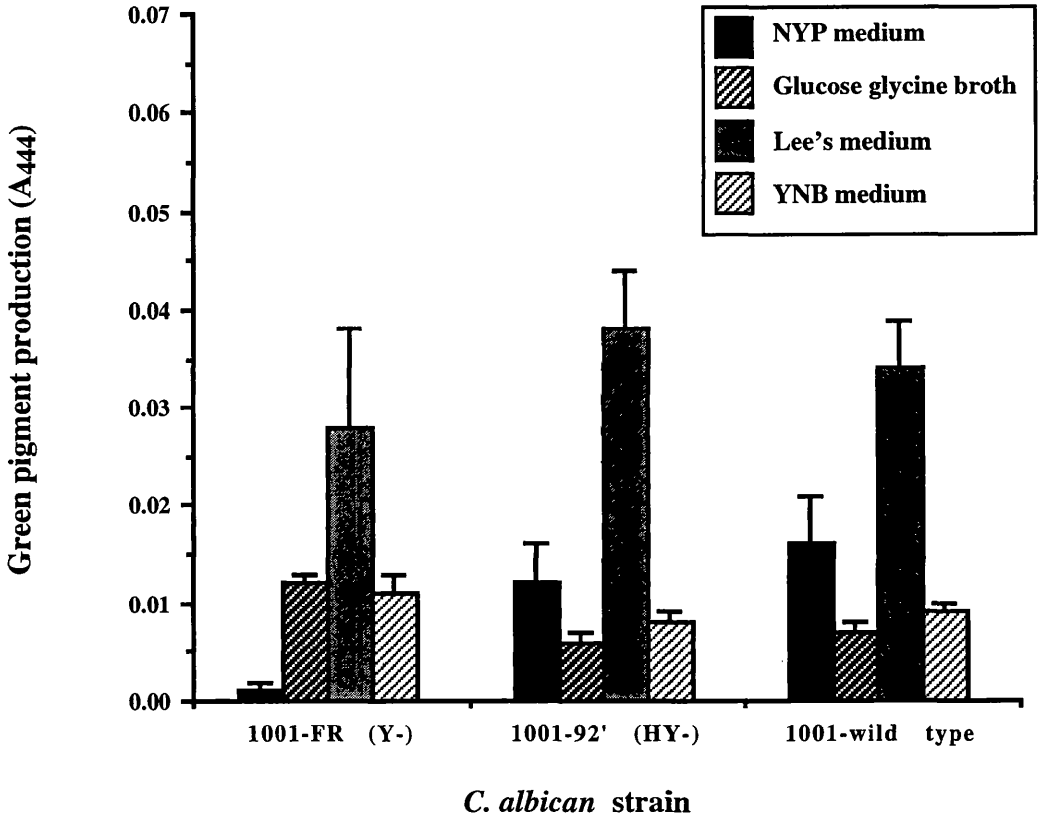

Green pigment production (A444)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
NYP medium
Glucose glycine broth
Lee's medium
YNB medium
1001-FR (Y-)
1001-92' (HY-)
1001-wild type
C. albican strain

Table 27.

Green pigment production by *C. albicans* on Sabouraud dextrose blood agar.

C. albicans strain	Growth	Morphological form (%)	Grey-green zone thickness (cm)
GRI 681	+	100 Y	0.15
GRI 682	+	100 Y	0.13
GDH 2023	+	100 Y	0.10
GDH 2346	+	100 Y	0.13
1001-Wild Type	+++	100 Y	0.10
1001-FR(Y-)	+	48 HY	0.10
1001-92'(HY-)	+++	100 Y	0.10
ATTC 10261	++	100 Y	0.15
HOG 301	+++	81 HY	0.20

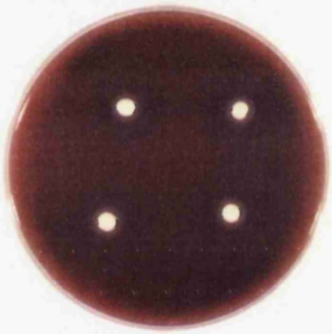
HY= Hypha form; Y = Yeast form.

Figure 39.

C. albicans **strains GRI 681, GRI 682, GDH 2023, and GDH 2346 grown on Sabouraud dextrose blood agar at 37˚C for 24h.**

GRI 682

GRI 681



GDH 2023

GDH 2346

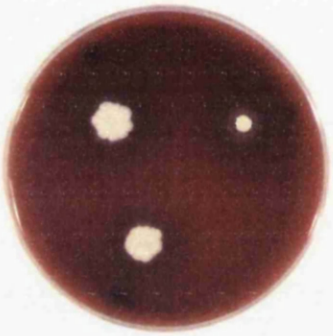
Figure 40.

C. albicans **strains 1001-wild type and its mutants 1001-FR(Y^-) and 1001-92'(HY^-) grown on Sabouraud dextrose blood agar at 37°C for 24h.**

Figure 41.

C. albicans **strain ATTC 10261 and its mutant HOG 301 grown on Sabouraud dextrose blood agar at 37°C for 24h.**

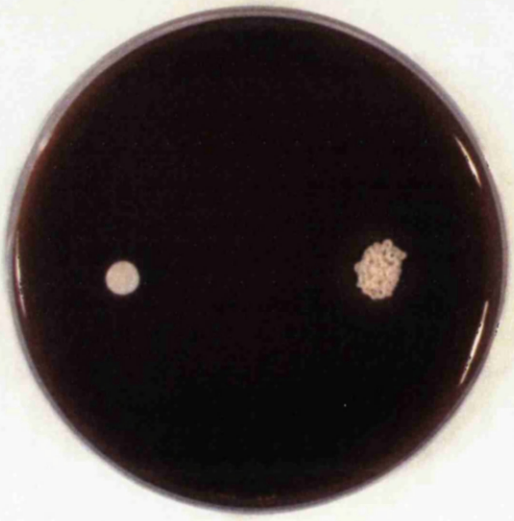
1001-WILD TYPE



1001-FR[Y⁻]

1001-92'[HY⁻]

ATTC 10261



HOG 301

7. Factors affecting green pigment production

In this part of the project, some factors which may affect green pigment production by *C. albicans* were investigated. For example, pigment production during growth on different carbon sources was monitored , and the effects of changes in incubation conditions such as temperature were studied.

7.1. Green pigment production by *C. albicans* in YNB medium containing a high galactose concentration or low glucose concentration

Production of a green pigment under other environmental conditions was noted previously in this laboratory (McCourtie and Douglas, 1985) during a study of *C. albicans* adhesion. This earlier report indicated that the green pigment was secreted by *C. albicans* during prolonged incubation in medium containing 500 mM galactose and that the green colour was particularly noticeable in culture supernatants of strain GDH 2346.

7.1.1 Green pigment production in YNB medium containing 500 mM galactose as carbon source

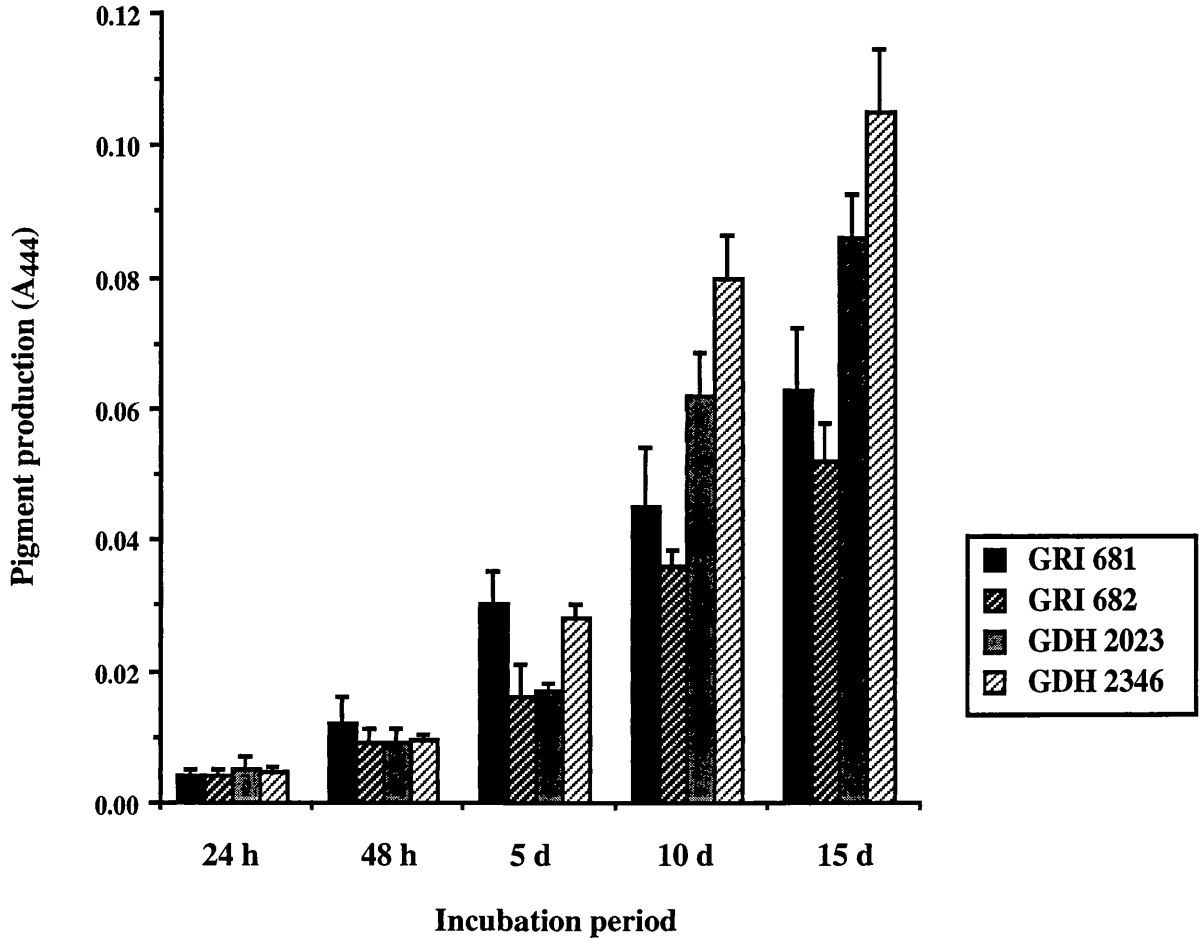
The yeasts were grown in yeast nitrogen base medium (Difco) containing 500 mM galactose for 24h, 48h, 5d, 10d, and 15d at 37°C. Figure 42 shows that green pigment production by four strains increased sharply with time and maximum readings were noted after 15 days. The highest absorbance readings were obtained with culture supernates of strain GDH 2346; these were markedly higher than those of the other strains after 10 and 15 days.

Pure yeast-form growth was observed with all four strains tested except for an occasional hyphal form, especially with strain GRI 681 (< 2 % hyphae) after 10 days' incubation. Growth of the cultures was measured by means of optical density readings at 550 nm (Figs 44A and 44B); after prolonged incubation, all strains gave higher readings in 500 mM galactose-containing medium than in 50 mM glucose-containing medium.

Figure 42.

Green pigment production by *C. albicans* strains incubated in YNB medium containing 500 mM galactose at 37˚C for different periods.

Pigment production was determined by measuring the absorbance of culture supernates at 444 nm. Data represent the means of A_{444} values ($\pm$ SEM) from four experiments done in duplicate.


0.12
0.10
0.08
0.06
0.04
0.02
0.00
Pigment production (A444)
24 h
48 h
5 d
10 d
15 d
Incubation period
GRI 681
GRI 682
GDH 2023
GDH 2346

In a separate experiment, *C. albicans* 'Outbreak' strain was tested for green pigment production in YNB medium containing 500 mM galactose after 48 h, 5 d and 15 d at 37°C. Similar results were obtained with this strain. The quantity of pigment produced increased sharply during prolonged incubation in 500 mM galactose and, as before, growth was greater in medium containing galactose than in medium containing glucose. Pure yeast cultures were observed throughout the incubation period.

7.1.2. Green pigment production in YNB medium containing 50 mM glucose as carbon source

For comparison, *C. albicans* strains were grown in YNB medium containing 50 mM glucose as the carbon source for 24h, 48h, 5d, 10d, and 15d at 37°C. Green pigment production increased with time, again reaching a maximum after 15 days incubation, but was substantially lower, overall, than that observed in galactose medium (Fig. 43). As before, strain GDH 2346 seemed to produce more pigment than any other strain. All strains grew in the yeast form apart from strain GRI 681 which occasionally showed 6 - 19 % hyphal forms after 10 - 15 days incubation.

A separate experiment confirmed that *C. albicans* 'Outbreak' strain also showed less pigment production and less growth in medium containing 50 mM glucose. Overall, these results demonstrate that *C. albicans* secretes a green pigment during prolonged incubation in medium containing either 500 mM galactose or 50 mM glucose, but that more pigment is produced in galactose medium.

7.2. Effect of temperature on green pigment production

Temperature has great influence on the induction of hyphal growth by *C.albicans*. For this reason , an investigation was carried out into the effect of temperature for 2, 5, and 10 days on green pigment production. Four strains of

Figure 43.

Green pigment production by *C. albicans* strains in YNB medium containing 50 mM glucose at 37°C for different incubation periods.

Pigment production was determined by measuring the absorbance of culture supernates at 444 nm.

Data represent the means of A_{444} values ($\pm$ SEM) from four experiments done in duplicate.

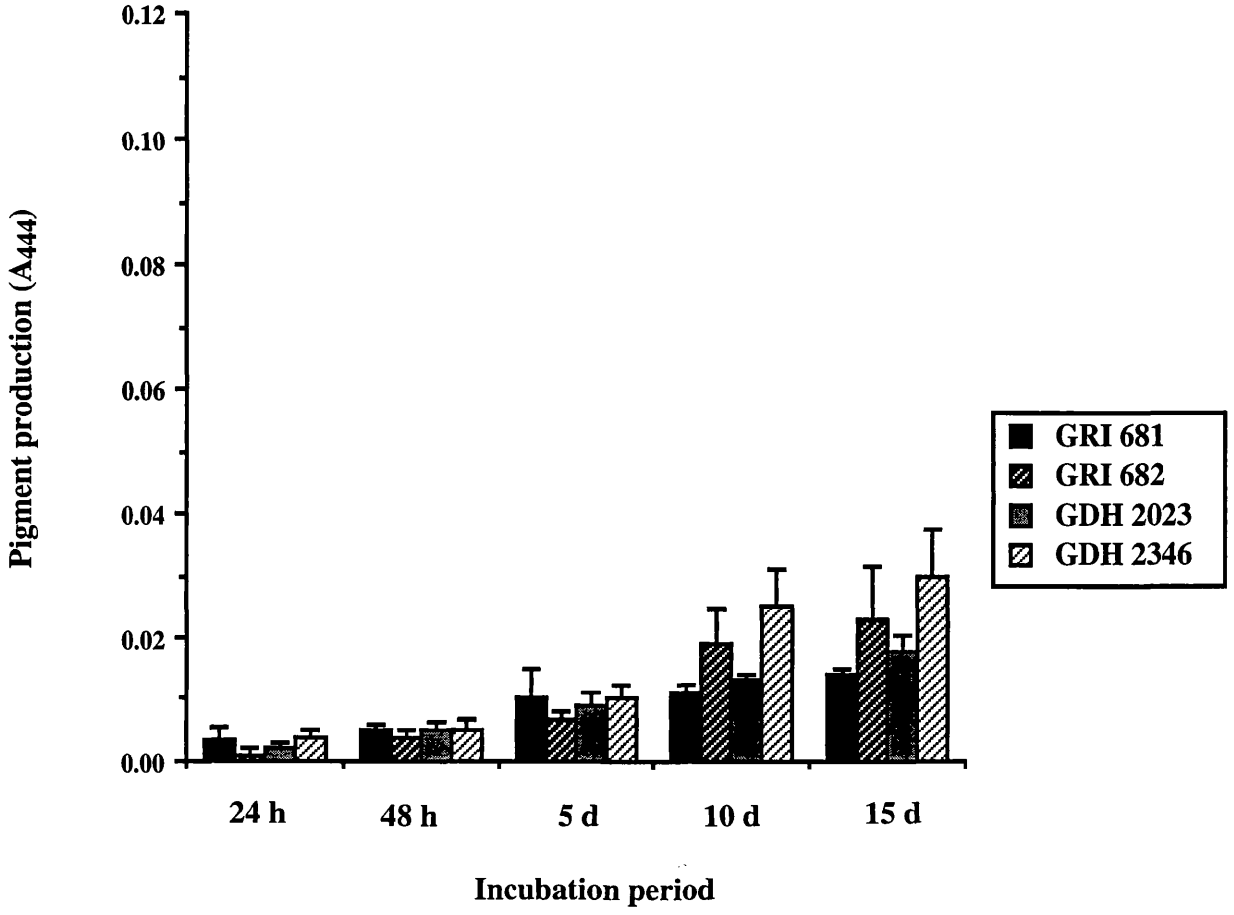

0.12
0.10
0.08
0.06
0.04
0.02
0.00
Pigment production (A444)
24 h
48 h
5 d
10 d
15 d
Incubation period
GRI 681
GRI 682
GDH 2023
GDH 2346

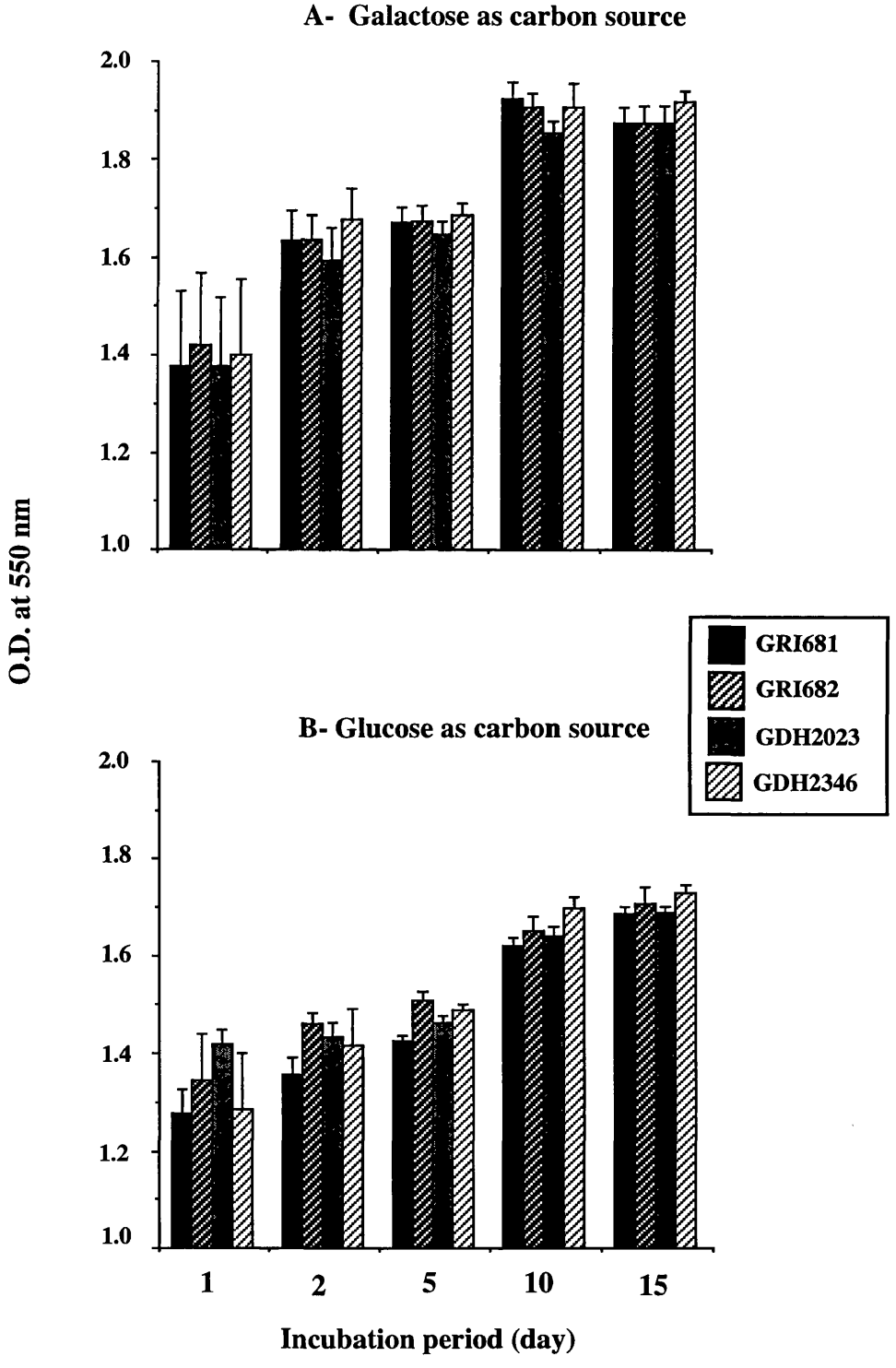
Figure 44.

Growth of *C. albicans* strains in YNB medium containing galactose or glucose as the carbon source at 37°C.

(A) 500 mM galactose.

(B) 50 mM glucose.

Data represent means of four optical density readings at 550 nm.


A- Galactose as carbon source
2.0
1.8
1.6
1.4
1.2
1.0
O.D. at 550 nm
GRI681
GRI682
GDH2023
GDH2346
B- Glucose as carbon source
2.0
1.8
1.6
1.4
1.2
1.0
1
2
5
10
15
Incubation period (day)

C. albicans were grown in YNB medium containing either 50 mM glucose or 500 mM galactose as the carbon source. Three flasks of each strain were incubated at 25°C, 30°C, and 37°C.

All four strains were capable of producing green pigment in YNB medium with 500 mM galactose or 50 mM glucose as a carbon source at 25°C, 30°C or 37°C, but the amount of pigment produced was higher when galactose (Fig. 45) was used rather than glucose (Fig. 46) at all temperatures tested. These observations confirm the results of the previous section. Pigment production was higher after incubation for 10 days than after 2 or 5 days, as also noted earlier. Generally, strains GRI 681, GRI 682 and GDH 2023 showed no significant difference between all three temperatures, unlike strain GDH 2346 which gave highest pigment production at 37°C (Figs. 45 and 46).

Growth of *C. albicans* in YNB medium containing either glucose or galactose at 25°C, 30°C and 37°C (Figs. 47 and 48) was measured at 550 nm. This showed that the four strains tested grow well ($O.D_{550}$ 1.5 to 2.0) at all three temperatures. The highest optical density readings were recorded were after 10 days incubation. No significant difference was noted between growth at the three temperatures or between growth on galactose or glucose as the carbon source.

7.2. Effect of static and shaking culture conditions on green pigment production

Strains GRI 681, GRI 682, GDH 2023, and GDH 2346 were inoculated into high galactose-containing YNB medium or low glucose-containing YNB medium. These cultures were incubated at 37°C either statically or on an orbital shaker (100 r.p.m.) for 10 days. Pigment production was measured after 2, 5 and 10 days.

Greater pigment production was found in the aerated cultures for all four strains. Under these conditions, GDH 2346 produced more pigment than the other three strains when either galactose or glucose was used as a carbon source. Using

Figure 45.

Effect of temperature (25°C, 30°C or 37°C) on green pigment production by *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 grown in YNB medium containing 500 mM galactose.

Pigment production was determined by measuring the absorbance of culture supernates at 444 nm.

Data represent the means of A_{444} values ($\pm$ SEM) from four experiments.

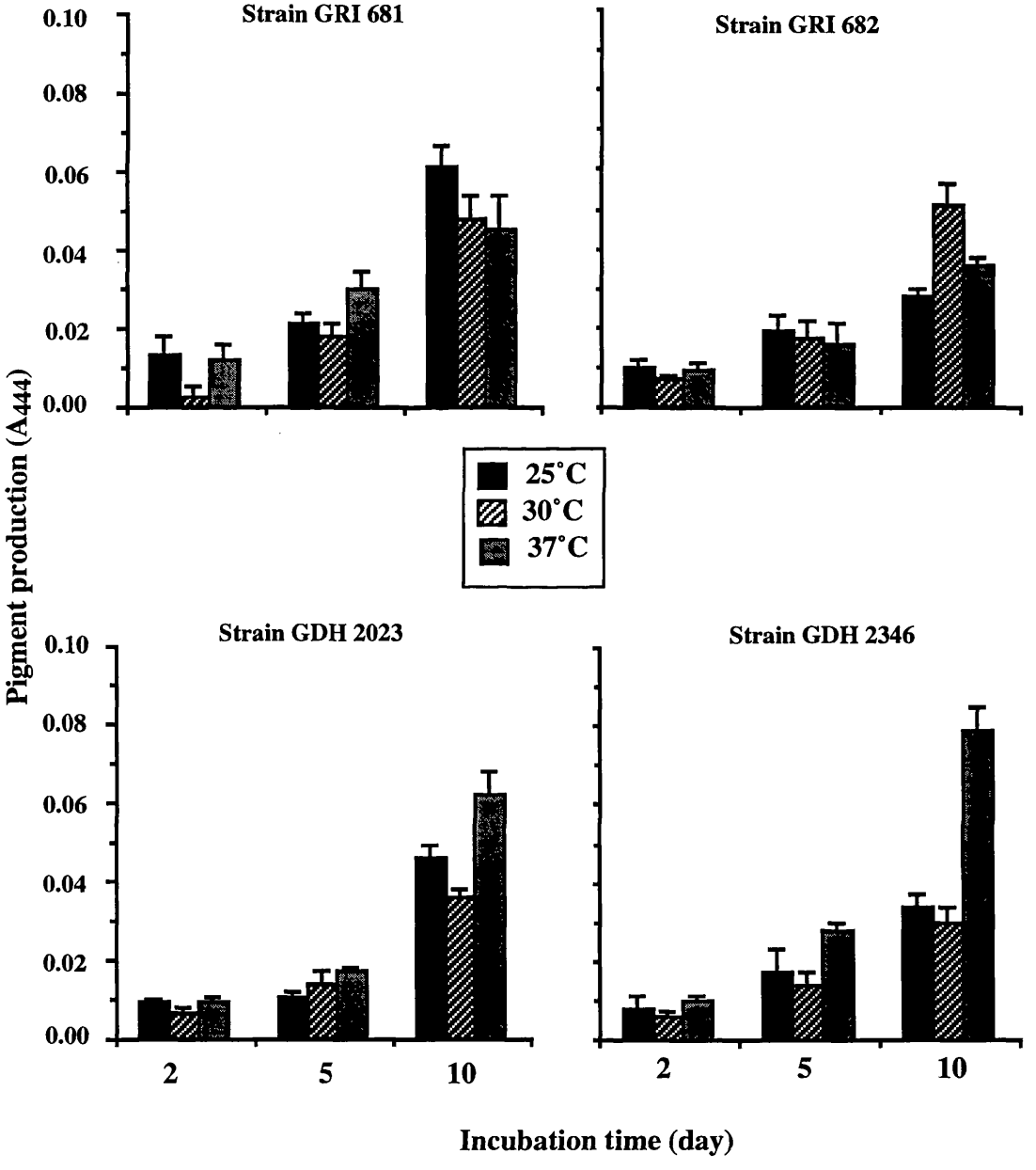

Strain GRI 681
Strain GRI 682
Strain GDH 2023
Strain GDH 2346
25°C
30°C
37°C
Pigment production (A444)
0.10
0.08
0.06
0.04
0.02
0.00
2
5
10
Incubation time (day)

Figure 46.

Effect of temperature (25°C, 30°C or 37°C) on green pigment production by *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 grown in YNB medium containing 50 mM glucose.

Pigment production was determined by measuring the absorbance of culture supernates at 444 nm. Data represent the means of A_{444} values (+ SEM) from four experiments.

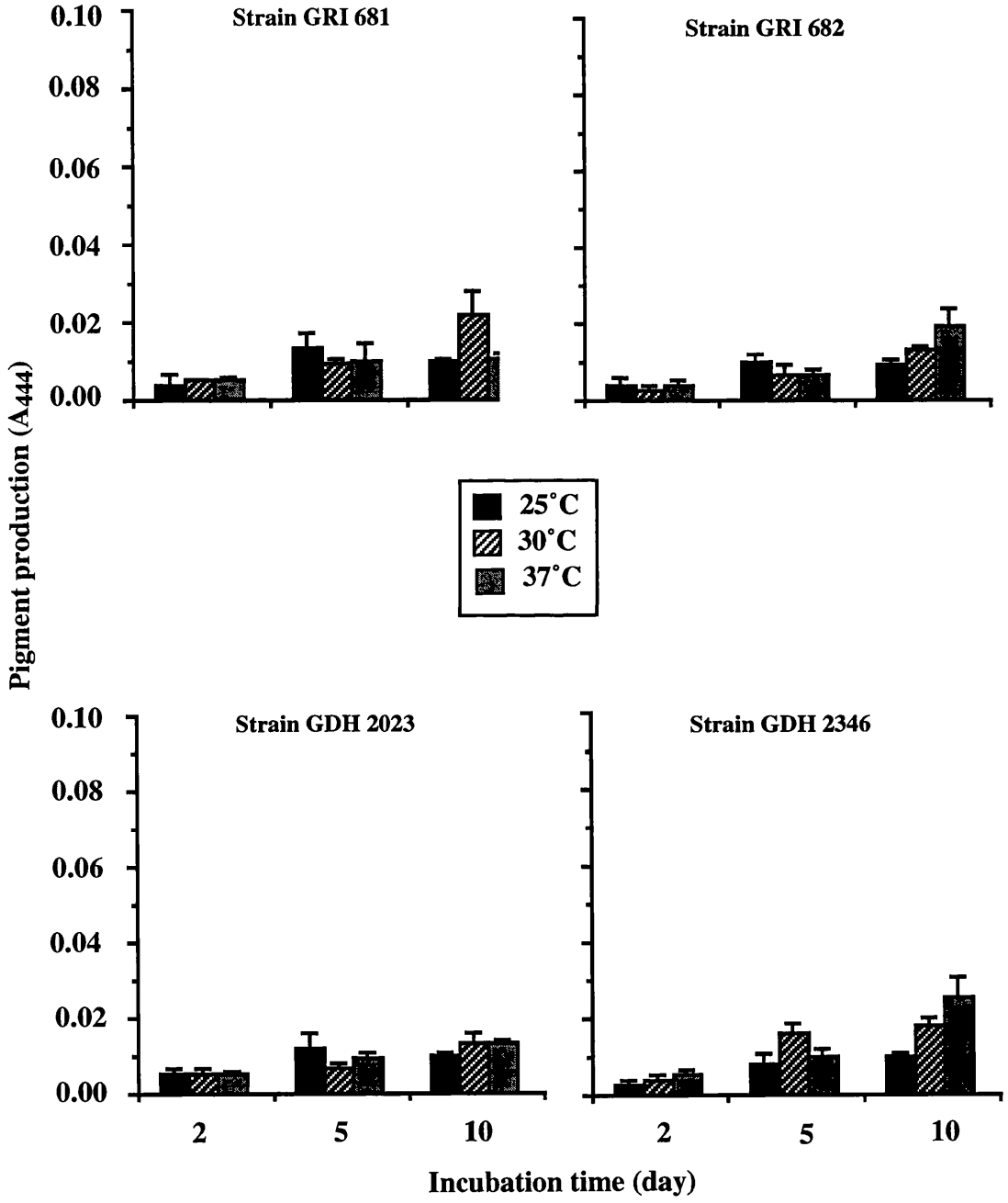

Strain GRI 681
Strain GRI 682
Strain GDH 2023
Strain GDH 2346
0.10
0.08
0.06
0.04
0.02
0.00
Pigment production (A444)
25°C
30°C
37°C
2
5
10
Incubation time (day)

Figure 47.

Growth of *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 in YNB medium containing galactose as the carbon source at different temperatures.

Data represent means of optical density readings from four experiments.

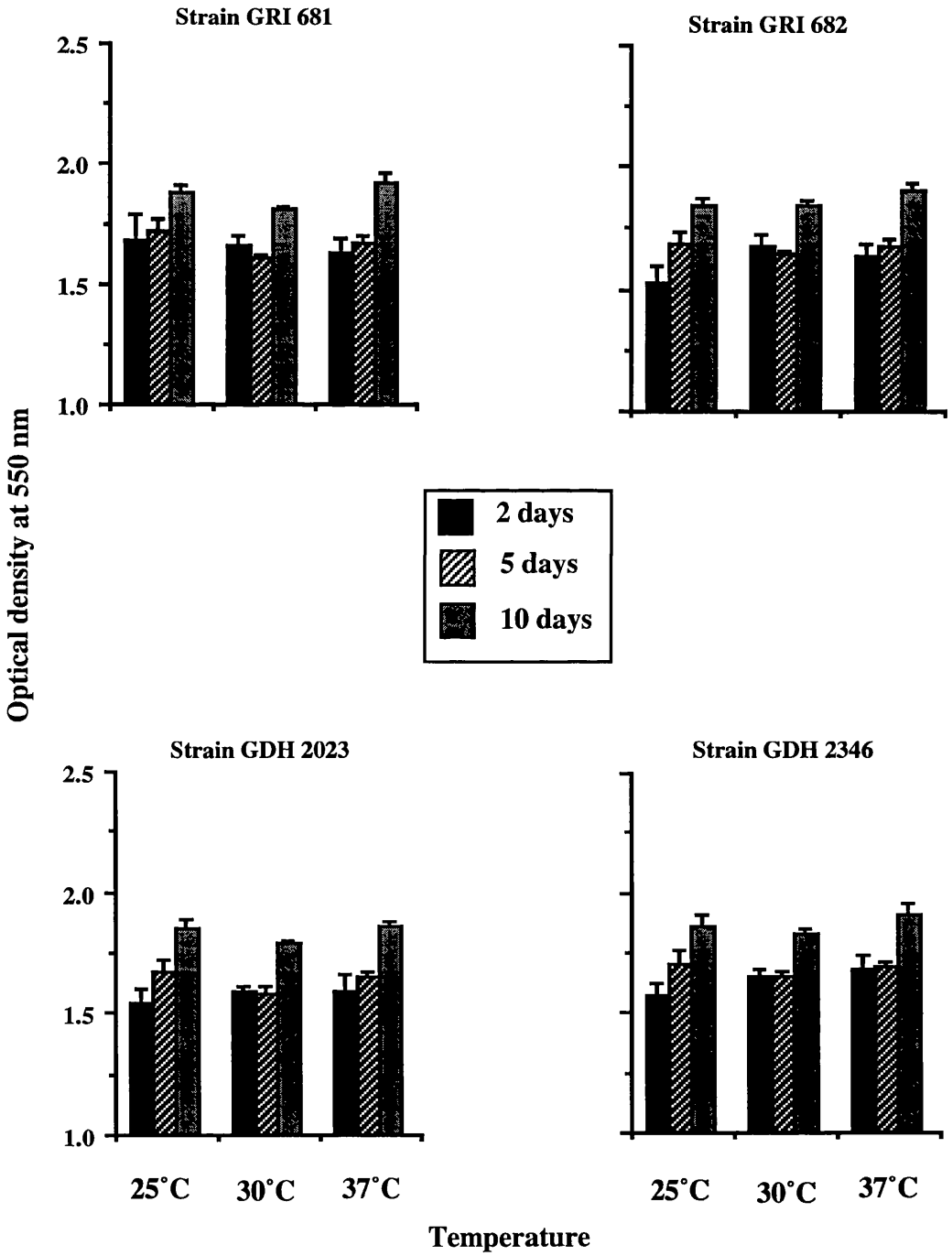
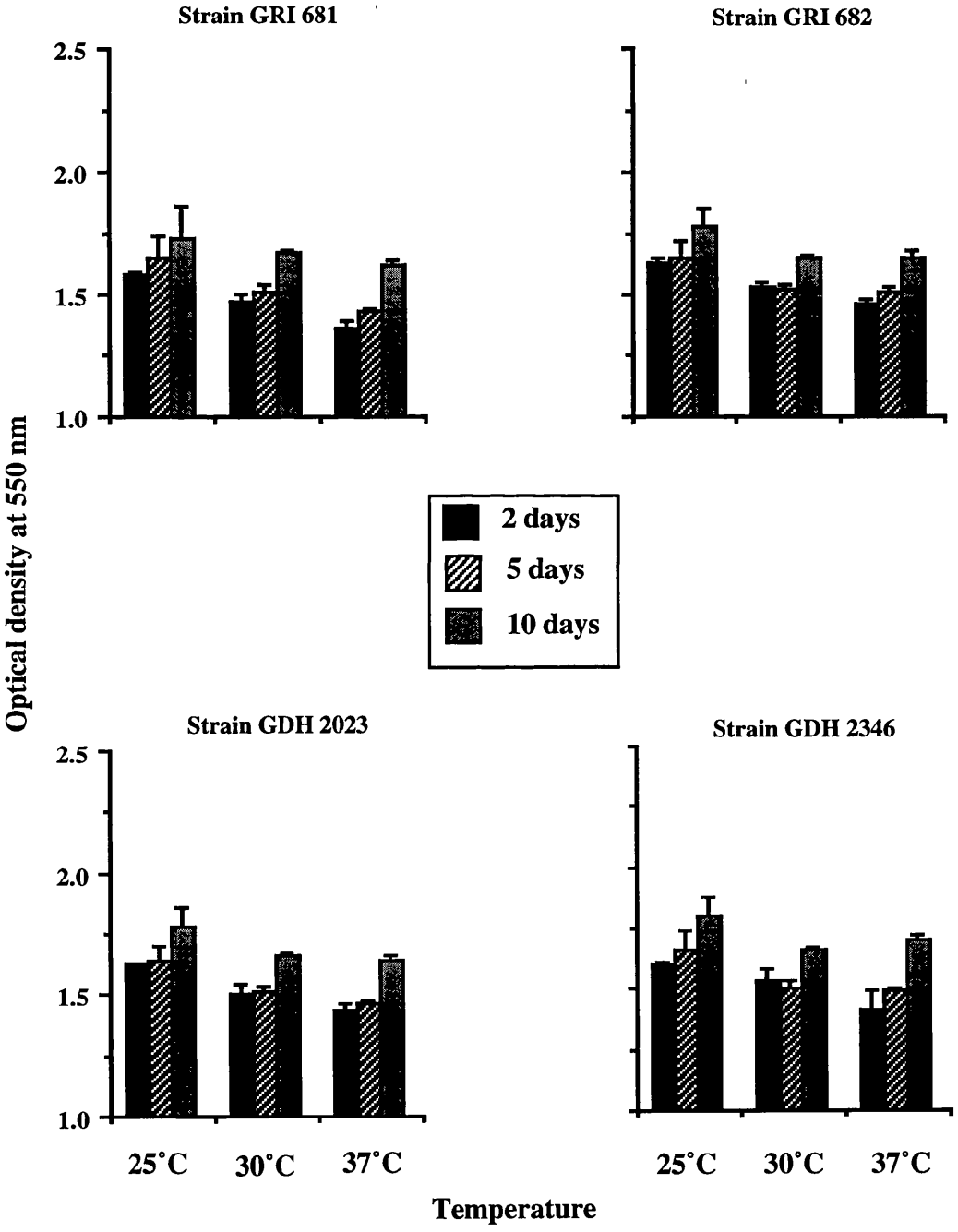

Strain GRI 681
Strain GRI 682
2.5
2.0
1.5
1.0
Optical density at 550 nm
2 days
5 days
10 days
Strain GDH 2023
Strain GDH 2346
25°C
30°C
37°C
25°C
30°C
37°C
Temperature

Figure 48.

Growth of *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 in YNB medium containing glucose as the carbon source at different temperatures.

Data represent means of optical density readings from four experiments.


Strain GRI 681
Strain GRI 682
2.5
2.0
1.5
1.0
Optical density at 550 nm
2 days
5 days
10 days
Strain GDH 2023
Strain GDH 2346
25°C
30°C
37°C
25°C
30°C
37°C
Temperature

galactose rather than glucose as a carbon source induced pigment production even under static conditions. An increase in pigment production was observed with galactose-grown, shaken cultures when the incubation time was increased from 2d to 10d; this was not observed with static cultures. There was some increase in pigment production with time for glucose-grown, static cultures, but only with strains GRI 681 and GDH 2023 (Figs. 49 and 50).

Aerated cultures of all four strains on either carbon source showed greater growth than the static cultures (Fig. 51), which gave maximum optical density readings of 1.4 and 1.3 with galactose and glucose, respectively. Microscopical examination indicated the complete absence of hyphae in aerated cultures. However, strains GRI 681 and GDH 2346 both had a tendency for hyphal growth in static cultures (Fig. 52).

8. Green pigment production in deferrated yeast nitrogen base containing various iron concentrations

The aim of these experiments was to study the effect of growth in medium containing low concentrations of iron on green pigment production. The synthesis of a green pigment by *C. albicans* appears to be regulated in a manner similar to that of siderophore production . Previously in this laboratory it was noted that, at low, but not high concentrations of iron, six strains of *C. albicans* (GRI 681, GRI 682, GDH 2023, GDH 2346, NCPF 3153 and 'Outbreak' strain) synthesised a green pigment when grown in YNB containing 50 mM glucose and supplemented with 0.026 - 0.8 μM iron (Sweet and Douglas, 1991a). The medium used was a modification of yeast nitrogen base, prepared from individual constituents. Deferration of the medium was accomplished by using Chelex 100 ion-exchange resin. Prior analysis of the medium components by graphite furnace atomic absorption spectrometry is shown in Table 28. Deferrated yeast nitrogen base medium (x10 concentrate) contains 4 μg/l iron; mixed vitamins

Figure 49.

Effect of shaking and static incubation conditions on green pigment production by *C. albicans* strains GRI 681 and GRI 682 in YNB medium containing either 500 mM galactose (Gal) or 50 mM glucose (Glc) at 37°C for different incubation times.

Data represent the means of A_{444} values ($\pm$ SEM) from four experiments.

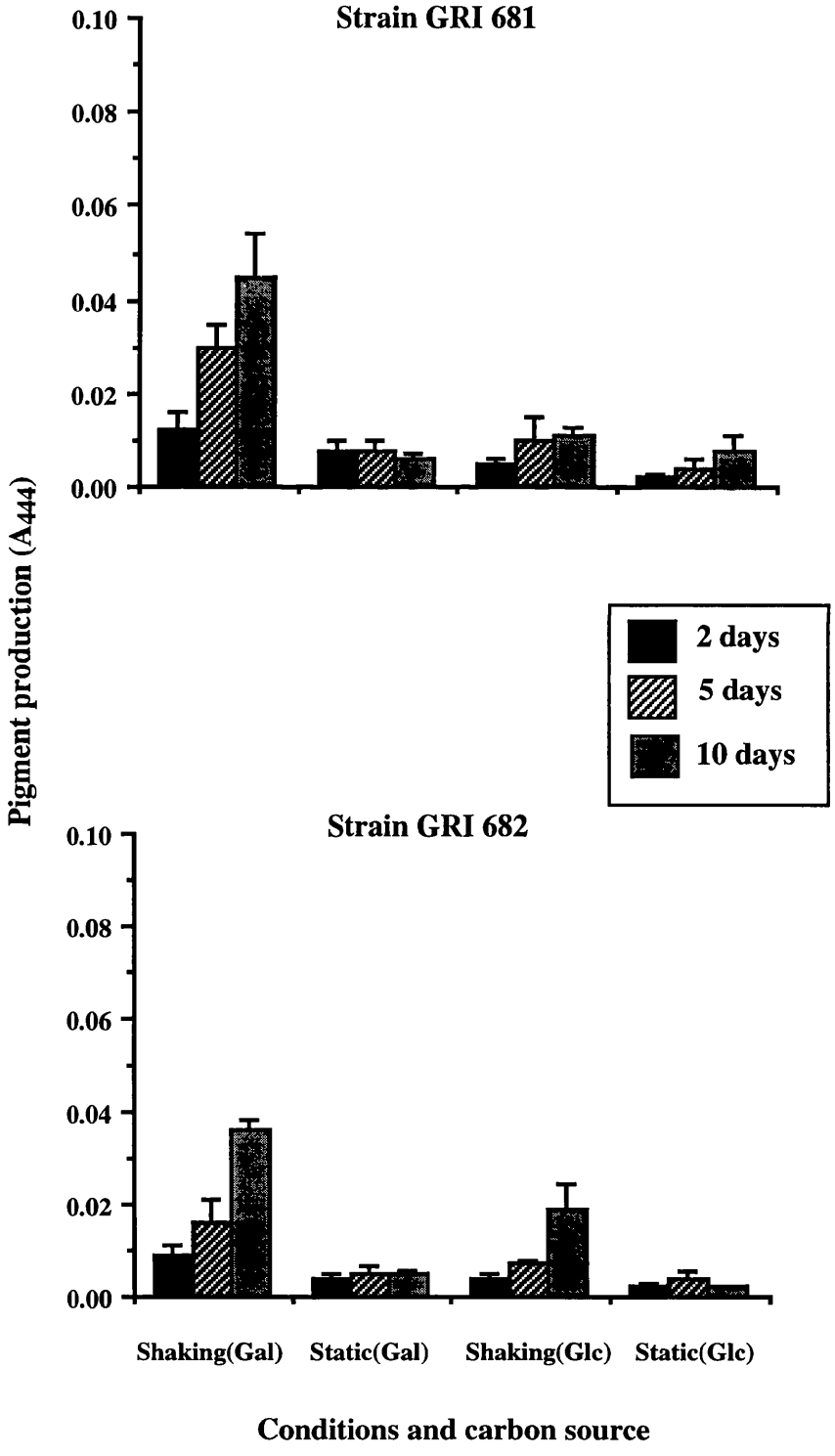

Strain GRI 681
0.10
0.08
0.06
0.04
0.02
0.00
2 days
5 days
10 days
Strain GRI 682
0.10
0.08
0.06
0.04
0.02
0.00
Pigment production (A444)
Shaking(Gal)
Static(Gal)
Shaking(Glc)
Static(Glc)
Conditions and carbon source

Figure 50.

Effect of shaking and static incubation conditions on green pigment production by *C. albicans* strains GDH 2023 and GDH 2346 in YNB medium containing either 500 mM galactose (Gal) or 50 mM glucose (Glc) at 37°C for different incubation times.

Data represent the means of A_{444} values ($\pm$ SEM) from four experiments.

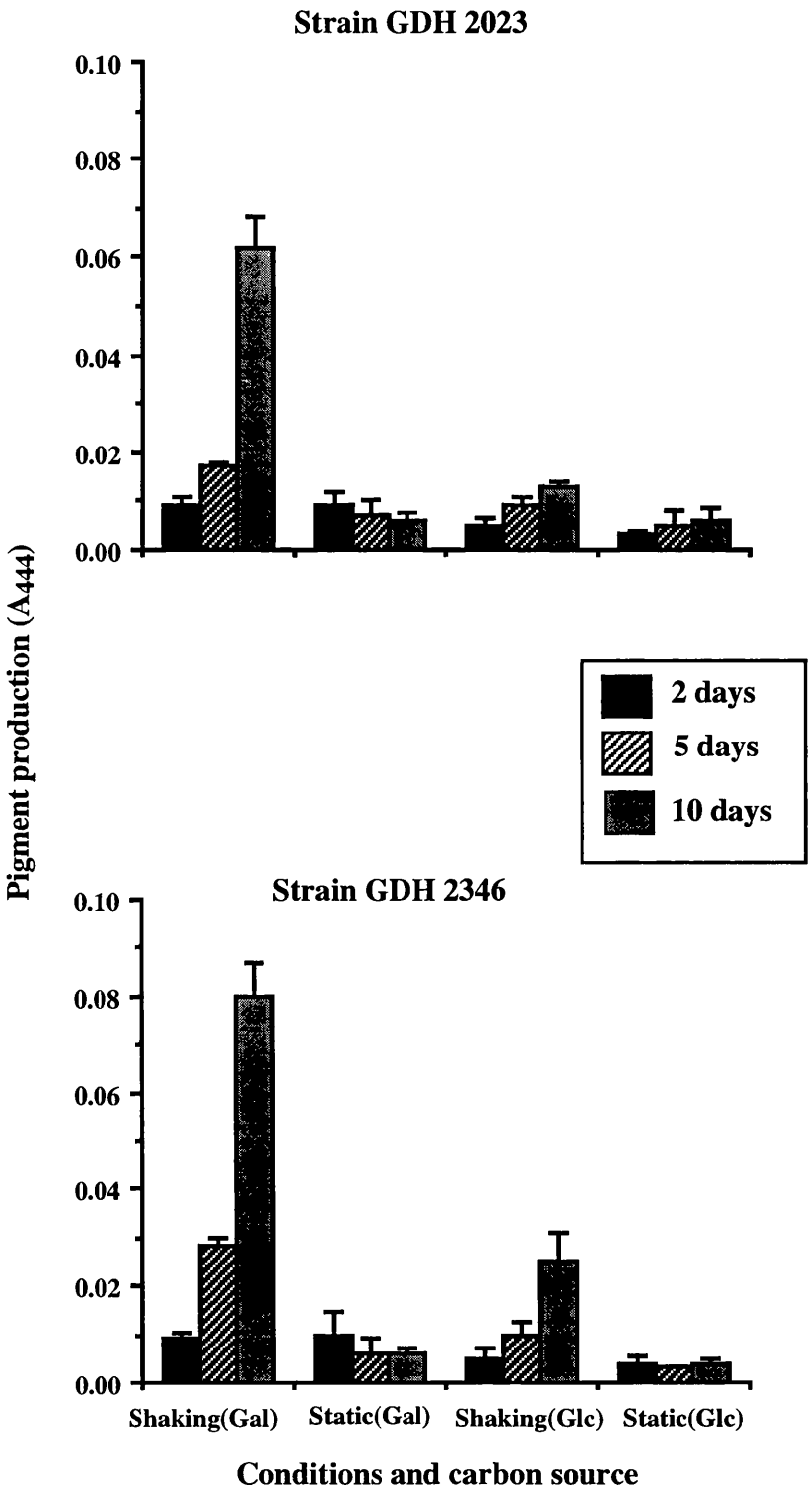

Strain GDH 2023
0.10
0.08
0.06
0.04
0.02
0.00
Pigment production (A444)
2 days
5 days
10 days
Strain GDH 2346
0.10
0.08
0.06
0.04
0.02
0.00
Shaking(Gal)
Static(Gal)
Shaking(Glc)
Static(Glc)
Conditions and carbon source

Figure 51.

Growth of *C. albicans* strains on YNB medium containing galactose or glucose and incubated statically at 37°C.

(A) 500 mM galactose.

(B) 50 mM glucose.

Data represent means of optical density readings from four experiments.

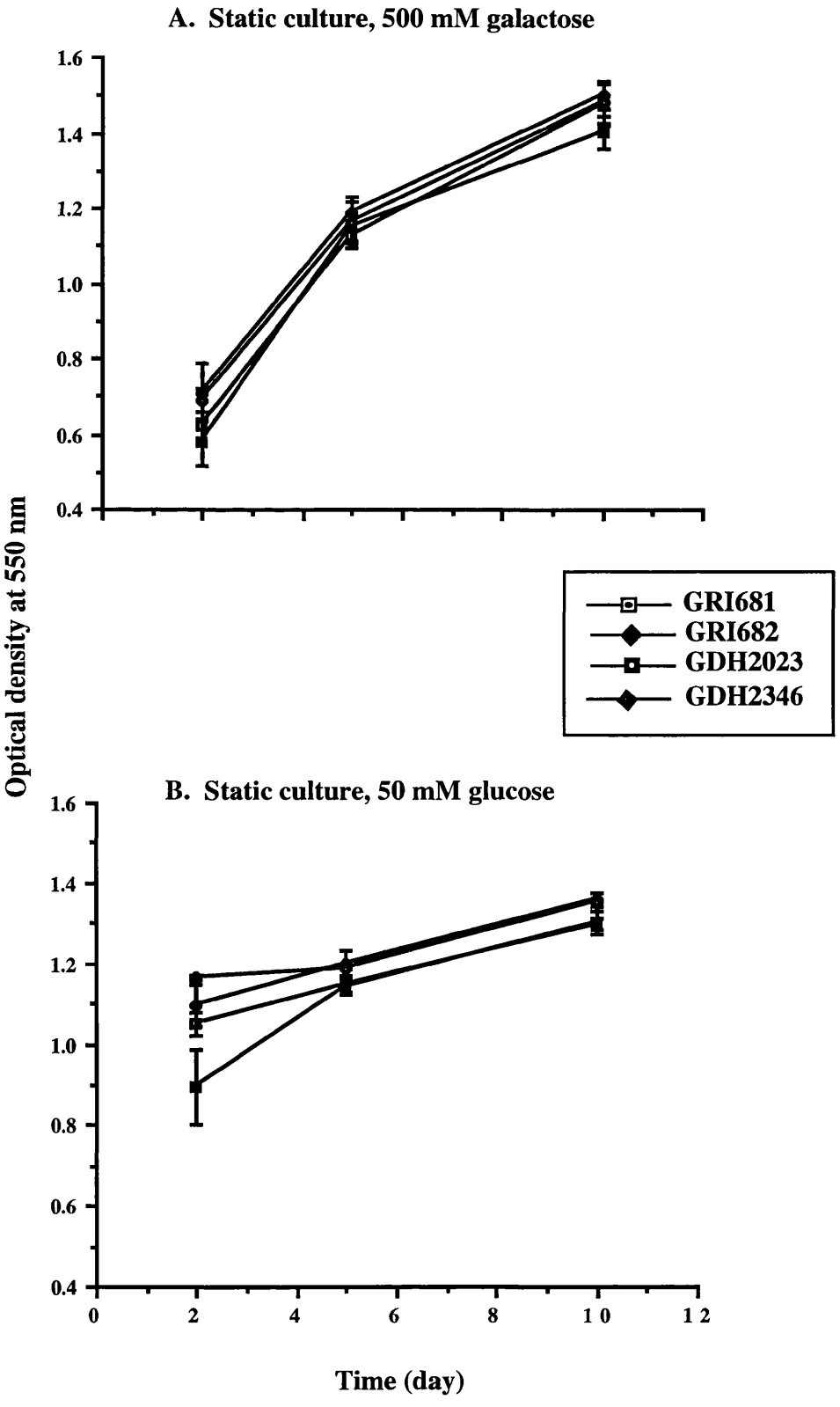

A. Static culture, 500 mM galactose
1.6
1.4
1.2
1.0
0.8
0.6
0.4
GRI681
GRI682
GDH2023
GDH2346
B. Static culture, 50 mM glucose
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0
2
4
6
8
10
12
Optical density at 550 nm
Time (day)

Figure 52.

Production of hyphae by *C. albicans* strains grown statically in YNB medium containing either 500 mM galactose (Gal) or 50 mM glucose (Glc) at 37˚C.

Data represent the means of four readings ± SEM

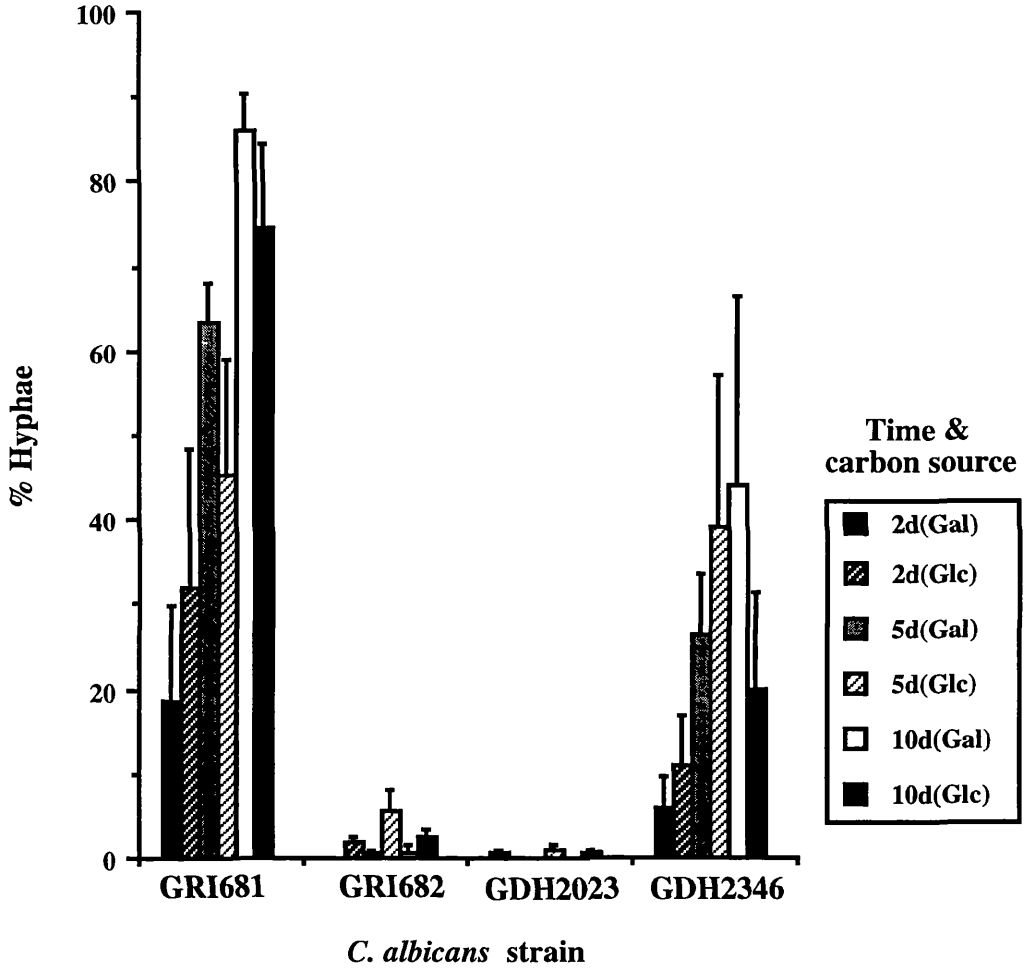

100
80
60
40
20
0
% Hyphae
GRI681
GRI682
GDH2023
GDH2346
C. albicans strain
Time & carbon source
2d(Gal)
2d(Glc)
5d(Gal)
5d(Glc)
10d(Gal)
10d(Glc)

Table 28.

Analysis of medium components for iron by graphite furnace atomic absorption spectrometry (courtesy of Glasgow Royal Infirmary).

Sample	Iron concentration (μg/l)
Deferrated yeast nitrogen base (x10 concentrate)	4
Deferrated yeast nitrogen base (normal strength)	5
Glucose (1M)	7
Mixed vitamins (x200 concentrate), containing biotin, calcium pantothenate, folic acid, inositol, niacin, p-Aminobenzoic acid, pyridoxin hydrochloride, riboflavin and thiamine hydrochloride.	<2
Deionized water	<2
Potassium phosphate, dibasic (x10 concentrate)	<2
Magnesium sulphate (1M)	5
Glucose glycine broth (normal strength)	61

(x200 concentrate), deionized water and potassium phosphate (x10 concentrate) all gave values of <2 μg/l iron.

8.1. Green pigment production in deferrated YNB containing various iron concentrations with glucose as carbon source

C. albicans 'Outbreak' strain was grown in deferrated YNB containing 50 mM glucose and various iron concentrations (0.026 - 0.8 μM). After incubation at 37°C for 7 and 14 days, cultures were examined for green pigment production (Fig. 53 and 58). As judged by A_{444} values, the highest pigment production was noted at the lowest iron concentration (0.026 μM). The same result was obtained for both incubation periods and a visible yellow-greenish colour was observed at low iron concentration (Fig. 57). Growth of *C. albicans* at different iron concentrations was measured at 550 nm (Fig. 54); this showed that the organism grew well (as pure yeast-form cultures) in 0.026 - 0.8μM iron, with the lowest optical density reading at 0.026 μM iron.

8.2. Green pigment production in deferrated YNB containing various iron concentrations with galactose as carbon source

C. albicans 'Outbreak' strain was grown in deferrated YNB containing various iron concentrations (0.026 - 0.8μM), and 500 mM galactose as the carbon source. The results (Fig. 55 and 58) showed that 500 mM galactose did not induce green pigment production as had 50 mM glucose when used as the carbon source. Incubation periods of one or two weeks gave little pigment production (A_{444} values < 0.02) in all galactose-containing media with different iron concentrations; over the same time periods, glucose-containing media gave substantial pigment production (A_{444} values of up to 0.12) with identical iron concentrations. Growth of *C. albicans* , measured at 550 nm (Fig. 56), was similar at iron concentrations of 0.026 μM and 0.8 μM, and pure yeast-form cultures were observed in all cases.

Figure 53.

Production of green pigment by *C. albicans* 'Outbreak' strain in deferrated YNB containing different iron concentrations and 50 mM glucose as carbon source.

Cultures were incubated at 37°C for 7 days (A) or 14 days (B). Data represent means of four A_{444} values ± SEM

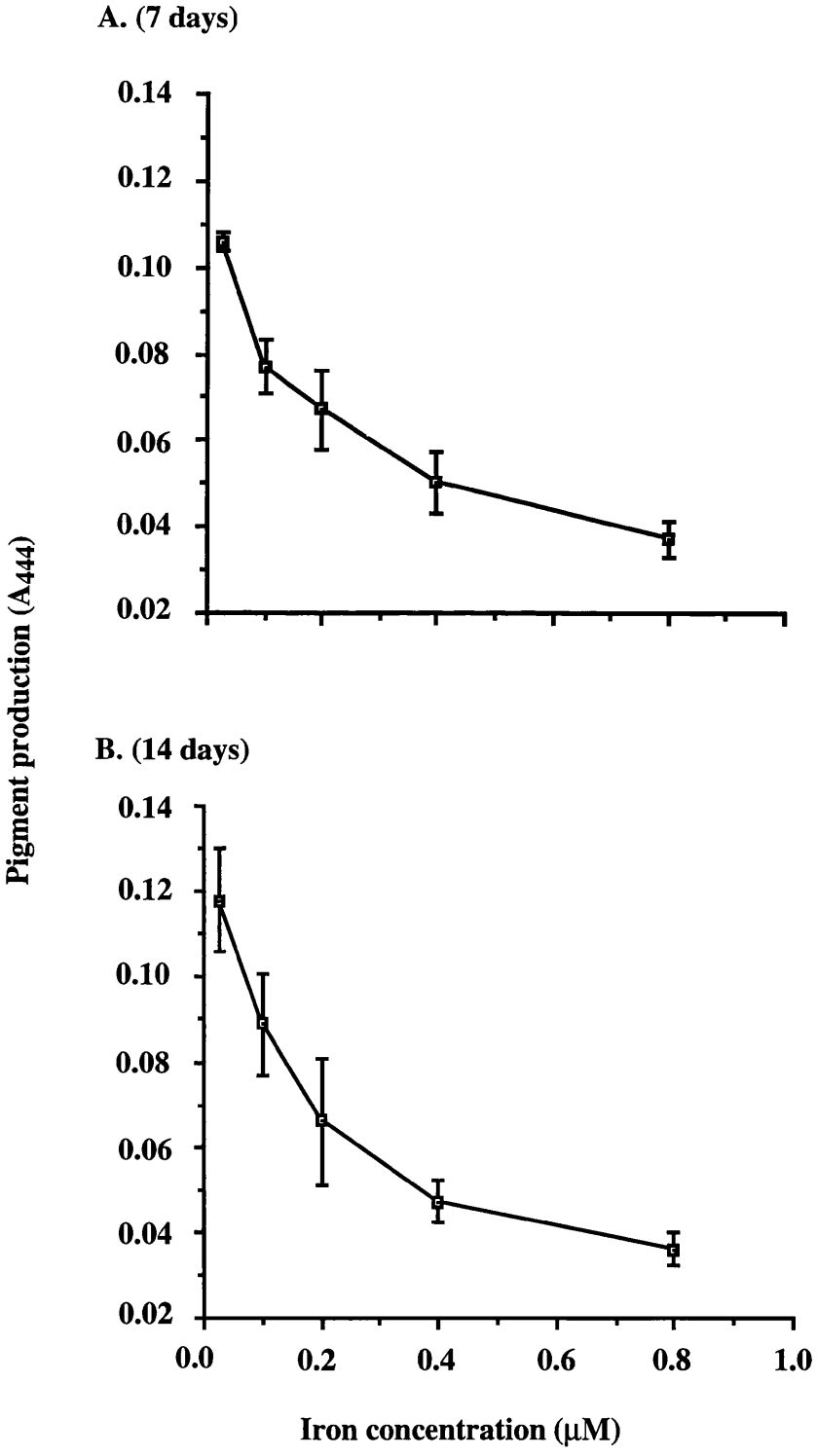

A. (7 days)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
B. (14 days)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.0
0.2
0.4
0.6
0.8
1.0
Pigment production (A444)
Iron concentration (μM)

Figure 54.

Growth of *C. albicans* 'Outbreak' strain on YNB containing different concentrations of iron and 50 mM glucose.

Cultures were incubated at 37°C for 2 days (A), 7 days (B) or 14 days (C). Data represent means of four OD_{550} readings $\pm$ SEM.

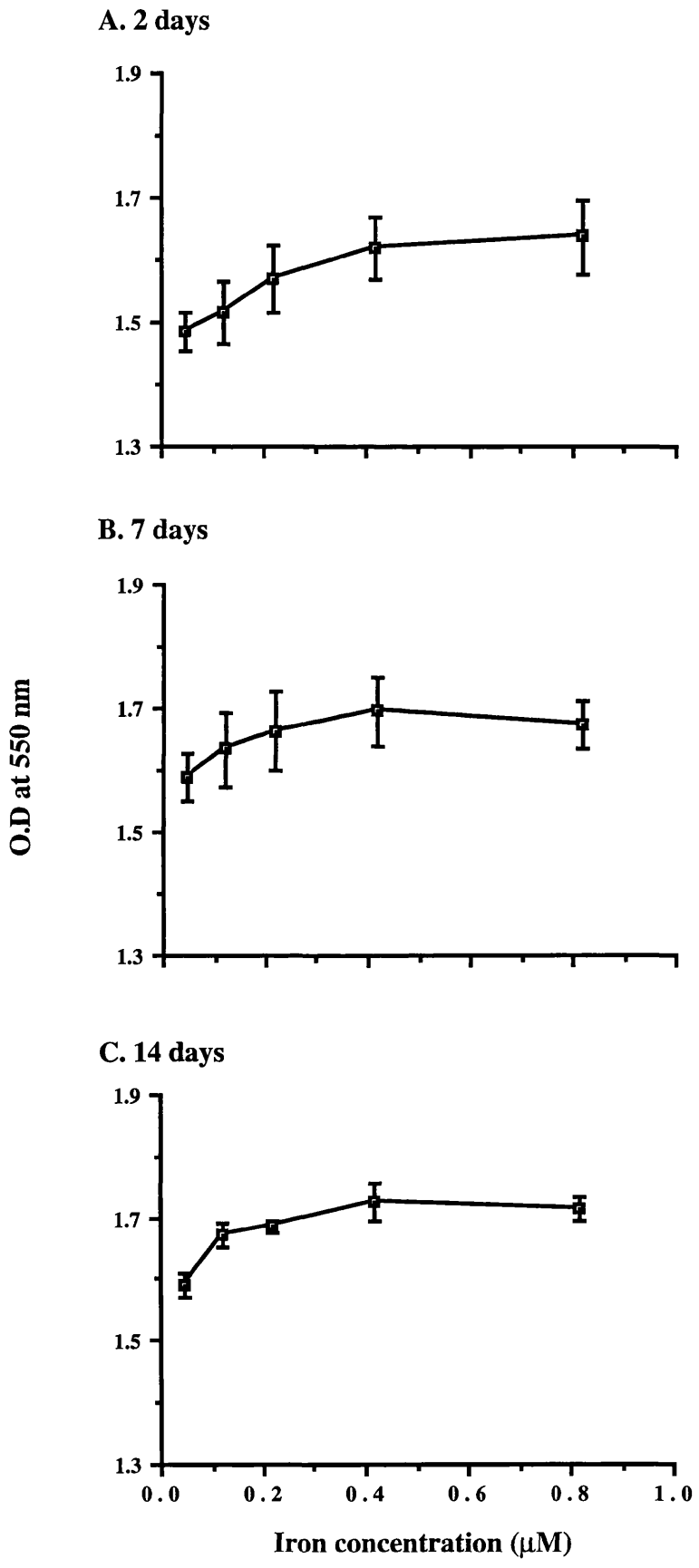

A. 2 days
1.9
1.7
1.5
1.3
B. 7 days
1.9
1.7
1.5
1.3
O.D at 550 nm
C. 14 days
1.9
1.7
1.5
1.3
0.0
0.2
0.4
0.6
0.8
1.0
Iron concentration (μM)

Figure 55.

Production of green pigment by *C. albicans* 'Outbreak' strain in deferrated YNB containing different iron concentrations and 500 mM galactose.

Cultures were incubated at 37°C for 7 days (A) and 14 days (B), then centrifuged. Pigment production was assessed by determining A_{444} values of culture supernates. Data represent means of four reading from different supernates ± SEM.

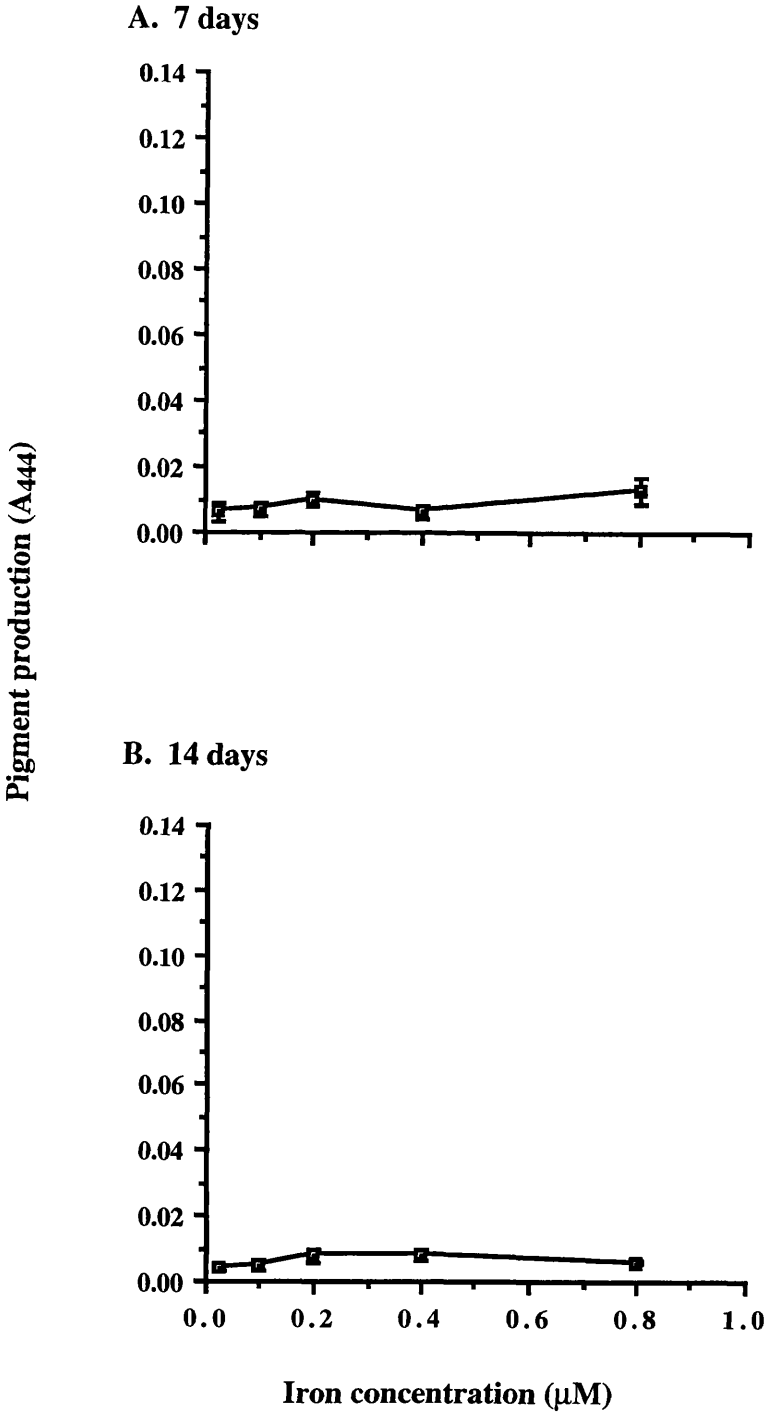

A. 7 days
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
B. 14 days
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.0
0.2
0.4
0.6
0.8
1.0
Pigment production (A444)
Iron concentration (μM)

Figure 56.

Growth of *C. albicans* 'Outbreak' strain on YNB containing different concentrations of iron and 500 mM galactose.

Cultures were incubated at 37°C for 2 days (A), 7 days (B) or 14 days (C). Data represent means of four OD_{550} readings ± SEM.

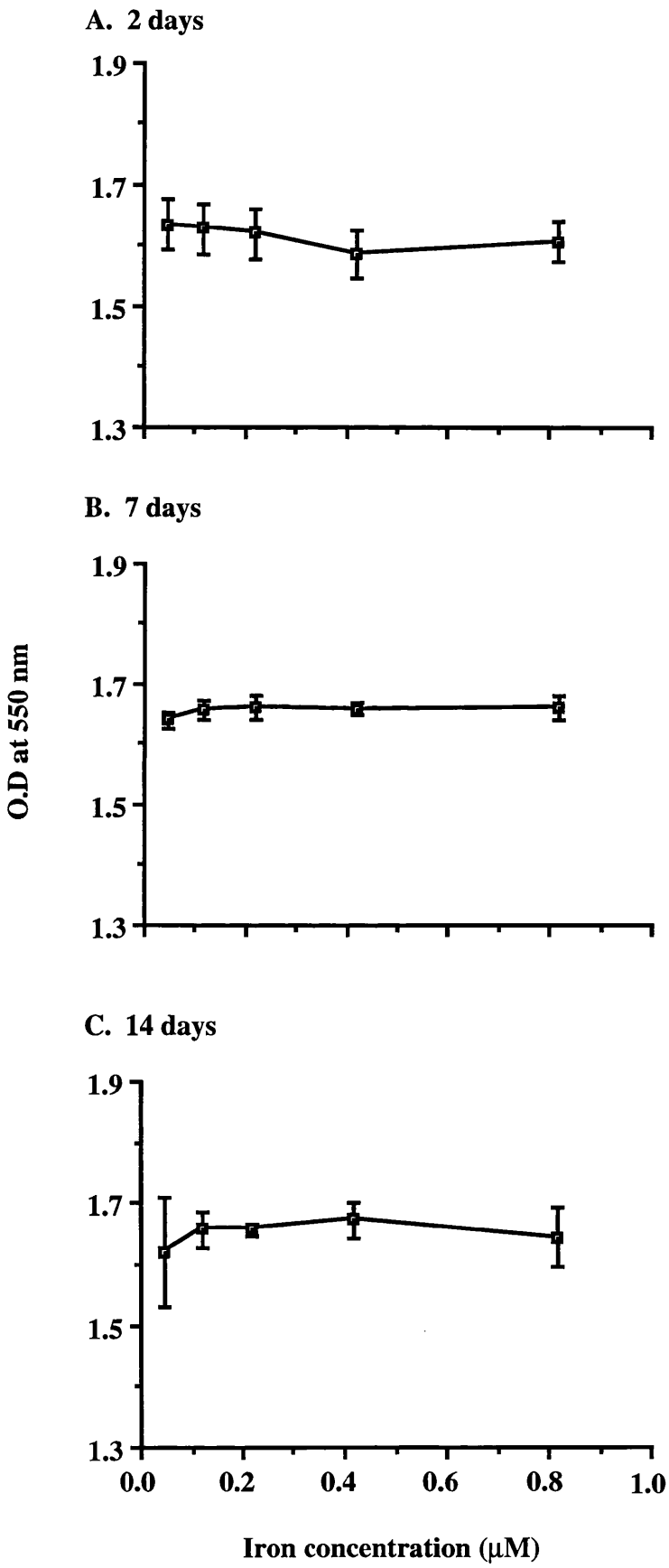

A. 2 days
1.9
1.7
1.5
1.3
B. 7 days
O.D at 550 nm
1.9
1.7
1.5
1.3
C. 14 days
1.9
1.7
1.5
1.3
0.0
0.2
0.4
0.6
0.8
1.0
Iron concentration (μM)

Figure 57.

Green pigment production by *C. albicans* 'Outbreak' strain after incubation at 37°C for 14 days.

The organism was grown in deferrated YNB containing 50 mM glucose and different iron concentrations (0.026 - 0.8 μM). After 14 days at 37°C, samples of each culture were centrifuged at 150 rpm and inspected visually. The photograph shows the appearance of samples from medium containing 0.026 μM iron (1), 0.1 μM iron (2), 0.2 μM iron (3), 0.4 μM iron (4) and 0.8 μM iron (5) after centrifugation.


1
2
3
4
5

Figure 58.

Green pigment production by *C. albicans* 'outbreak' strain after incubation at 37°C for 5 days.

Photograph shows samples of uninoculated medium (1) or culture supernates after incubation at 37°C for 5 days (2-4) as follows:

1. Uninoculated deferrated YNB containing 50 mM glucose and 0.026 μM iron .
2. Culture supernate of deferrated YNB containing 50 mM glucose and 0.026 μM iron .
3. Culture supernate of deferrated YNB containing 50 mM glucose and 0.8 μM iron.
4. Culture supernate of deferrated YNB containing 500 mM galactose and 0.026μM iron .

1
2
3
4

9. Effect of various salts and trace elements on green pigment production

In view of the effect of iron-limitation in inducing the synthesis of a green pigment, further experiments were carried out to investigate whether other important salts or trace elements also affected green pigment production. In this study *C. albicans* strains were grown in defined medium supplemented with various concentrations of phosphate, magnesium, manganese, zinc and copper. Glucose (50 mM) was used as the carbon source.

9.1. Green pigment production in yeast nitrogen base medium with different phosphate concentrations

Three strains of *C. albicans* were grown in YNB containing 0 - 0.5 mM potassium phosphate at 37°C with shaking for 2 and 5 days; controls contained 500 mM galactose or 50 mM glucose with 7 mM phosphate (the concentration of phosphate in YNB powder). Green pigment production was not induced by growing *C. albicans* under phosphate limitation (Fig. 59), the highest pigment production being noted when the organism was grown at normal phosphate concentrations (7 mM) in either carbon source. Generally, very low pigment production was noted in media which had no added phosphate and which therefore contained only traces of phosphate associated with the other chemicals which compose yeast nitrogen base. The results indicate that phosphate is necessary for pigment production and that low concentrations do not increase pigment synthesis.

Growth of *C. albicans* with different concentrations of phosphate was measured at 550 nm (Fig. 60). There was a steady increase with increasing phosphate concentrations, all cultures being entirely in the yeast form. Optimum growth was noted in media containing the normal phosphate concentration (7 mM) and 500 mM galactose as the carbon source. Little or no growth was obtained in medium with no added phosphate.

Figure 59.

Green pigment production by *C. albicans* strain GRI 681, GDH 2023 and GDH 2346 grown in YNB medium containing 50 mM glucose supplemented with different concentrations of phosphate.

Cultures were incubated at 37°C for 2 or 5 days. Control cultures were grown in YNB with either 50 mM glucose or 500 mM galactose and 7 mM phosphate. After incubation, cultures were centrifuged and A_{444} values of supernates determined.

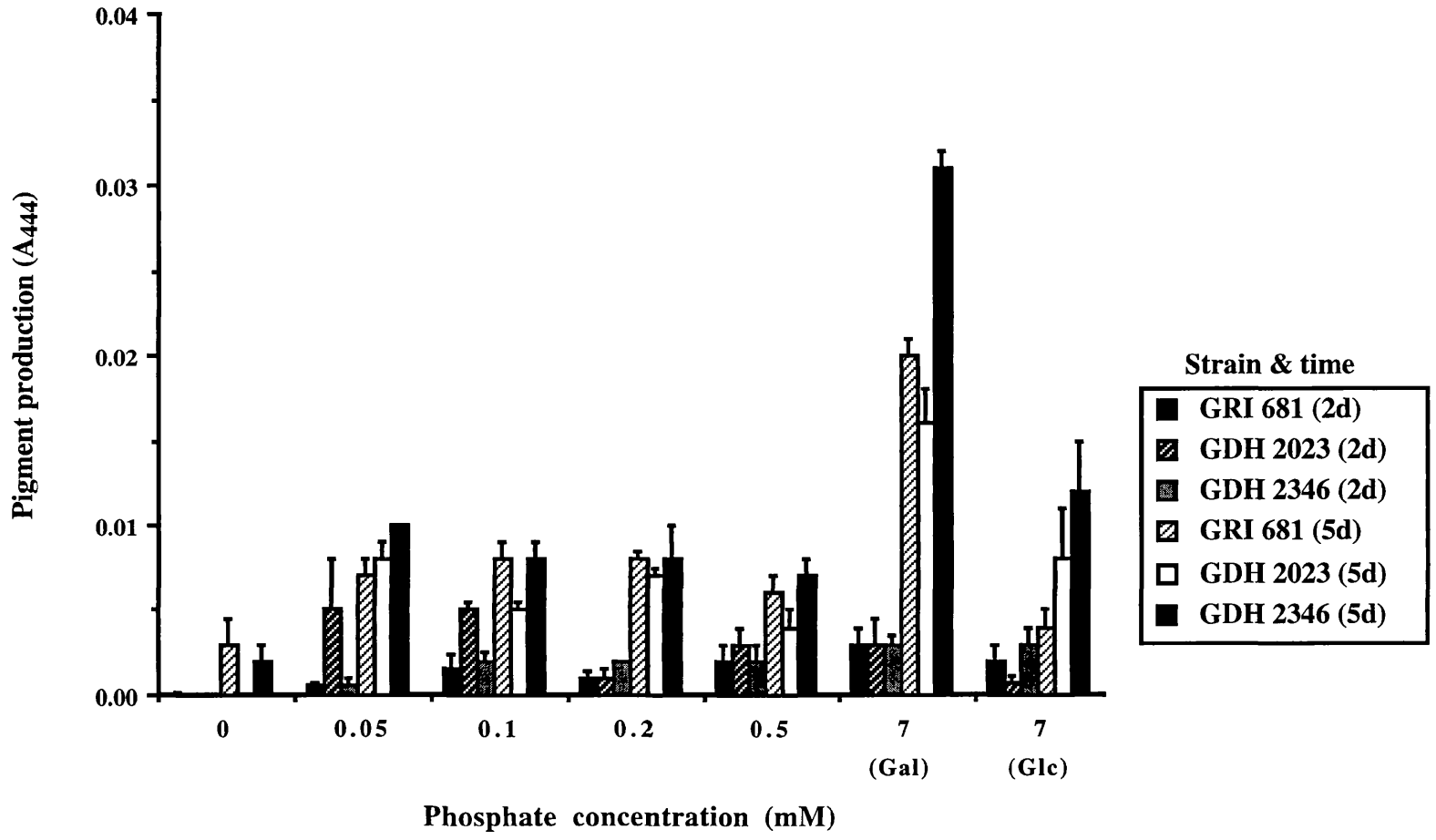
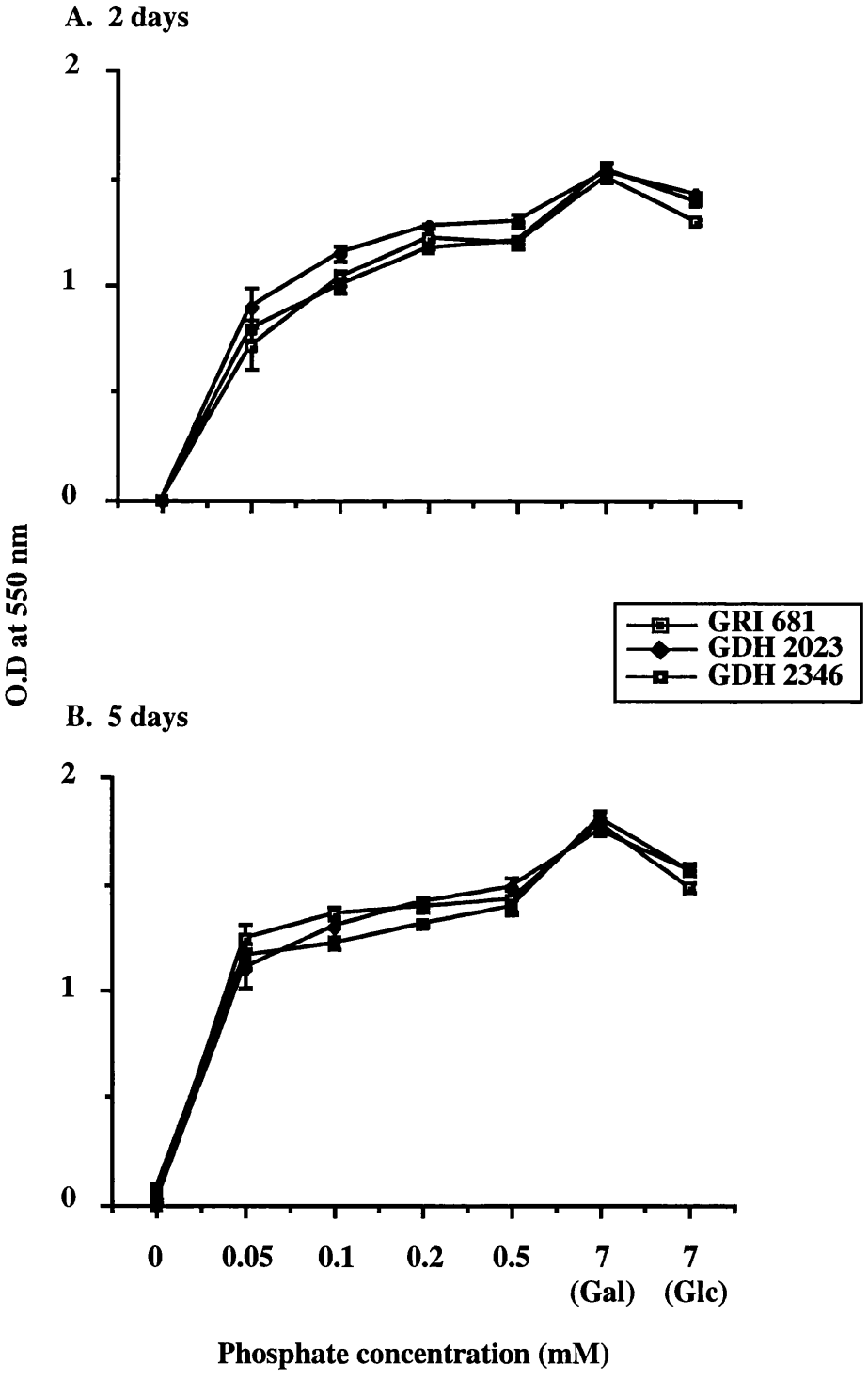

0.04
0.03
0.02
0.01
0.00
Pigment production (A444)
0
0.05
0.1
0.2
0.5
7
(Gal)
7
(Glc)
Phosphate concentration (mM)
Strain & time
GRI 681 (2d)
GDH 2023 (2d)
GDH 2346 (2d)
GRI 681 (5d)
GDH 2023 (5d)
GDH 2346 (5d)

Figure 60.

Growth of *C. albicans* strains GRI 681, GDH 2023 and GDH 2346 in glucose-containing YNB media supplemented with various concentrations of phosphate.

Cultures were incubated at 37°C for 2 days (A) or 5 days (B). Control cultures were grown in YNB with either 50 mM glucose or 500 mM galactose and 7 mM phosphate. Data represent means of four OD_{550} readings $\pm$ SEM.


A. 2 days
2
1
0
O.D at 550 nm
GRI 681
GDH 2023
GDH 2346
B. 5 days
2
1
0
0
0.05
0.1
0.2
0.5
7 (Gal)
7 (Glc)
Phosphate concentration (mM)

9.2. Green pigment production in yeast nitrogen base medium with different magnesium concentrations

Yeast nitrogen base medium was made up from individual constituents as outlined in the manufacturer's manual and $MgSO_4$ added to a final concentration of 0 - 1 mM. Batches of medium were inoculated with three strains of *C. albicans* and the cultures were incubated at 37°C for 2 and 5 days. Control cultures contained 500 mM galactose or 50 mM glucose with 2 mM magnesium (the concentration of magnesium in YNB powder). A low concentration of magnesium, unlike iron, did not induce green pigment production (Fig. 61); pigment synthesis was greatest with the two controls containing galactose or glucose as carbon sources and 2 mM magnesium. Magnesium was necessary for growth (Fig. 62), 2 mM being the optimum concentration. All three *C. albicans* strains tested gave 100% yeast-form cultures after 2 or 5 days. Little growth was observed in medium without added magnesium.

9.3. Green pigment production in yeast nitrogen base medium with different manganese concentrations

Three strains of *C. albicans* were grown in defined medium supplemented with $MnSO_4$ at concentrations ranging from 0-1 μM. Control cultures contained 500 mM galactose or 50 mM glucose with 1.79 μM manganese (the concentration of manganese in YNB powder). For each strain examined under these conditions, little or no pigment production was seen at any of the given manganese concentrations (Fig. 63). The highest values were recorded when each strain was grown in YNB containing 500 mM galactose; these were typical of those obtained after 5 days' incubation at 37°C of a galactose-grown culture (compare with Figs. 59 and 61). The control cultures contained manganese at a concentration of 1.79 μM, which is not growth limiting. However, good growth was obtained with all three strains in media containing all of the manganese concentrations tested (Fig. 64), suggesting that sufficient Mn^{2+} was present as a contaminant of other

Figure 61.

Green pigment production by *C. albicans* strain GRI 681, GDH 2023 and GDH 2346 grown in YNB medium containing 50 mM glucose supplemented with different concentrations of magnesium.

Cultures were incubated at 37°C for 2 or 5 days. Control cultures were grown in YNB with either 50 mM glucose or 500 mM galactose and 2 mM magnesium. After incubation, cultures were centrifuged and A444 values of supernates determined.

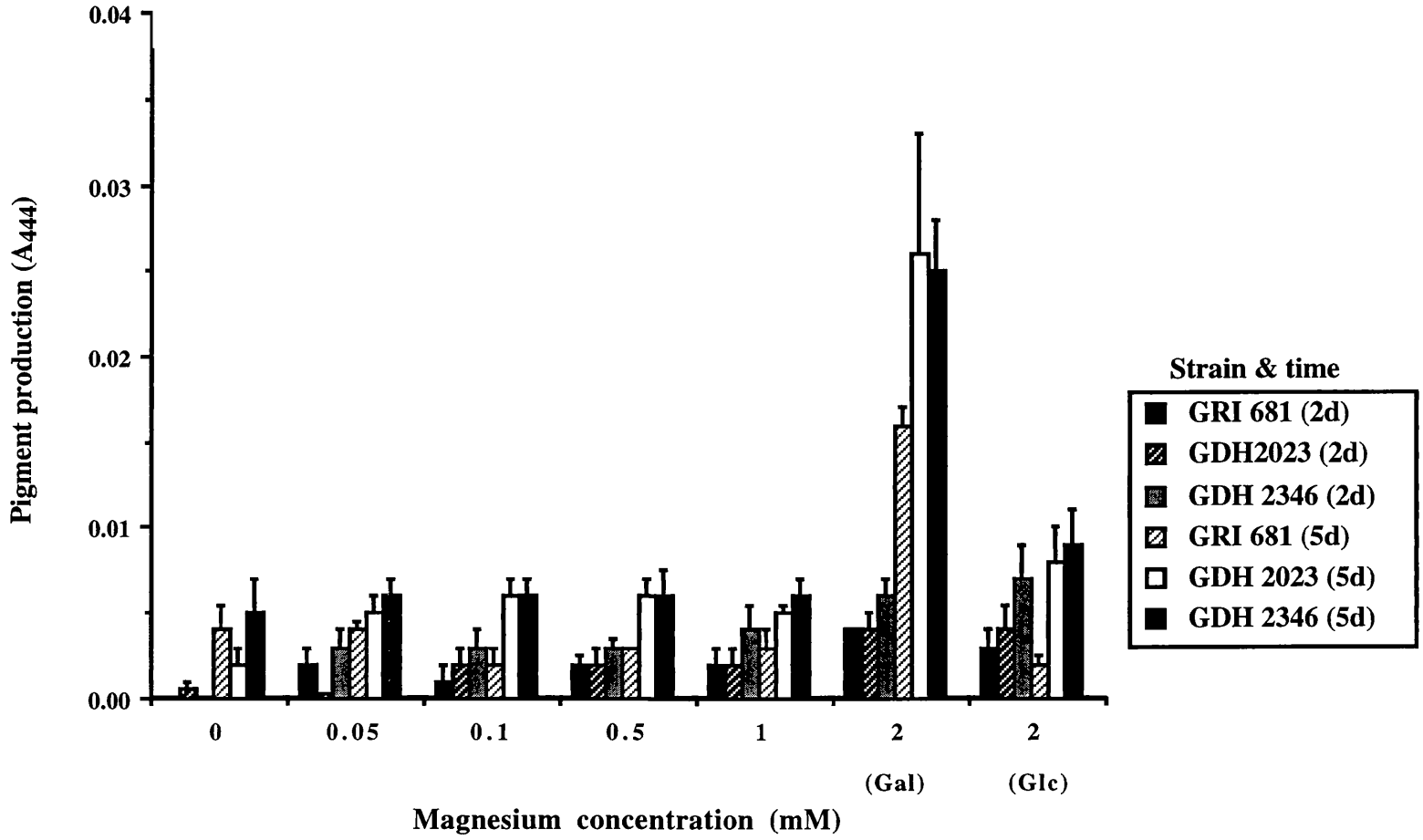

0.04
0.03
0.02
0.01
0.00
Pigment production (A444)
0
0.05
0.1
0.5
1
2
(Gal)
2
(Glc)
Magnesium concentration (mM)
Strain & time
GRI 681 (2d)
GDH2023 (2d)
GDH 2346 (2d)
GRI 681 (5d)
GDH 2023 (5d)
GDH 2346 (5d)

Figure 62.

Growth of *C. albicans* strains GRI 681, GDH 2023 and GDH 2346 in glucose-containing YNB media supplemented with various concentrations of magnesium.

Cultures were incubated at 37°C for 2 days (A) or 5 days (B). Control cultures were grown in YNB with either 50 mM glucose or 500 mM galactose and 2 mM magnesium. Data represent means of four OD_{550} reading $\pm$ SEM

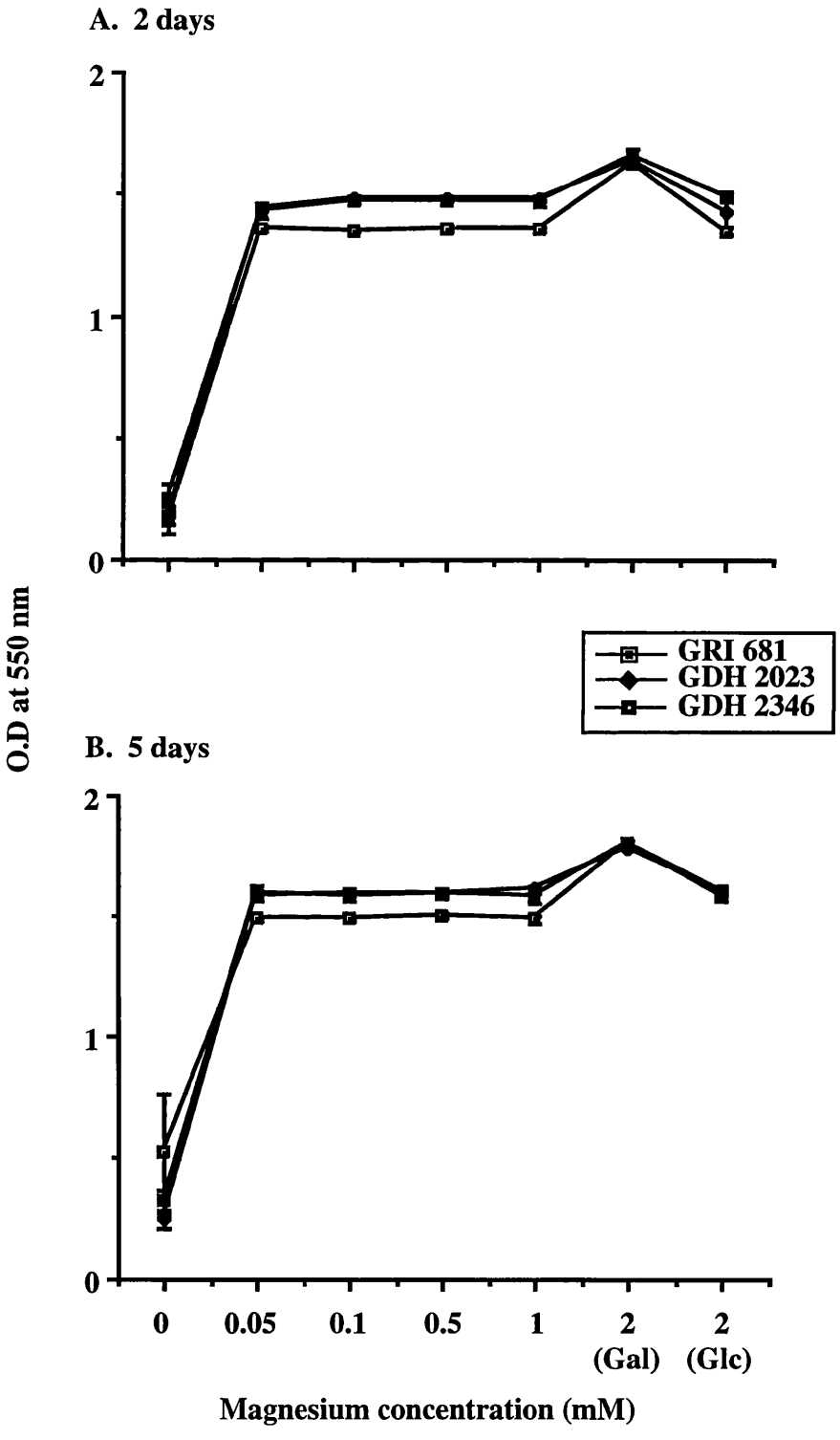

A. 2 days
2
1
0
GRI 681
GDH 2023
GDH 2346
B. 5 days
2
1
0
O.D at 550 nm
0
0.05
0.1
0.5
1
2 (Gal)
2 (Glc)
Magnesium concentration (mM)

Figure 63.

Green pigment production by *C. albicans* strain GRI 681, 'Outbreak' and GDH 2346 grown in YNB medium containing 50 mM glucose supplemented with different concentrations of manganese.

Cultures were incubated at 37°C for 2 or 5 days. Control cultures were grown in YNB with either 50 mM glucose or 500 mM galactose and 1.79 μM manganese. After incubation, cultures were centrifuged and A_{444} values of supernates determined.

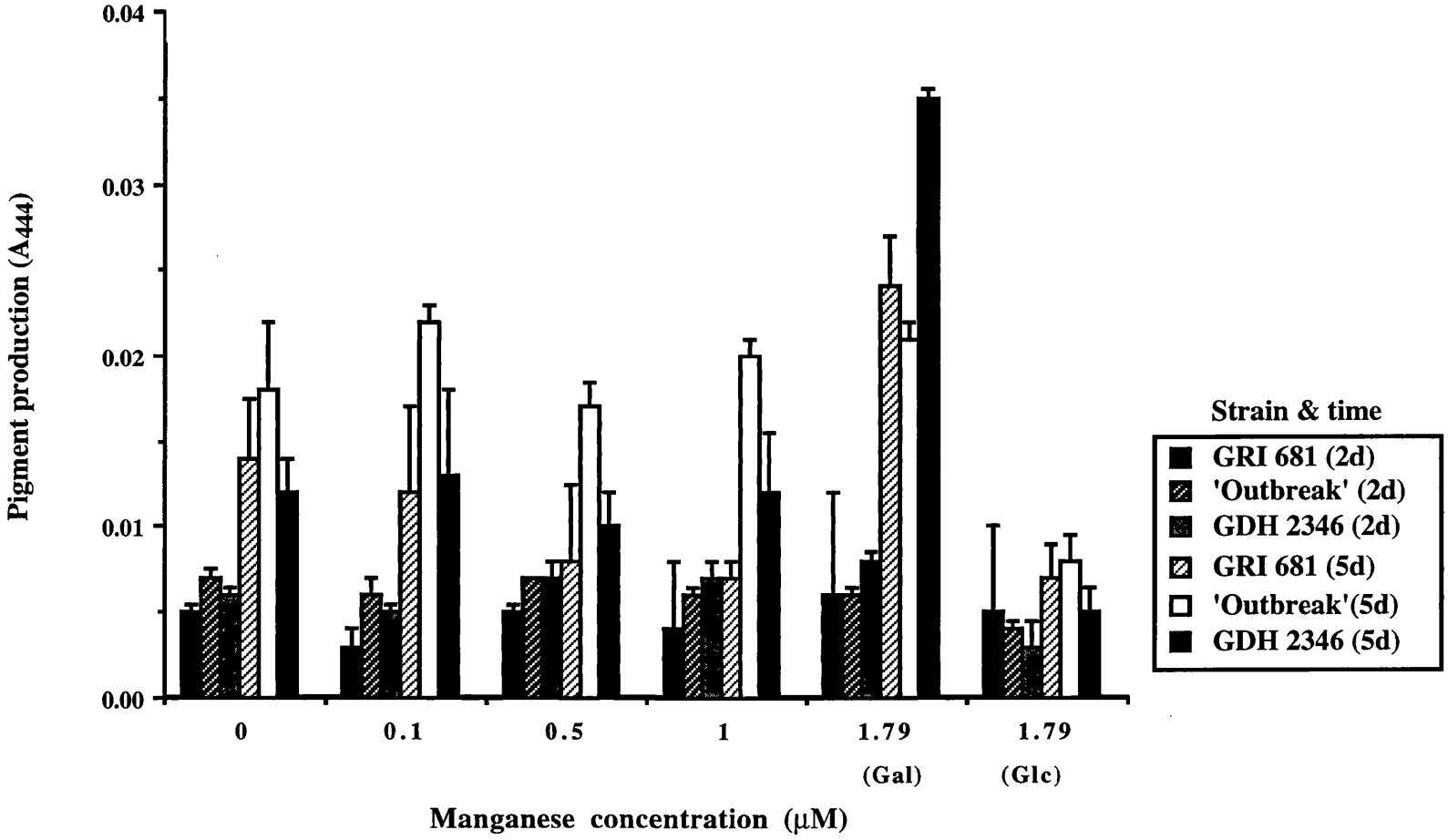
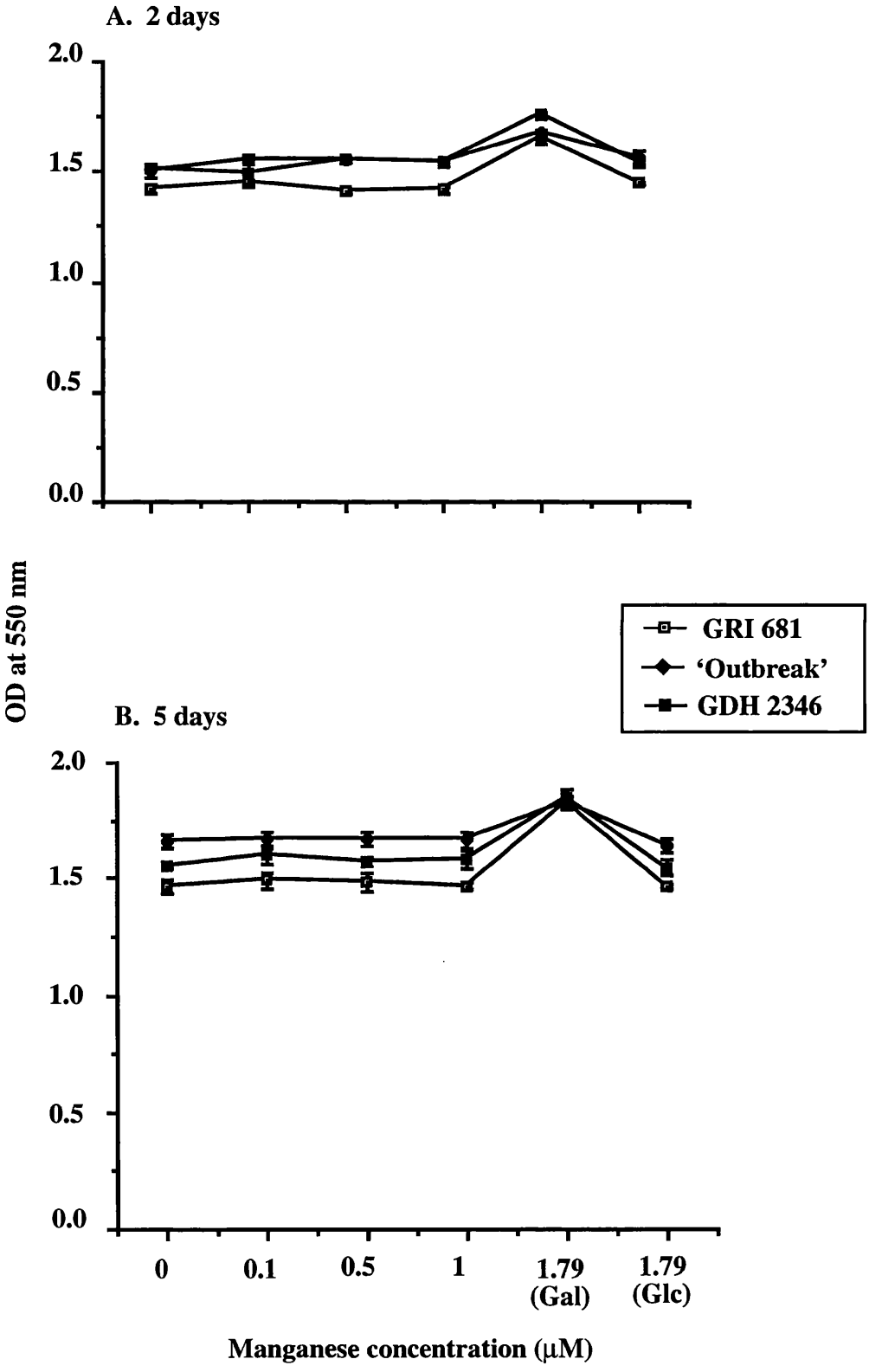

0.04
0.03
0.02
0.01
0.00
Pigment production (A444)
0
0.1
0.5
1
1.79
(Gal)
1.79
(Glc)
Manganese concentration (μM)
Strain & time
GRI 681 (2d)
'Outbreak' (2d)
GDH 2346 (2d)
GRI 681 (5d)
'Outbreak'(5d)
GDH 2346 (5d)

Figure 64.

Growth of *C. albicans* strains GRI 681, 'Outbreak' and GDH 2346 in glucose-containing YNB media supplemented with various concentrations of manganese.

Cultures were incubated at 37°C for 2 (A) or 5 days (B). Control cultures were grown in YNB with either 50 mM glucose or 500 mM galactose and 1.79 μM manganese. Data represent means of four OD_{550} reading ± SEM.


A. 2 days
2.0
1.5
1.0
0.5
0.0
OD at 550 nm
GRI 681
'Outbreak'
GDH 2346
B. 5 days
2.0
1.5
1.0
0.5
0.0
0
0.1
0.5
1
1.79 (Gal)
1.79 (Glc)
Manganese concentration (μM)

medium components to supply the organisms' needs even in the absence of exogenous manganese. All cultures consisted exclusively of yeast-form cells.

9.4. Green pigment production in yeast nitrogen base medium with different zinc concentrations

In this study, the same three strains of *C. albicans* were grown in media containing $ZnSO_4$ concentrations ranging from 0-1 μM. Cultures were incubated as before at 37°C with shaking for 2 and 5 days. Control cultures contained 500 mM galactose or 50 mM glucose with 1.39 μM zinc (the concentration of zinc in YNB powder). Different zinc concentrations had a minimal effect on green pigment production as compared with the effect of iron limitation (Fig. 65). Media containing zinc concentrations of 0.5 or 1μM appeared to promote the greatest pigment synthesis after 5 days . However, good growth was obtained with all three strains even in media containing no added zinc, especially after 5 days (Fig. 66). This is again probably due to traces of zinc salts present in other medium components. All cultures consisted exclusively of yeast-form cells.

9.5. Green pigment production in yeast nitrogen base medium with different copper concentrations

The effect of copper on green pigment production was also investigated. This time, the three strains of *C. albicans* were grown in YNB media containing $CuSO_4$ concentrations ranging from 0 to 0.1 μM, and the cultures were incubated at 37°C with shaking for 2 or 5 days. Control cultures contained 500 mM galactose or 50 mM glucose with 0.16 μM copper (the concentration of copper in YNB powder). Different copper concentrations appeared to have a minimal effect on pigment production which was substantially less than that observed under conditions of iron limitation (Fig. 67). However, all strains tested showed good growth even in the absence of added copper (Fig. 68), suggesting that other medium components were supplying sufficient copper salts to fulfil the

Figure 65.

Green pigment production by *C. albicans* strain GRI 681, 'Outbreak' and GDH 2346 grown in YNB medium containing 50 mM glucose supplemented with different concentrations of zinc.

Cultures were incubated at 37°C for 2 or 5 days. Control cultures were grown in YNB with either 50 mM glucose or 500 mM galactose and 1.39 μM zinc. After incubation, cultures were centrifuged and A_{444} values of supernates determined.

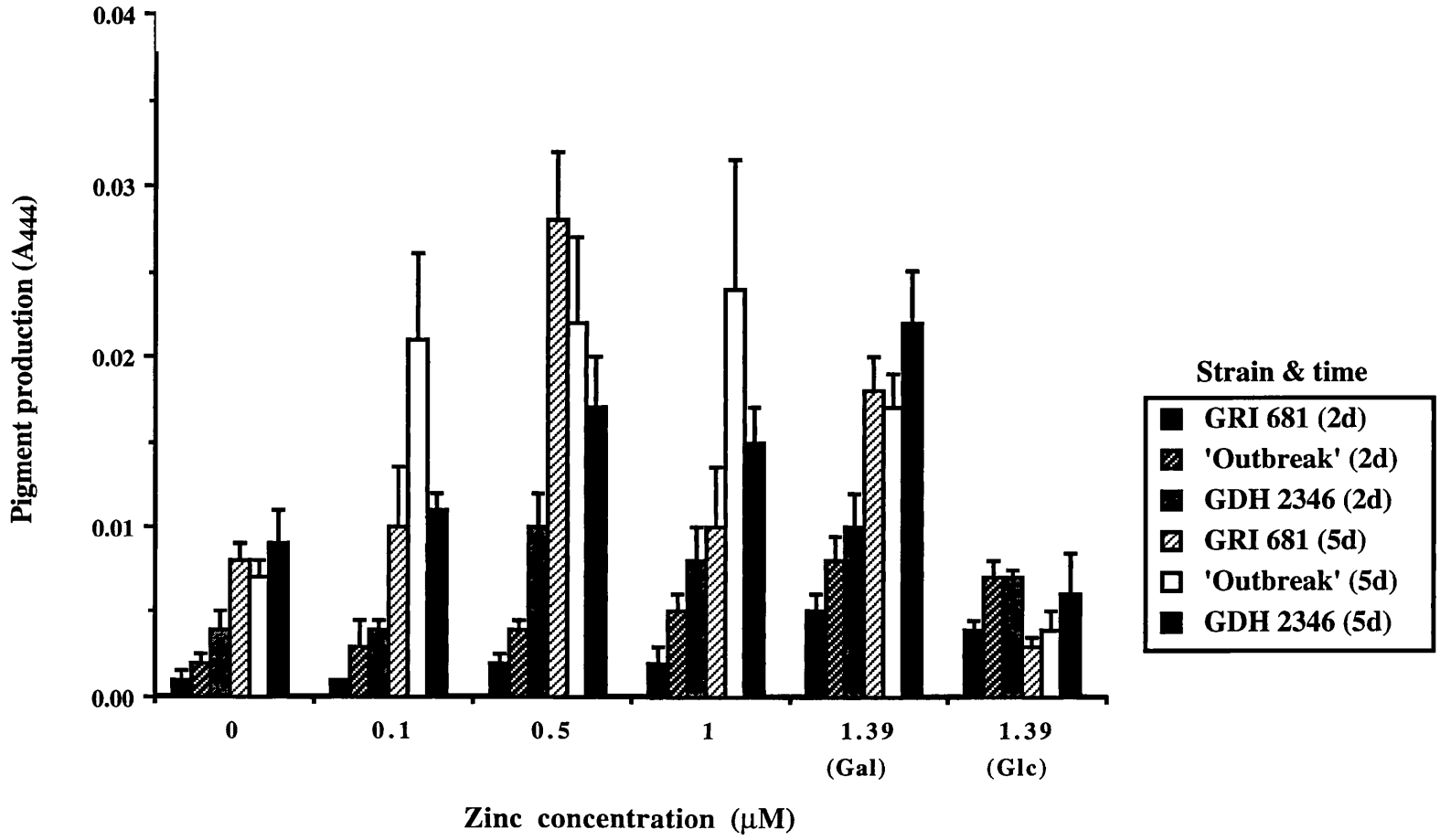

0.04
0.03
0.02
0.01
0.00
Pigment production (A444)
0
0.1
0.5
1
1.39 (Gal)
1.39 (Glc)
Zinc concentration (μM)
Strain & time
GRI 681 (2d)
'Outbreak' (2d)
GDH 2346 (2d)
GRI 681 (5d)
'Outbreak' (5d)
GDH 2346 (5d)

Figure 66.

Growth of *C. albicans* strains GRI 681, 'Outbreak' and GDH 2346 in glucose-containing YNB media supplemented with various concentrations of zinc.

Cultures were incubated at 37°C for 2 (A) or 5 days (B). Control cultures were grown in YNB with either 50 mM glucose or 500 mM galactose and 1.39 μM zinc. Data represent means of four OD_{550} readings ± SEM.

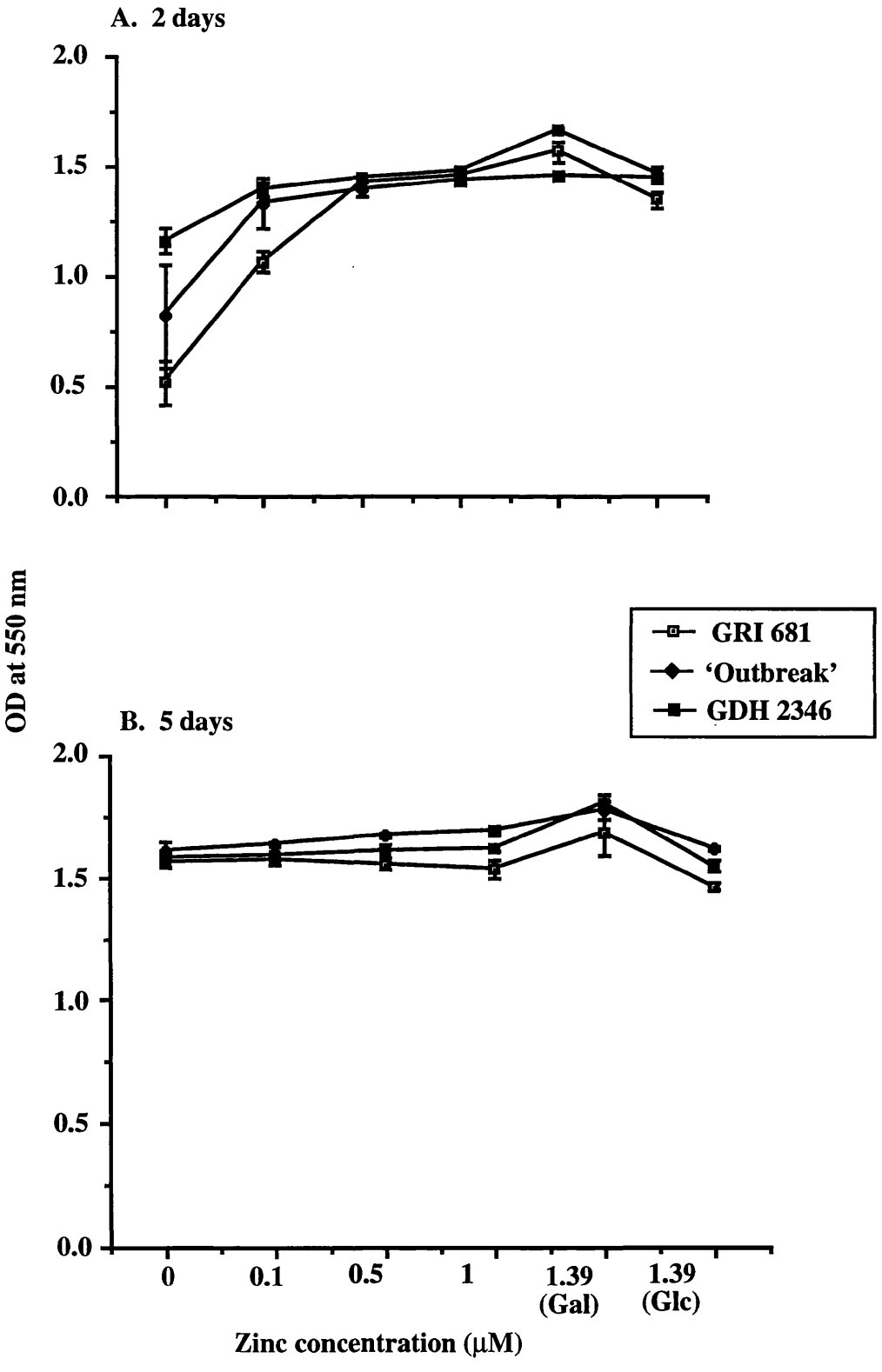

A. 2 days
2.0
1.5
1.0
0.5
0.0
OD at 550 nm
GRI 681
'Outbreak'
GDH 2346
B. 5 days
2.0
1.5
1.0
0.5
0.0
0
0.1
0.5
1
1.39 (Gal)
1.39 (Glc)
Zinc concentration (μM)

Figure 67.

Green pigment production by *C. albicans* strain GRI 681, 'Outbreak' and GDH 2346 grown in YNB medium containing 50 mM glucose supplemented with different concentrations of copper.

Cultures were incubated at 37°C for 2 or 5 days. Control cultures were grown in YNB with either 50 mM glucose or 500 mM galactose and 0.16 μM copper. After incubation, cultures were centrifuged and A_{444} values of supernates determined.

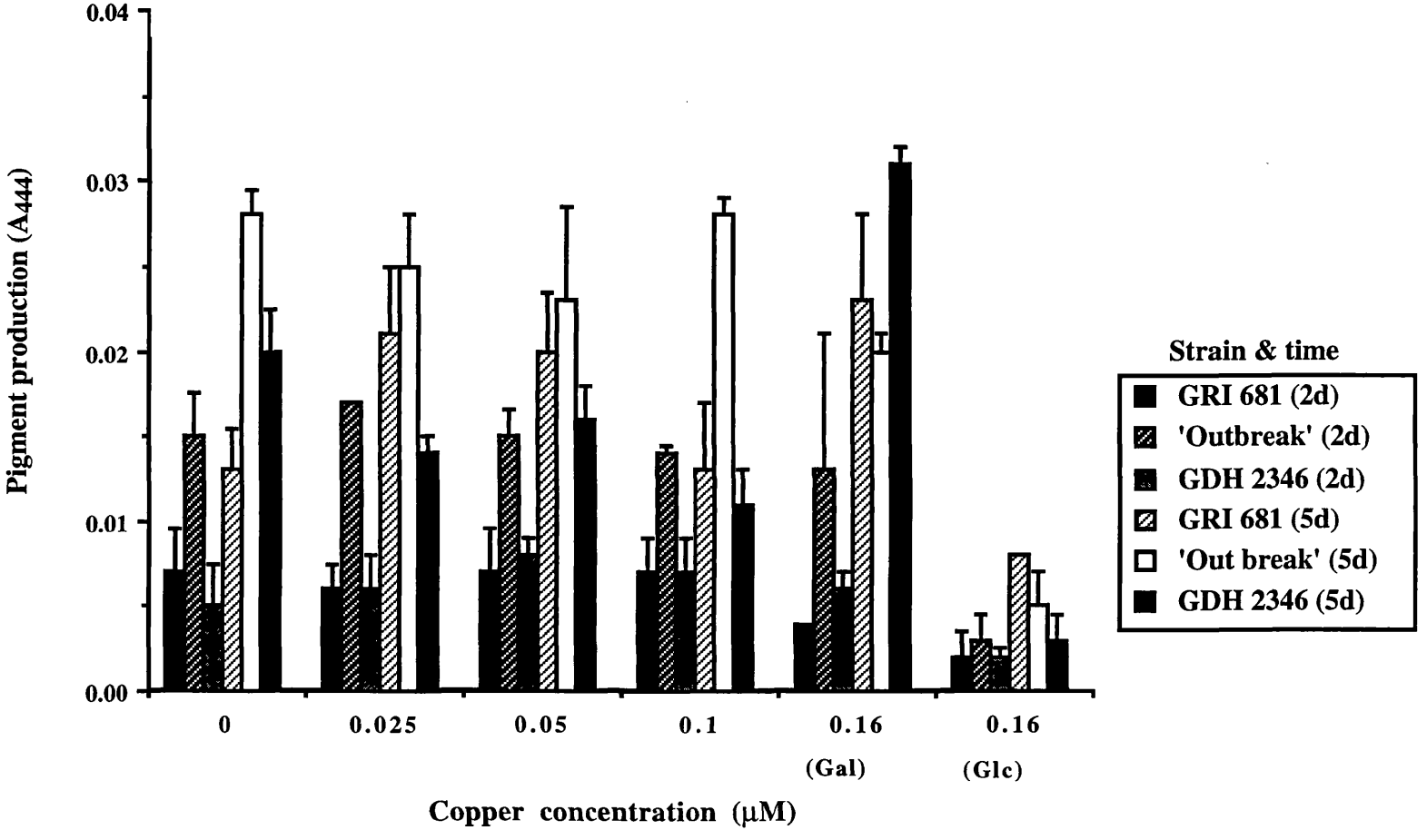
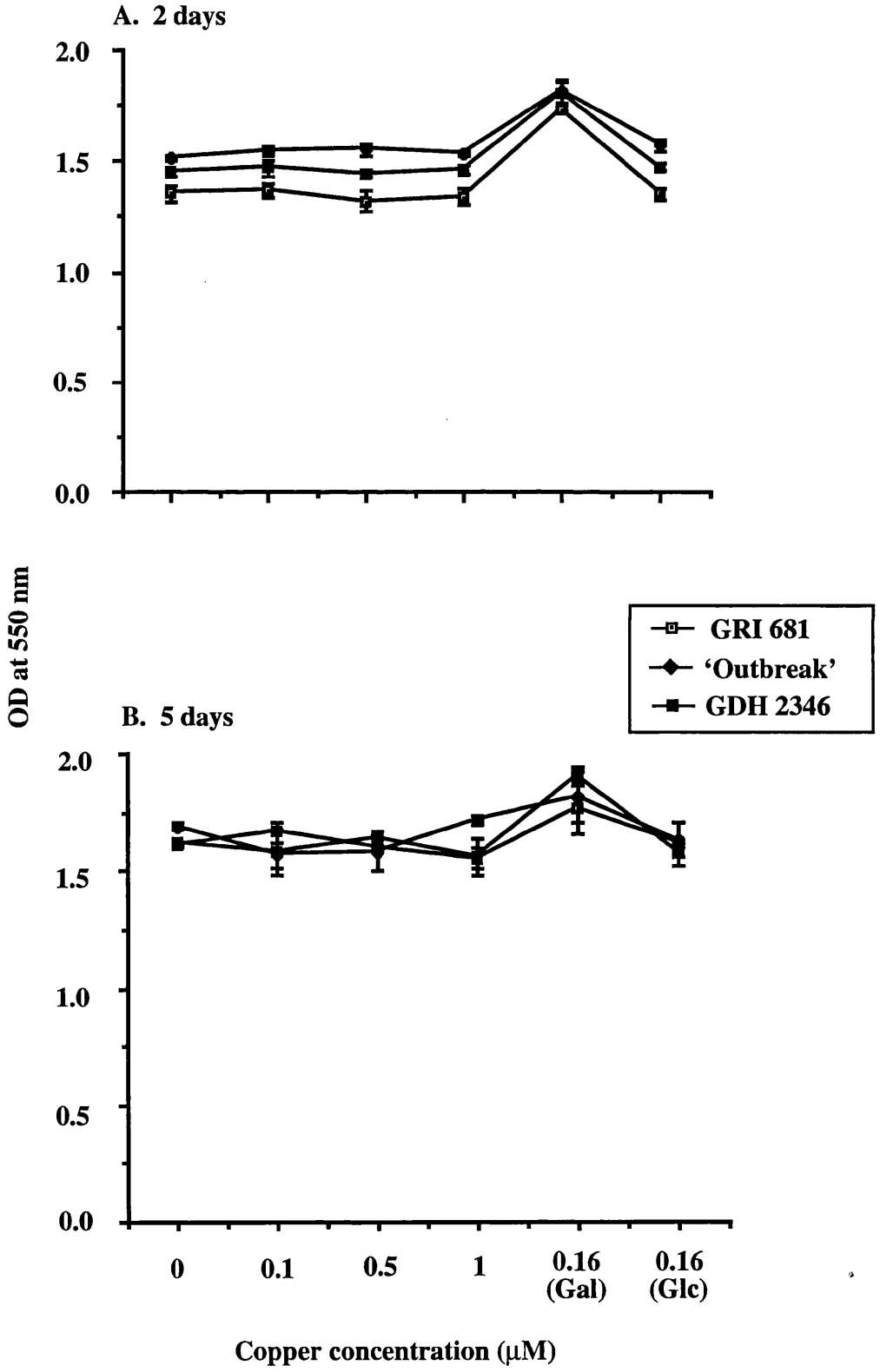

0.04
0.03
0.02
0.01
0.00
Pigment production (A444)
0
0.025
0.05
0.1
0.16
(Gal)
0.16
(Glc)
Copper concentration (μM)
Strain & time
GRI 681 (2d)
'Outbreak' (2d)
GDH 2346 (2d)
GRI 681 (5d)
'Out break' (5d)
GDH 2346 (5d)

Figure 68.

Growth of *C. albicans* strains GRI 681, 'Outbreak' and GDH 2346 in glucose- containing YNB media supplemented with various concentrations of copper.

Cultures were incubated at 37°C for 2 (A) or 5 days (B). Control cultures were grown in YNB with either 50 mM glucose or 500 mM galactose and 0.16 μM copper. Data represent means of four OD_{550} readings ± SEM.


A. 2 days
2.0
1.5
1.0
0.5
0.0
OD at 550 nm
GRI 681
'Outbreak'
GDH 2346
B. 5 days
2.0
1.5
1.0
0.5
0.0
0
0.1
0.5
1
0.16 (Gal)
0.16 (Glc)
Copper concentration (μM)

organisms' requirements. When cultures were examined microscopically they were seen to consist entirely of yeast cells and no hyphae were observed.

10. Pink pigment production by *C. albicans*

For comparison with the green pigment, pink pigment production by *C. albicans* was also studied. Pink pigment was obtained when *C. albicans* strains were grown in tryptophan medium supplied with $FeSO_4$. Previous studies revealed that *Candida* species can synthesise indole or tryptophan derivatives. For example, Schindler and Zahner (1971) observed that *C. lipolytica* produces reddish-brown pigments after the addition of tryptophan. Chaskes and Phillips (1974) showed that when *C. albicans* and two other medically important *Candida* species, *C. tropicalis* and *C. parapsilosis* , were cultured on glucose-salts-biotin media containing tryptophan as the major nitrogen source, they all produced a pink pigment. Here, the effect of iron supplementation of the medium and the use of different nitrogen sources on pink pigment production were investigated with *C. albicans*.

10.1. Pink pigment production in glucose-salts-biotin medium containing tryptophan as the major nitrogen source with or without added iron.

When cultured in a chemically defined medium having tryptophan as the major nitrogen source at 30°C for 24, 48, and 72 hours, *C. albicans* 'Outbreak' strain produced a pink pigment; this was only noted with media containing iron (0.2 g/litre $FeSO_4$). Quantitative measurements of pink pigment production at 535 nm showed large differences between two media tested. An OD_{535} reading of 0.224 ± 0.011 (mean $\pm$ SEM) was obtained with medium containing tryptophan as nitrogen source and supplied with iron, as compared with an OD_{535} reading of 0.036 ± 0.010 (mean $\pm$ SEM) for medium containing tryptophan as nitrogen source and no added iron (Fig. 69). Pure yeast forms were noted in both media but

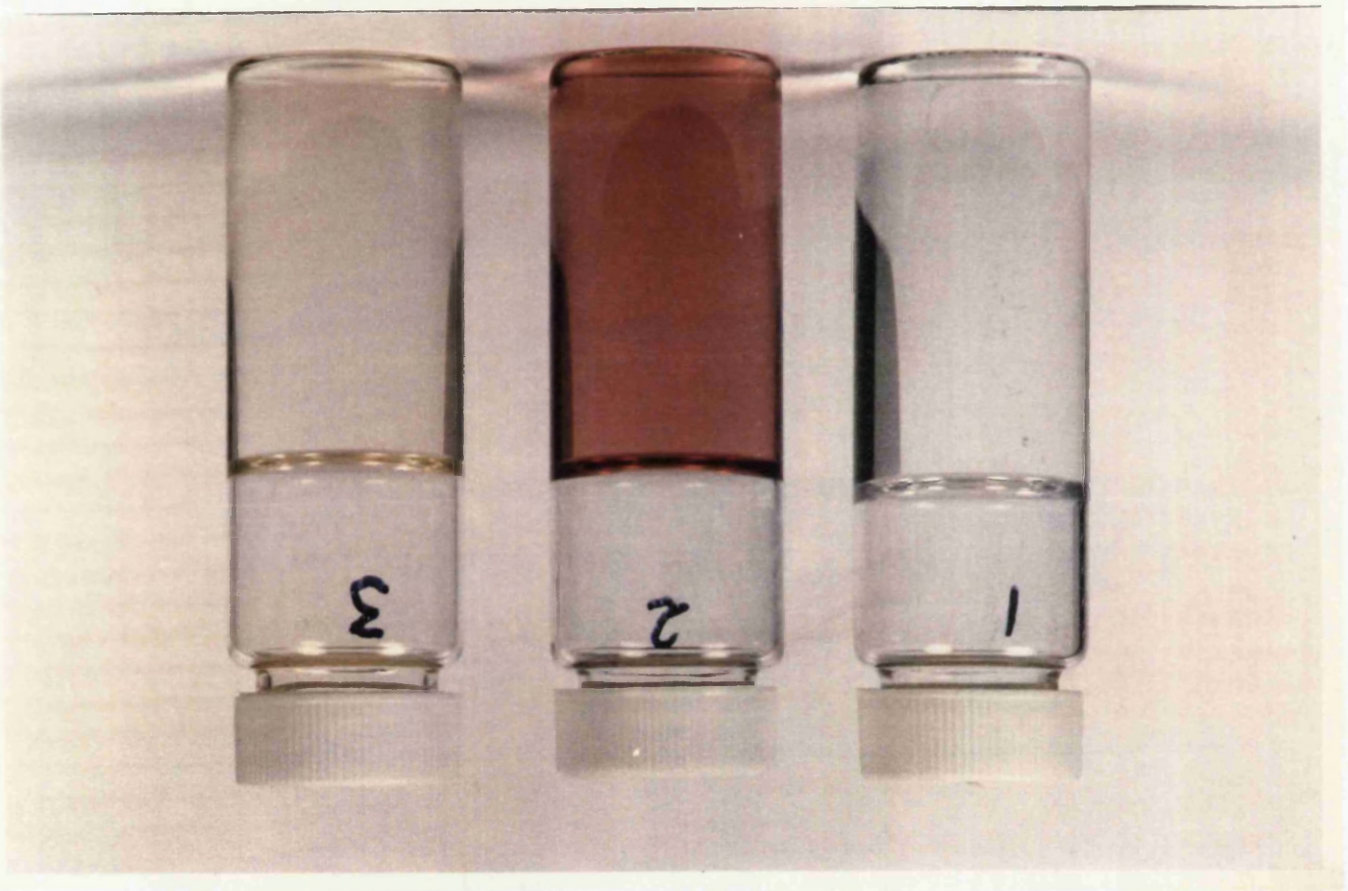
Figure 69.

Pink pigment production by *C. albicans* 'Outbreak' strain after growth on glucose-salts-biotin medium containing tryptophan as the major nitrogen source.

Sample 1. Sterile glucose-salts-biotin medium containing tryptophan as the major nitrogen source

Sample 2. Culture supernate showing pink pigment in glucose-salts-biotin medium containing tryptophan and iron (0.2g/litre $FeSO_4$) following incubation at 30°C for 24h.

Sample 3. Culture supernate showing absence of pink pigment in glucose-salts-biotin medium containing tryptophan but no iron following incubation at 30°C for 24h.


3
2
1

the culture containing pink pigment also had occasional giant yeast cells with a diameter two to three times that of normal yeasts.

10.2. Pink pigment production in glucose-salts-biotin medium containing proline as the major nitrogen source, with or without added iron

C. albicans 'Outbreak' strain was grown in glucose-salts-biotin medium containing proline as the nitrogen source, with or without added iron (0.2 g/litre $FeSO_4$) at 30°C for 24, 48, and 72 hours. No pigment was noted in this proline medium even in the presence of exogenous iron. These results indicate that tryptophan is the constituent responsible for the production of a pink pigment and that iron enhances pigment formation.

11. The effect of physical and chemical agents on the green and pink pigments

11.1. The effect of light

Filter-sterilised supernates containing pink or green pigment produced by *C.albicans* 'Outbreak' strain were placed in sterile glass tubes and exposed near a window to ordinary laboratory light/daylight.

Exposure to ordinary light had a marked effect on the green pigment, which is unstable and becomes colourless after a few days. Exposure of the sterile pink supernates for four months caused changes in colour eventually to light orange (Fig. 70)

11.2. The effect of adding acid or alkali

The effect of adding acid (1 M HCl) or alkali (1 M NaOH) to both green and pink pigments was studied (Chaskes and Phillips, 1974). The addition of either acid or alkali had no effect on the green pigment in culture supernates which had an initial pH value of 2.92; the yellow-greenish colour was unchanged. On the other hand, a substantial effect was observed with the pink pigment in

Figure 70.

The effect of physical and chemical agents on the green and pink pigments.

Tube 1. Pink pigment produced by *C. albicans* 'Outbreak' strain in tryptophan medium containing iron (0.2 g $FeSO_4$ / litre) after 48 h incubation at 30°C.

Tube 2. Pink pigment changes to orange colour after exposure near window to ordinary laboratory light for four months.

Tube 3. Pink pigment changes to light orange colour after dropwise addition of 1 M NaOH.

Tube 4. Pink pigment regains pink colour after dropwise addition of 1 M HCl.

Tube 5. Green pigment (yellow-greenish) produced by *C. albicans* 'Outbreak' strain in deferrated YNB medium containing 0.026 μM iron after 5 d incubation at 37°C.

Tube 6. Green pigment becomes completely colourless after exposure near window to ordinary laboratory light for few days.

Tube 7. No change in colour of green pigment following dropwise addition of 1 M NaOH.

Tube 8. No change in colour of green pigment following dropwise addition of 1 M HCl.

1
2
3
4
5
6
7
8

culture supernates with an initial pH of 3.5; the pink pigment was sensitive to alkali and on addition of sodium hydroxide it converted to a light orange colour, returning to pink with the addition of acid (Fig. 70)

12. Extraction of green and pink pigments

12.1. Extraction of green pigment

Culture supernates containing the green pigment produced by *C. albicans* 'Outbreak' strain (the highest pigment producer) were collected after growth of the organism in deferrated YNB containing 50 mM glucose and 0.026 μM iron (optimum conditions for green pigment production). The cell pellets were retained and both these and culture supernates were freeze-dried. Different solvents were then examined for the best extraction of green pigment.

The green pigment was found in culture supernates and not in the cells; it was soluble in acetone, water and acetic acid. The pigment appeared to be almost insoluble in amyl alcohol and ethyl acetate (Table 29). Based on these findings, green pigment was prepared by extracting 500 mg of freeze-dried culture supernate with 100 ml acetone; a rotary evaporator was used to concentrate the pigment. Pigment was also extracted in bulk using ethanol as a solvent. Both acetone-extracted and ethanol-extracted material was analysed by thin-layer chromatography.

12.2. Extraction of pink pigment

Culture supernates containing pink pigment were obtained from *C. albicans* 'Outbreak' strain after growth in glucose-salts-biotin medium containing tryptophan under optimum conditions for pink pigment production. Both culture supernates and cell pellets were freeze-dried. The pink pigment was soluble in water and ethanol, and was found in culture supernates but not in cells.

Table 29. **Extraction of green pigment using different solvents.**

	Absorbance (A_{444}) of extract from	
Solvent used for extraction	**Culture supernate**	**Cell pellet**
Acetone	0.052	0
Acetic acid	0.066	0
Ethyl acetate	0.007	0.005
Water	0.056	0.023
Amyl alcohol	0.039	0

13. Thin layer chromatography (TLC)

13.1. TLC of the green pigment

Crude pigment preparations (acetone extracts) and freeze-dried supernates were subjected to TLC on silica plates containing a fluorescent indicator. Various solvents were tested for their ability to separate the green pigment and *Rf* values were recorded (Table 30). The butanol: ethanol: water (BEW) system separated culture supernates into two components visible under UV light, a yellow fluorescent band with an *Rf* value of 0.40-0.46 and a blue fluorescent band with an *Rf* value of 0.65-0.70 (Fig. 71). Chromatography of acetone-extracted or ethanol-extracted material in this solvent, however, gave poor resolution of individual components.

The butanol: acetic acid : water (BAW) system also revealed several bands (Fig. 72) when freeze-dried supernates were applied to the TLC plates. There was a yellow visible band with an *Rf* value of 0.39 and three UV bands, namely a blue fluorescent band (*Rf* 0.52- 0.54), a yellow fluorescent band (*Rf* 0.39), and a thin yellow fluorescent band (*Rf* 0.42-0.44). Poor resolution was obtained with the acetone-extracted green pigment in this solvent.

The green pigment separated into two fluorescent bands and one visible band in the propanol : ethyl acetate : water (7:1:2) system (Fig. 73); these comprised a blue fluorescent band (*Rf* 0.67-0.68), a yellow fluorescent band (*Rf* 0.55) and a yellow visible band (*Rf* 0.55). Ethanol : water : NH_3 (18:1:1) gave good separation of the green pigment (Fig. 74) with a visible yellow band (*Rf* 0.25-0.30) and three fluorescent bands (a very thin yellow fluorescent band, *Rf* 0.44-0.51; a thick yellow fluorescent band, *Rf* 0.25-0.30; and a blue fluorescent band, *Rf* 0.75-0.76). In methanol: water : pyridine (20:5:1), a yellow fluorescent band (*Rf* 0.79-0.80) appeared to migrate ahead of a blue fluorescent band (*Rf* 0.71-0.72) under UV light (Fig. 75), unlike previous solvent systems when a blue fluorescent bands travelled ahead of one or more yellow fluorescent bands.

Table 30. Separation of green pigment by TLC using different solvent systems.

Solvent system	Band visible under daylight	Band visible under UV light	*Rf* (x100)
Butanol: ethanol: water (4:1:1) [a]	Yellow		40-46 [b]
		Yellow fluorescent	40-46
		Blue fluorescent	65-70
Butanol: ethanol: water (4:1:1) [c]	Yellow		40-45
		Yellow fluorescent	40-45
		Blue fluorescent	64-67
Butanol: acetic acid: water (65:10:25) [a]	Yellow		39-39
		Upper yellow fluorescent	42-44
		Lower yellow fluorescent	39-39
		Blue fluorescent	52-54
Butanol: acetic acid: water (65:10:25) [c]	-		
		Yellow fluorescent, trailing	56-70
Butanol: pyridine: water (1:1:1) [a]	Yellow		66-72
		Upper yellow fluorescent	80-76
		Lower yellow fluorescent	72-66
		Blue fluorescent	89-81
Methanol: water: pyridine (20:5:1) [a]	Yellow		79-80
		Yellow fluorescent	79-80
		Blue fluorescent	71-72
Propanol: ethyl acetate: water (7:1:2) [a]	Yellow		55-55
		Yellow fluorescent	55-55
		Blue fluorescent	67-68
Ethyl acetate: pyridine: water (12:5:4) [a]	Yellow		63-63
		White fluorescent	63-63
		No blue fluorescent	-
Acetic acid: conc. HCl: water (30:3:10) [a]	Yellow		72-72
		Yellow trailing	72-72
		No blue fluorescent	-
Ethanol: water: NH_3 (18:1:1) [a]	Yellow		25-30
		Lower yellow fluorescent	25-30
		Upper yellow fluorescent	44-51
		Blue fluorescent	75-76
Phenol saturated with water [a]	Yellow, trailing		73-76
		Blue fluorescent	9-9

Cont. Table 30.

Solvent system	Bands visible under daylight	Bands visible under UV light	*Rf* (x100)
Phenol sat. H_2O : ethanol: water (15:4:1) [a]	-		-
		Blue fluorescent	29-27
Chloroform: formic acid: water (125:73:3) [a]	-		-
		Blue fluorescent, trailing	-
Chloroform: acetic acid: water (125:73:3) [a]	Yellow		17-16
		Yellow fluorescent	17-16
		Lower blue fluorescent	24-22
		Upper blue fluorescent	40-41
5% ammonium formate and 0.5% formic acid [a]	Yellow		36-36
		Yellow fluorescent	36-36
		Blue fluorescent	21-20
Peteroleum ether: diethyl ether: acetic acid (70:30:1) [a]	No separation was noted	-	-
Chloroform: methanol: water : acetic acid (75:25:3:8) [a]	Yellow		51-54
		Yellow fluorescent	51-54
		Blue fluorescent	75-81

[a] Sample applied was freeze-dried culture supernate of deferrated YNB with 0.026 µM iron containing a high concentration of green pigment (A_{444} value of up to 1.232) dissolved in deionized distilled water (1 mg / 1ml). A 20µL volume was applied to TLC plates.

[b] Figures represent values obtained from two separate plates.

[c] Sample applied was acetone-extracted green pigment.

Figure 71.

Thin layer chromatography of the green pigment (culture supernate) in butanol : ethanol : water (4:1:1)

The chromatogram was viewed under UV illumination.

A. Blue fluorescent band (*Rf* 0.65-0.70).

B. Yellow fluorescent band (*Rf* 0.40-0.46).

C. Origin.

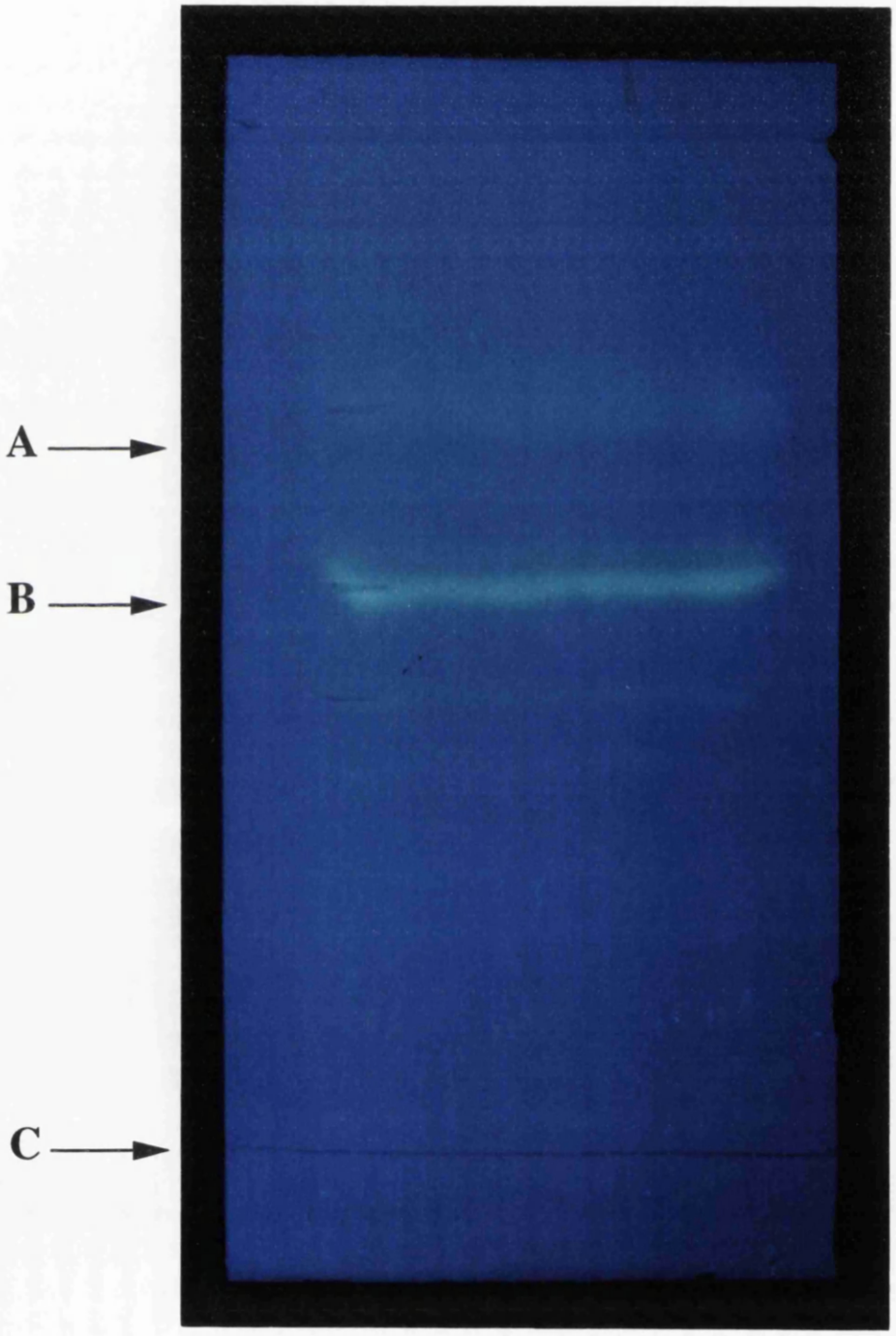

A
B
C

Figure 72.

Thin layer chromatography of the green pigment (culture supernate) in butanol : acetic acid : water (65:10:25)

The chromatogram was viewed under UV illumination.

A. Blue fluorescent band (*Rf* 0.52-0.54).

B. Yellow fluorescent band (*Rf* 0.42-0.44).

C. Yellow fluorescent band (*Rf* 0.39).

D. Origin.

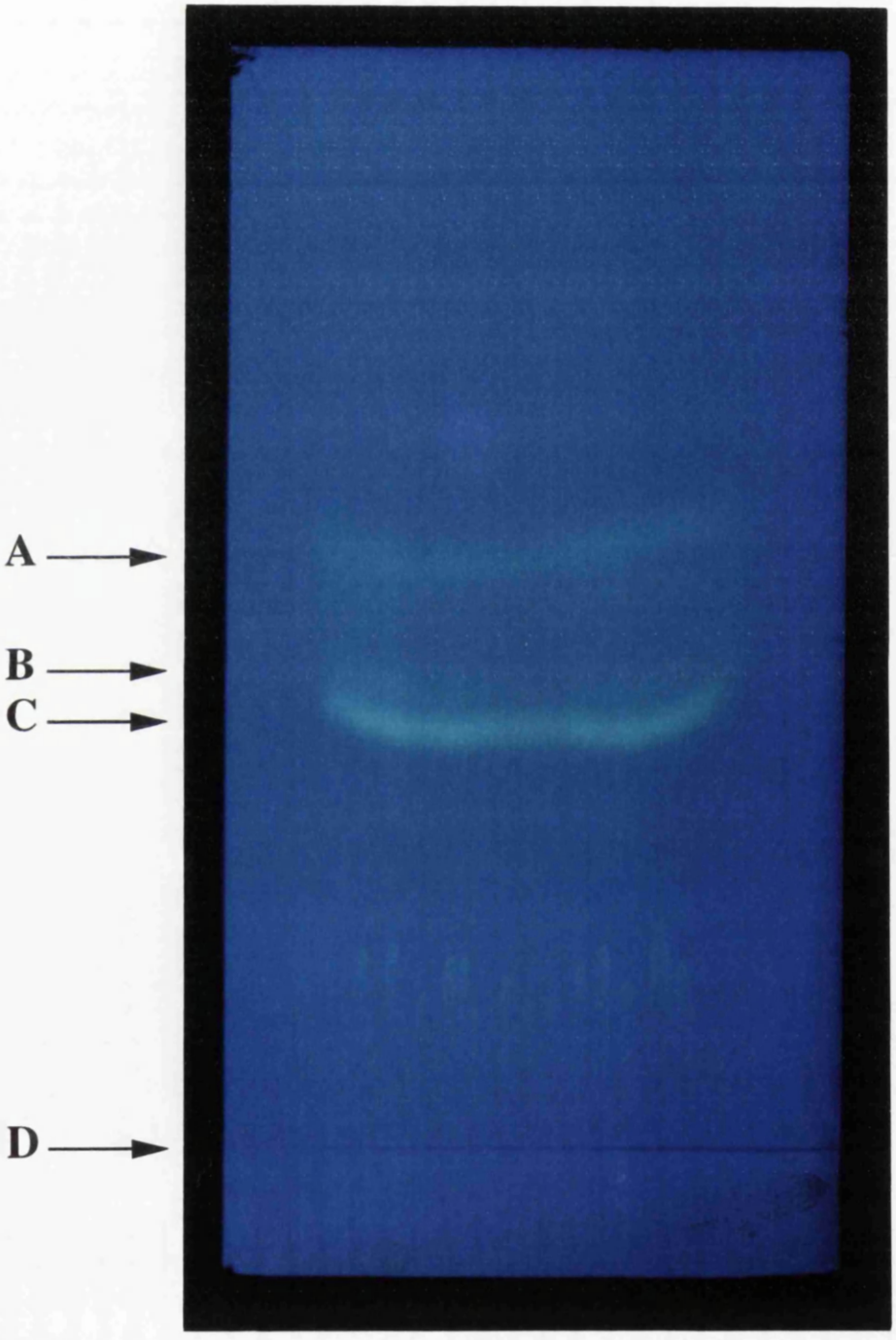

A
B
C
D

Figure 73.

Thin layer chromatography of the green pigment (culture supernate) in n-propanol : ethyl acetate : water (7:1:2)

The chromatogram was viewed under UV illumination.

A. Blue fluorescent band (*Rf* 0.67-0.68).

B. Yellow fluorescent band (*Rf* 0.55).

C. Origin.

Thin layer Chromatography, solvent system of
n-propanol, ethyl acetate and water
(7:1:2)

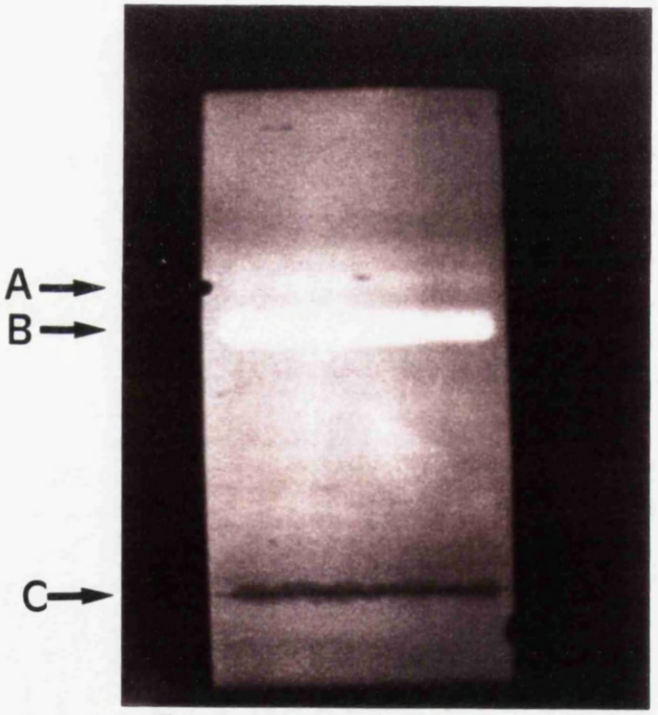


Figure 74.

Thin layer chromatography of the green pigment (culture supernate) in ethanol : water : NH_3 (18:1:1)

The chromatogram was viewed under UV illumination.

A. Blue fluorescent band (*Rf* 0.75-0.76).

B. Yellow fluorescent band (*Rf* 0.44-0.51).

C. Yellow fluorescent band (*Rf* 0.25-0.30).

D. Origin.

Thin layer Chromatography, solvent system of
Ethanol, water and NH_3
(18:1:1:)

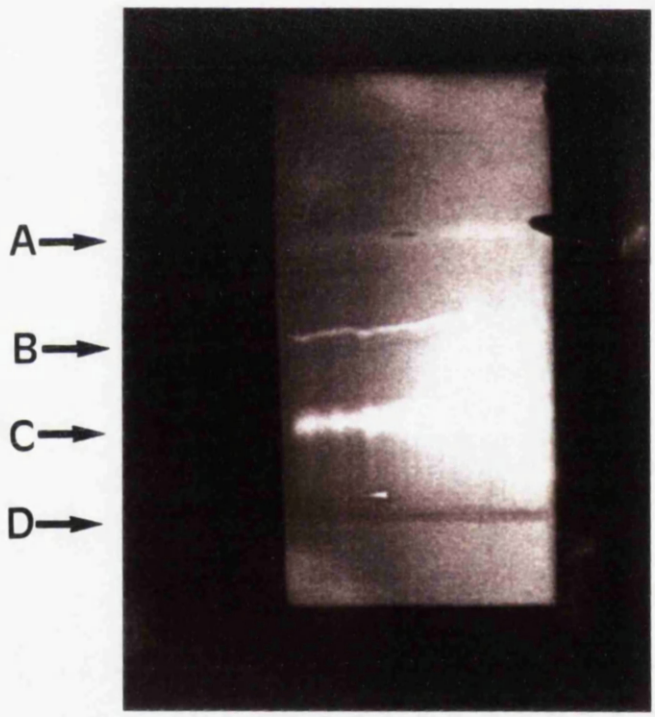


Figure 75.

Thin layer chromatography of the green pigment (culture supernate) in methanol : water : pyridine (20:5:1)

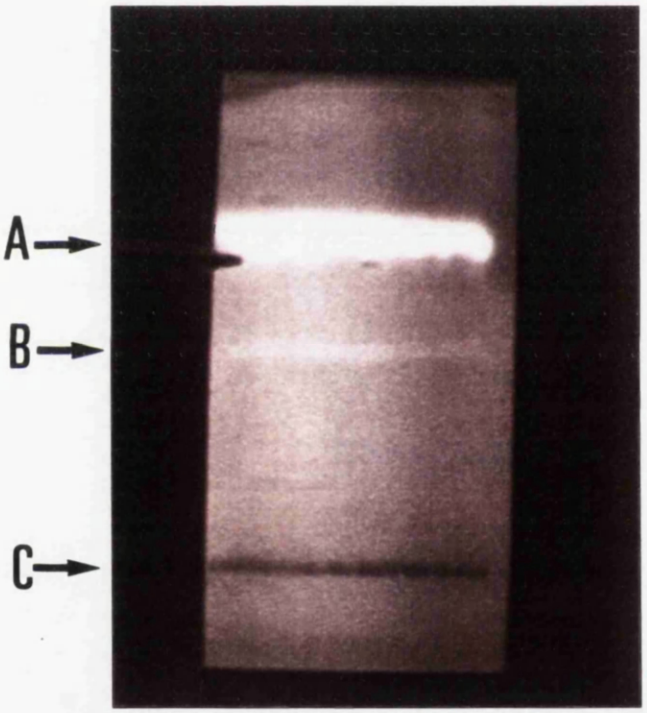
The chromatogram was viewed under UV illumination.

A. Yellow fluorescent band (Rf 0.79-0.80).

B. Blue fluorescent band (Rf 0.71-0.72).

C. Origin.

Thin layer Chromatography, solvent system of
Methanol, water and pyridine
(20:5:1)



A solvent system of 5% ammonium formate and 0.5% formic acid which is used for the separation of catechol-type siderophores produced a visible yellow band (*Rf* 0.36) and two components under UV light, a yellow fluorescent band (*Rf* 0.36) and a blue fluorescent band (*Rf* 0.20-0.21). Petroleum ether : diethyl ether : acetic acid (70:30:1), a solvent system useful for the separation of cholesteryl esters, free fatty acids and cholesterol, showed no separation of the green pigment. Visualisation of components by spraying with 50% sulphuric acid and heating at 120°C for 15-30 min showed that the entire sample had remained at the origin of the chromatogram. Finally, a solvent system of chloroform: methanol: water: acetic acid (75:25:3:8) which is used for separation of phospholipids produced one visible yellow band (*Rf* 0.51-0.54) and two fluorescent components, a blue fluorescent band (*Rf* 0.75-0.81) and a yellow fluorescent band (*Rf* 0.51-0.54). When plates were sprayed with 50% sulphuric acid and heated at 120°C for 15-30 min, no brown spots were noted in positions corresponding to those of the blue or yellow fluorescent components. However, when YNB medium was chromatographed as a control, and the plate treated similarly, a brown spot with an *Rf* value of 0.29-0.30 was observed. These results indicate that the green pigment is unlikely to be lipid in nature (Fig. 79)

Overall, the results of this part of the study show that freeze-dried culture supernates contain a green pigment which can be separated into one visible yellow component (*Rf* values differ depending on the solvent) or two fluorescent components (blue and yellow, respectively) under UV light. The blue fluorescent band in most solvents had a higher *Rf* value than the yellow fluorescent band. The yellow fluorescent band matched and appeared to be identical with the visible yellow band. With some solvents, a very thin yellow fluorescent band appeared. In the case of the solvent system chloroform: acetic acid : water (125:73:3), there were two blue fluorescent bands which travelled ahead of the yellow fluorescent band.

13.2. TLC of culture supernates of yeasts grown in deferrated YNB medium with different iron concentrations

Cultures supernates of *C. albicans* 'Outbreak' strain grown in deferrated YNB medium containing different iron concentrations (0.026, 0.1, 0.2, 0.4, and 0.8 μM) were also analysed by TLC. Freeze-dried supernates which appeared to contain different concentrations of the green pigment, were dissolved in deionized, distilled water and applied to TLC plates containing a fluorescent indicator. Samples of sterile YNB medium and glucose were included as controls; butanol: ethanol : water (4:1:1) was used as the solvent system . When the TLC plates were viewed under UV illumination, all supernates which visibly contained green pigment produced a yellow fluorescent spot with an *Rf* value of 0.40-0.46 and a blue fluorescent spot with an *Rf* value of 0.65-0.70 (Fig. 76). A particularly strong yellow fluorescent and visible spot was noted with supernate containing 0.026 μM iron. No yellow or blue fluorescent (or visible) spots were observed with the controls. These results indicate that the highest pigment production was observed in YNB medium containing 0.026 μM iron and that the green pigment is completely absent from uninoculated YNB medium.

13.3. TLC of the pink pigment

Freeze-dried culture supernates of *C. albicans* 'Outbreak' strain grown in glucose-salts-biotin medium with tryptophan was subjected to TLC in butanol: ethanol : water (4:1:1). A pink component with an *Rf* value of 0.42-0.54 was observed when chromatograms were viewed under normal light. Three fluorescent components were noted under UV light, a purple fluorescent spot with an *Rf* value of 0.42-0.54, a yellow fluorescent spot with an *Rf* value of 0.36-0.38 and a second yellow fluorescent spot with an *Rf* value of 0.62-0.65 (Figs. 77 and 81).

When ethanol extracts of freeze-dried cells or culture supernates were chromatographed on TLC plates, it was clear that the pink pigment was found in culture supernates but not in cells of *C. albicans*. A pink band was visible with

Figure 76.

TLC of green pigment in culture supernates obtained from yeasts grown in deferrated YNB medium with various iron concentrations.

The solvent system used was butanol : ethanol : water (4:1:1) and the chromatogram was viewed under UV illumination.

Samples were applied as follows:

Lane 1. Culture supernate from YNB containing 0.026 μM iron.

Lane 2. Culture supernate from YNB containing 0.1 μM iron.

Lane 3. Culture supernate from YNB containing 0.2 μM iron.

Lane 4. Culture supernate from YNB containing 0.4 μM iron.

Lane 5. Culture supernate from YNB containing 0.8 μM iron.

Lane 6. Yeast nitrogen base containing 50 mM glucose as carbon source.

Lane 7. Yeast nitrogen base containing no glucose.

Lane 8. Glucose (50 mM).

Components were identified as :

A. Blue fluorescent spot (Rf 0.65-0.70).

B. Yellow fluorescent spot (Rf 0.40-0.46).

C. Origin.

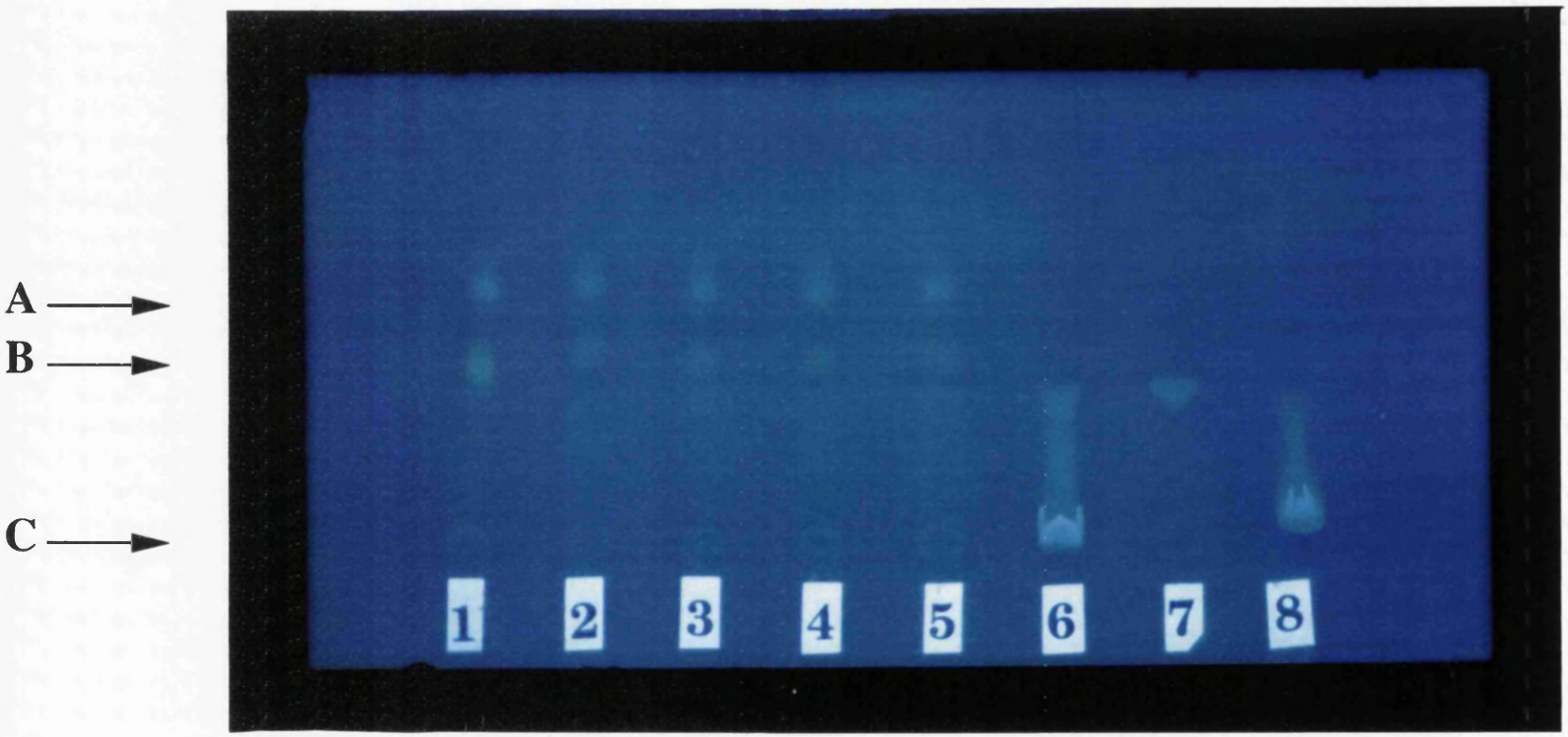

A
B
C
1
2
3
4
5
6
7
8

Figure 77.

TLC of the pink pigment (crude culture supernate)

A solvent system consisting of butanol : ethanol : water (4:1:1) was used and the chromatogram was viewed under UV illumination.

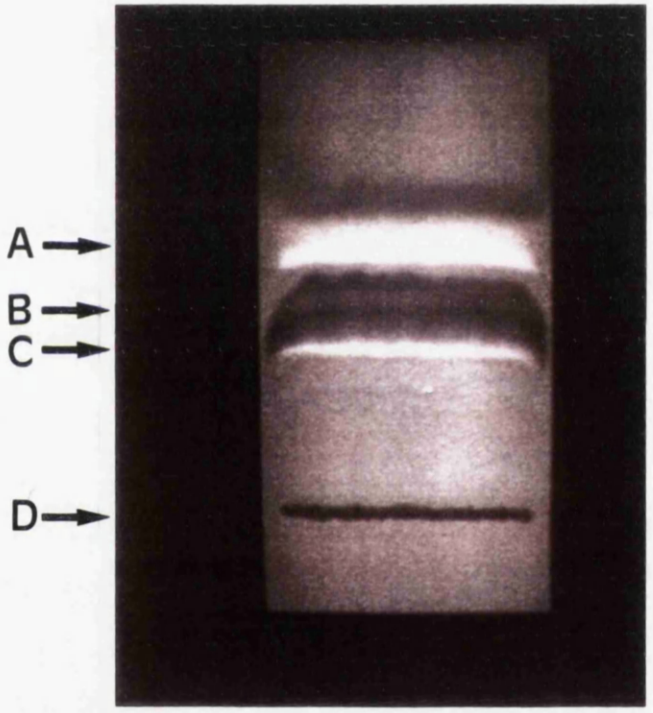
A. Yellow fluorescent band (*Rf* 0.62-0.65).

B. Purple fluorescent band (*Rf* 0.42-0.54).

C. Yellow fluorescent band (*Rf* 0.36-0.38).

D. Origin.

Thin layer Chromatography, solvent system of
Butanol, ethanol and water
(4:1:1)



ethanol-extracted supernate which had an Rf value of 0.40-0.42, and under UV light a purple fluorescent band with an Rf value of 0.40-0.42 was observed (Fig. 78). These results indicate that the pink pigment produced by *C. albicans* 'Outbreak' is completely different from the green pigment produced by the same strain. Separation of crude pink pigment preparations from culture supernates revealed a pink visible band and purple and yellow fluorescent bands when viewed under UV light. By contrast, crude preparations of the green pigment gave blue and yellow fluorescent bands with completely different Rf values.

13.4. Comparison of the green and pink pigments and a known siderophore using TLC

Samples of pink and green pigments (crude culture supernates) were applied to TLC plates, together with a known siderophore, desferal (at 1 and 5 mM), for comparison. A solvent system consisting of 5% ammonium formate plus 0.5% formic acid which is recommended for the separation of siderophore compounds (Rogers, 1973) or butanol: ethanol: water (4:1:1) was used. Plates were air-dried and viewed under UV light before spraying with either 0.1 M $FeCl_3$ in 0.1 M HCl or with CAS solution (Schwyn and Neilands, 1987).

With 5% ammonium formate plus 0.5% formic acid as the solvent, two fluorescent components were seen in the sample of green pigment following chromatography. These were a yellow fluorescent spot with an Rf value of 0.36 and a blue fluorescent spot with an Rf value of 0.20-0.21. No fluorescent spots were observed in the samples of pink pigment or desferal (Fig. 80). However, when the plate was sprayed with $FeCl_3$, brown spots appeared with both samples of desferal (1 and 5 mM) with Rf values of 0.30-0.32; at the same time no brown spots were observed in positions corresponding to the yellow or blue fluorescent components of the green pigment. The pink pigment was not separated into individual components in this system (Fig. 83).

Figure 78.

TLC of ethanol-extracted pink pigment from culture supernates of *C. albicans* 'Outbreak' strain.

A solvent system of butanol : ethanol : water (4:1:1) was used and the chromatogram was viewed under UV illumination. There was a single purple fluorescent band with an Rf value of 0.40-0.42.

Thin layer Chromatography, solvent system of
Butanol, ethanol and water
(4:1:1)

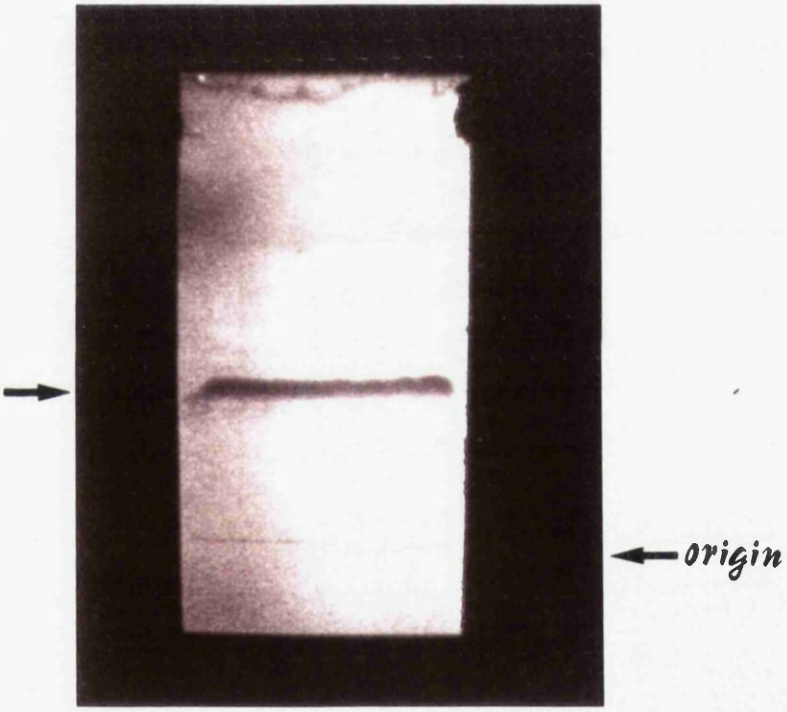


Figure 79.

TLC of yeast nitrogen base medium, pink pigment, and green pigment (crude culture supernate) in chloroform : methanol : water : acetic acid (75:25:3:8)

The chromatogram was viewed under normal light after spraying with 50% sulphuric acid then heated in an oven at 120°C for 15-30 min.

Samples were applied as follows:

Lane 1. Sterile deferrated YNB containing 50 mM glucose as carbon source.

Lane 2. Pink pigment in culture supernate obtained from yeasts grown in tryptophan medium.

Lane 3. Green pigment in culture supernate obtained from yeasts grown in deferrated YNB medium with 0.026 μM iron.

The chromatogram showed a brown spot (*Rf* 0.29-0.30) with deferrated YNB medium but no spot with the green pigment. However, three dark spots were evident with the pink pigment.

Thin layer Chromatography, solvent system of
Chloroform, methanol, water and acetic acid
(75:25:3:8)

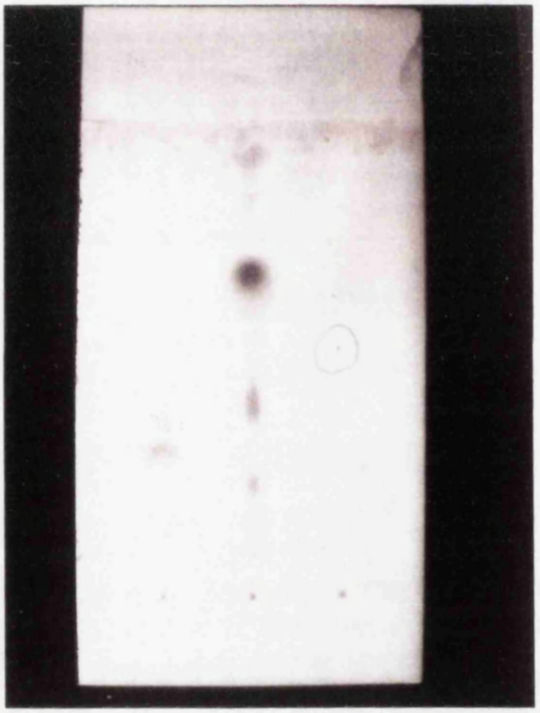


Figure 80.

TLC of desferal, and pink and green pigments

A 5% ammonium formate plus 0.5% formic acid was used as the solvent system and the chromatogram was viewed under UV illumination.

Samples were applied as follows :

Lane 1. Pink pigment from culture supernate of yeasts grown in tryptophan medium.

Lane 2. Green pigment from culture supernate of yeasts grown in deferrated YNB medium.

Lane 3. Desferal (5 mM).

Lane 4. Desferal (1 mM).

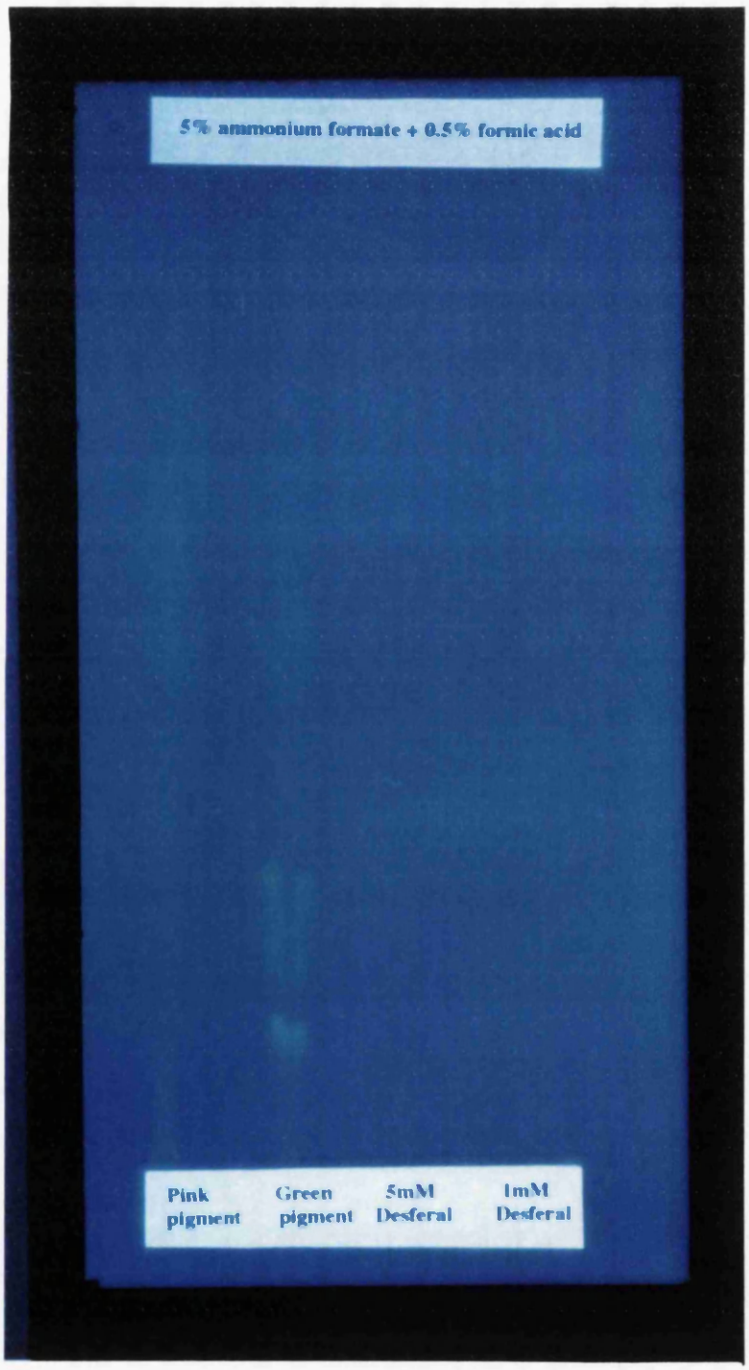
Components were identified as :

A. Blue fluorescent spot (Rf 0.20-0.21) with green pigment.

B. Yellow fluorescent spot (Rf 0.36-0.36) with green pigment.

C. Origin.

No fluorescent spots were observed with desferal or the pink pigment


5% ammonium formate + 0.5% formic acid
Pink pigment
Green pigment
5mM Desferal
1mM Desferal

A →

B →

C →

1 2 3 4

When a solvent system of butanol: ethanol: water (4:1:1) was used, no fluorescent spots were detected in either sample of desferal under UV light. However, both green and pink pigments separated into several fluorescent spots (Fig. 81). After spraying with $FeCl_3$, brown spots with *Rf* values of 0.30-0.32 again appeared in samples of desferal (Fig. 82), but not as components of the pigments. These results suggest that the green and pink pigments are unlikely to function as siderophores.

14. Absorption spectra of green and pink pigments

Automatic and manual wavelength scanning of the green and pink pigments was investigated using culture supernates and extracted pigments. The yellow and blue fluorescent components of the green pigment were also analysed following their isolation from TLC plates.

Culture supernates of *C. albicans* 'Outbreak' strain in deferrated YNB medium, containing either 0.026 μM iron (for optimal production of green pigment) or 0.8 μM iron, were subjected to automatic wavelength scanning at very low speed. When culture supernates containing 0.8 μM iron were read against sterile deferrated YNB, a peak at 360 nm with a reading of 0.035 was noted; another peak of 0.015 was recorded at 400 nm. There was no absorption at 520 nm. With culture supernates of deferrated YNB medium containing 0.026 μM iron, a peak at 360 nm with a reading of 0.140 was noted and another peak of 0.065 was recorded at 440 nm. Again, there was no absorption at 520 nm (Fig. 84).

Automatic wavelength scanning of acetone-extracted green pigment showed no absorption above 520 nm and a peak at 330 nm. On the other hand, ethanol-extracted green pigment showed no absorption at 460 nm and a peak at 350 nm (Fig. 85). However, acetone appeared to extract the green pigment more efficiently than did ethanol.

Figure 81.

TLC of desferal, and pink and green pigments

Butanol : ethanol : water (4:1:1) was used as the solvent system and the chromatogram was viewed under UV illumination.

Samples were applied as follows:

Lane 1. Pink pigment from culture supernate of yeasts grown in tryptophan medium.

Lane 2. Green pigment from culture supernate of yeasts grown in deferrated YNB medium.

Lane 3. Desferal (5 mM).

Lane 4. Desferal (1 mM).

Components were identified as:

A. Blue fluorescent spot (Rf 0.65-0.70) with green pigment.

B. Yellow fluorescent spot (Rf 0.40-0.46) with green pigment.

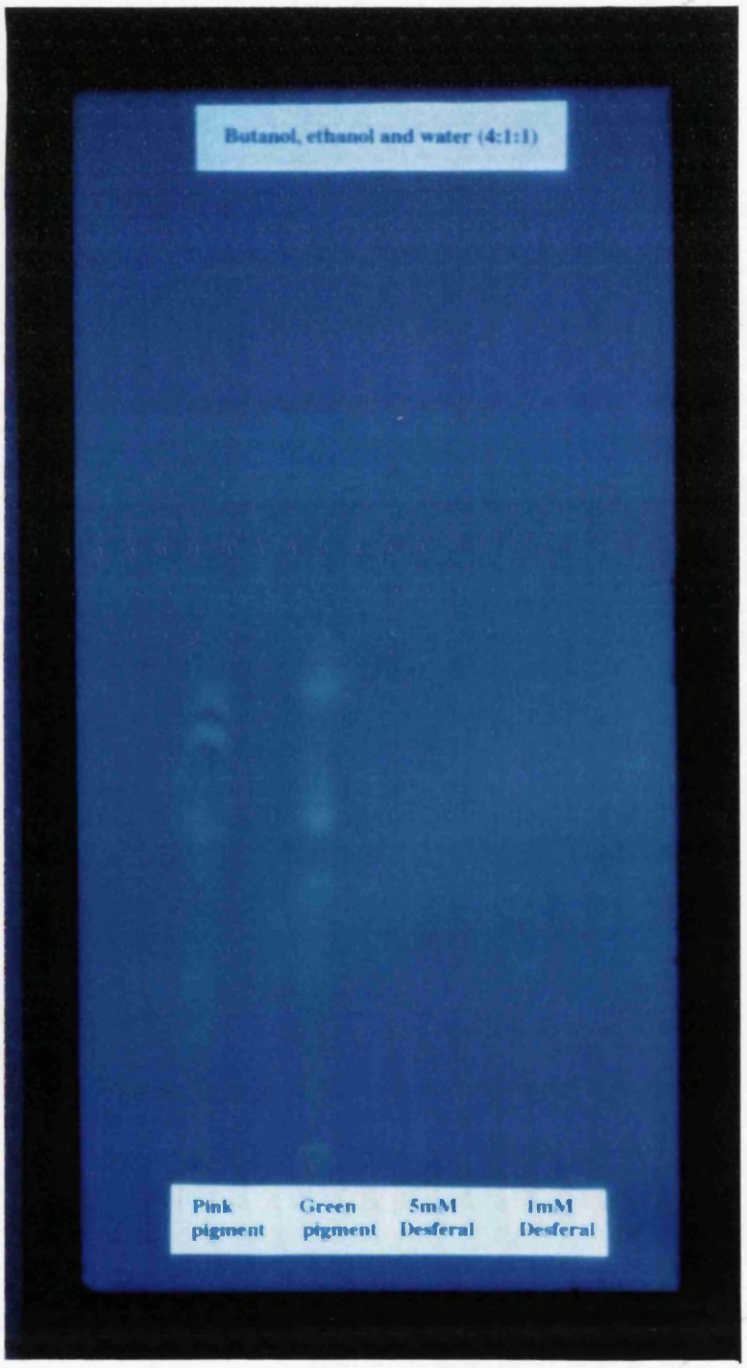
C. Yellow fluorescent spot (Rf 0.62-0.65) with pink pigment

D. Purple fluorescent spot (Rf 0.42-0.54) with pink pigment

E. Yellow fluorescent spot (Rf 0.36-0.38) with pink pigment

F. Origin

No fluorescent spots were observed with two samples of desferal.


Butanol, ethanol and water (4:1:1)
Pink pigment
Green pigment
5mM Desferal
1mM Desferal

C →
D →
E →
F →

← A
← B

1 2 3 4

Figure 82.

TLC of desferal , and pink and green pigments

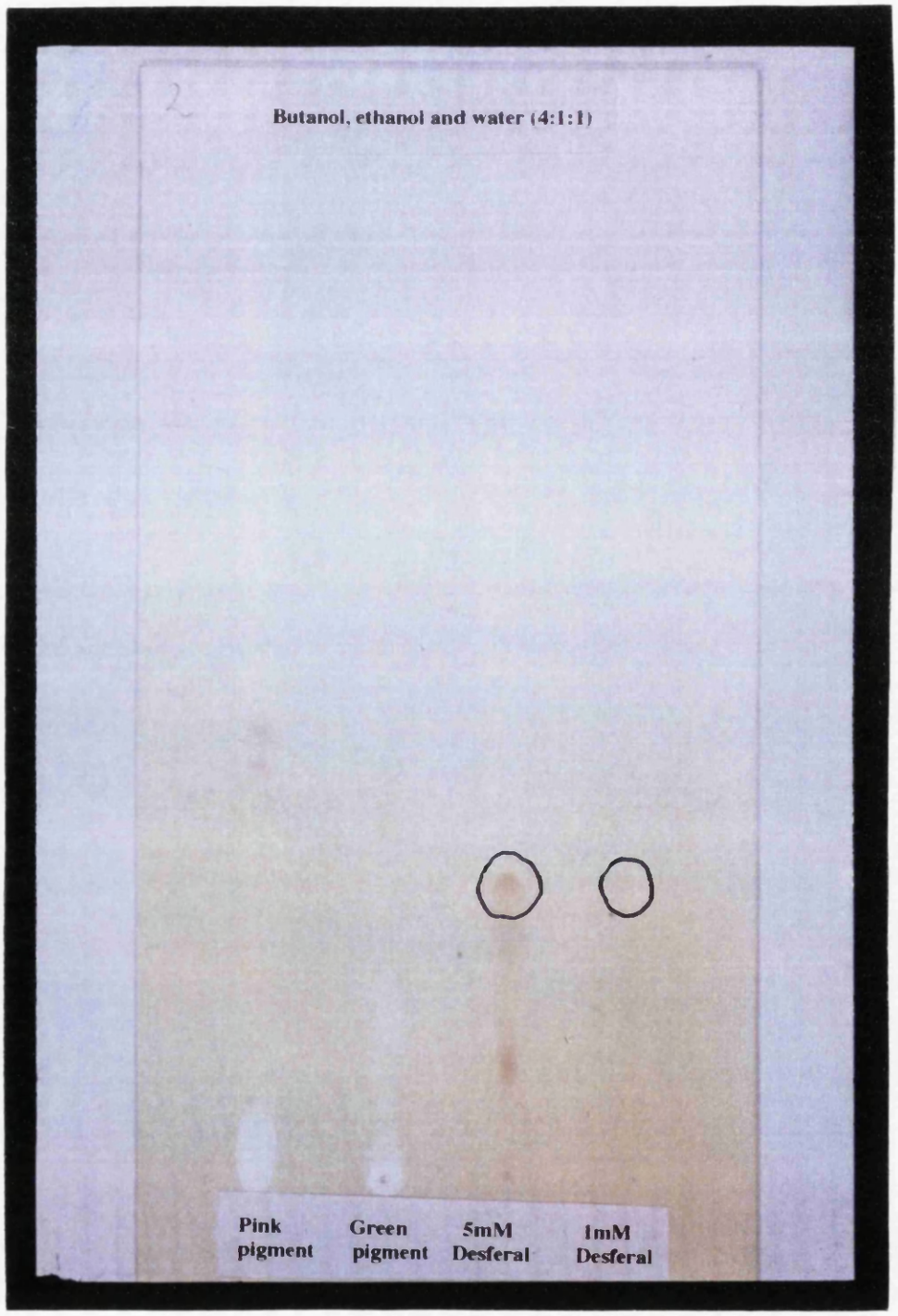
Butanol : ethanol : water (4:1:1) was used as the solvent system, and the chromatogram was viewed under normal light after spraying with $FeCl_3$. The position of siderophores is shown by the brown spots.

Lane 1. Pink pigment, no brown spots were observed.

Lane 2. Green pigment, no brown spots were observed.

Lane 3. Desferal (5 mM), brown spot with Rf value of 0.30-0.32

Lane 4. Desferal (1 mM), brown spot with Rf value of 0.30-0.32


2
Butanol, ethanol and water (4:1:1)
Pink pigment
Green pigment
5mM Desferal
1mM Desferal

1 2 3 4

Figure 83.

TLC of desferal, and pink and green pigments

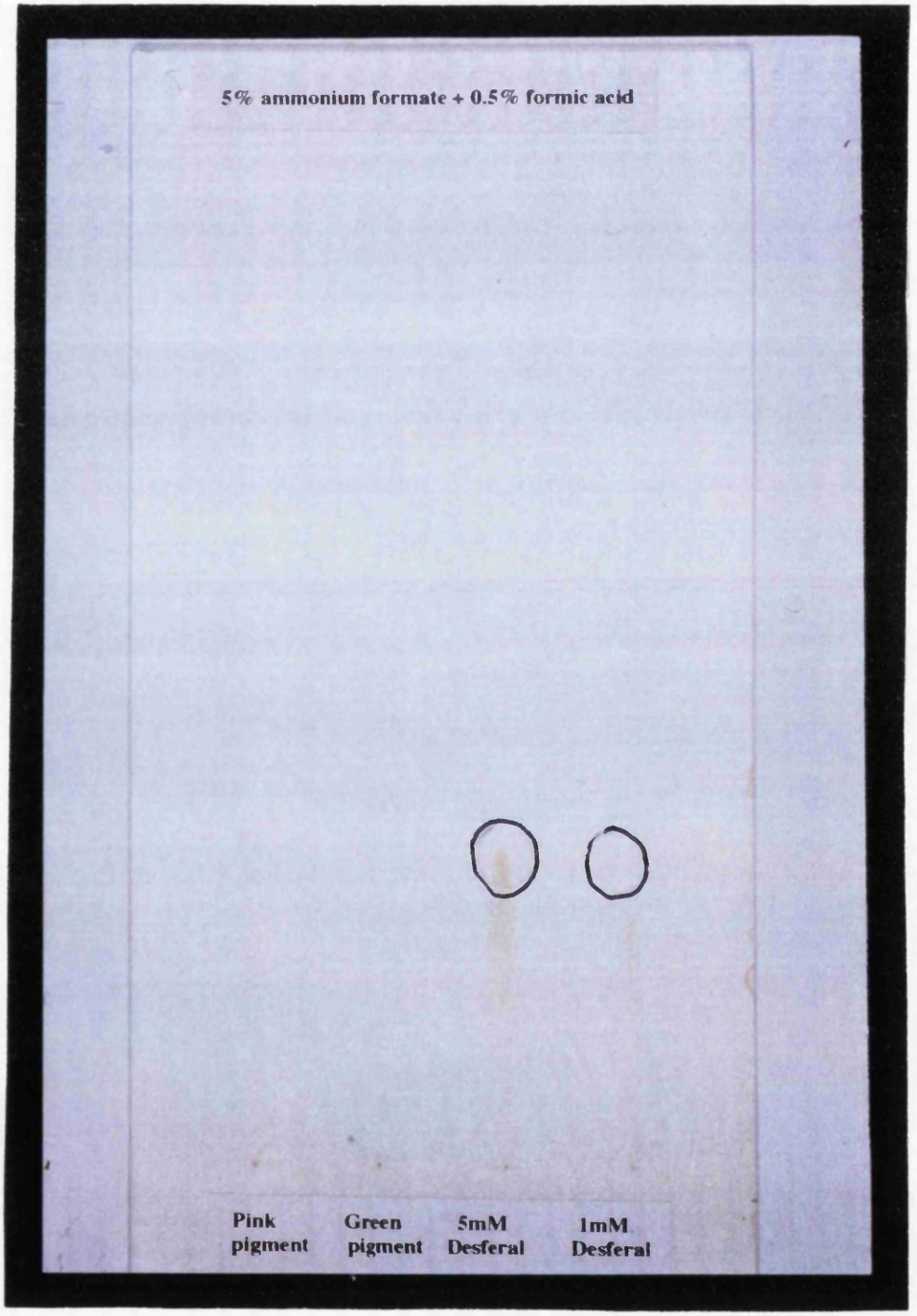
A solvent system of 5% ammonium formate plus 0.5% formic acid was used and the chromatogram was viewed under normal light after spraying with $FeCl_3$. The position of siderophores is shown by the brown spots.

Lane 1. Pink pigment, no brown spots were observed.

Lane 2. Green pigment, no brown spots were observed.

Lane 3. Desferal (5 mM), brown spot with *Rf* values of 0.30-0.32

Lane 4. Desferal (1 mM), brown spot *Rf* values of 0.30-0.32


5% ammonium formate + 0.5% formic acid
Pink pigment
Green pigment
5mM Desferal
1mM Desferal

1 2 3 4

Figure 84.

Automatic wavelength scanning of green pigment in culture supernate of deferrated YNB medium containing either 0.026 μM (A) or 0.8 μM (B) iron.

Samples were read against sterile deferrated YNB medium.

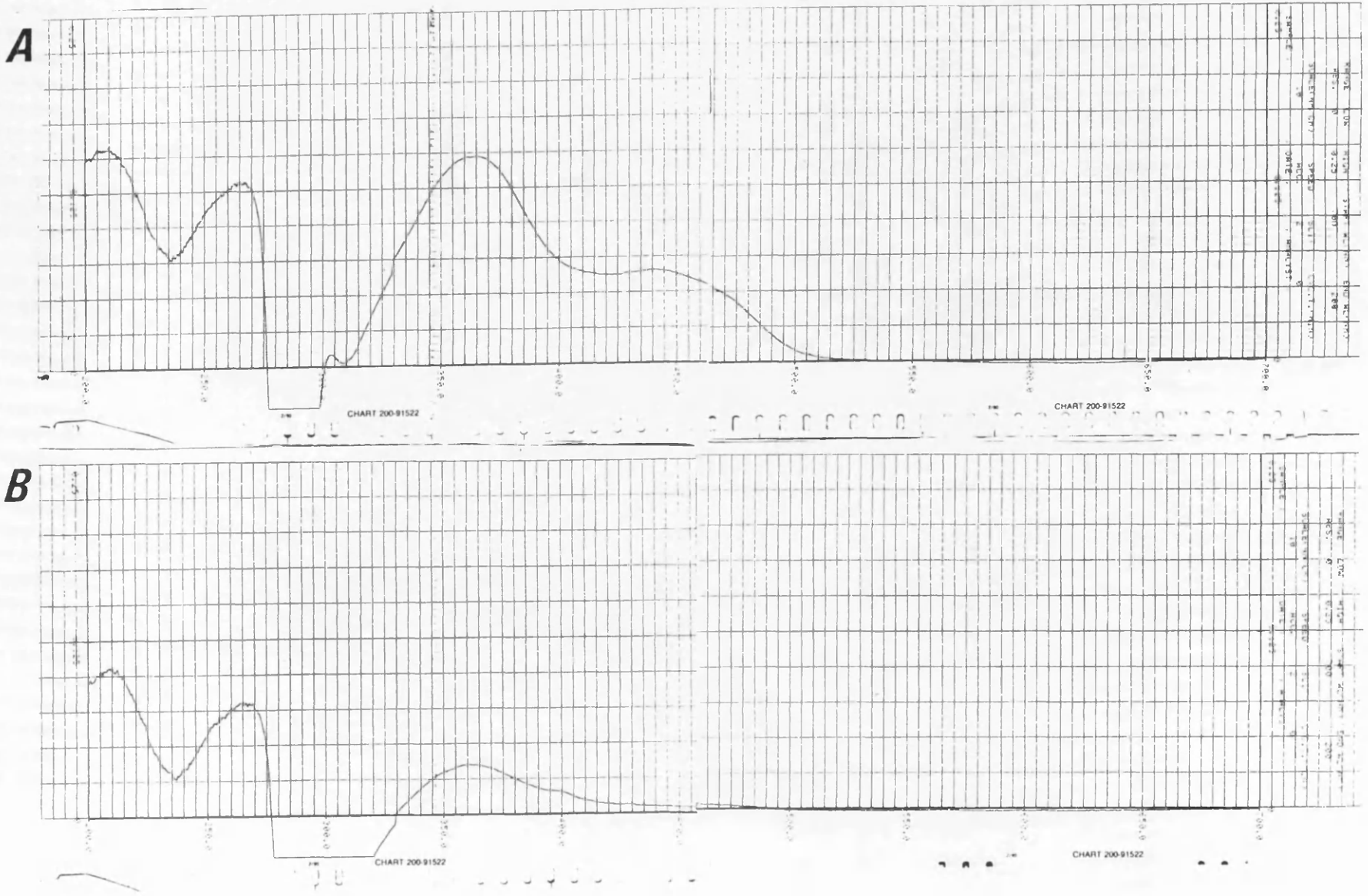
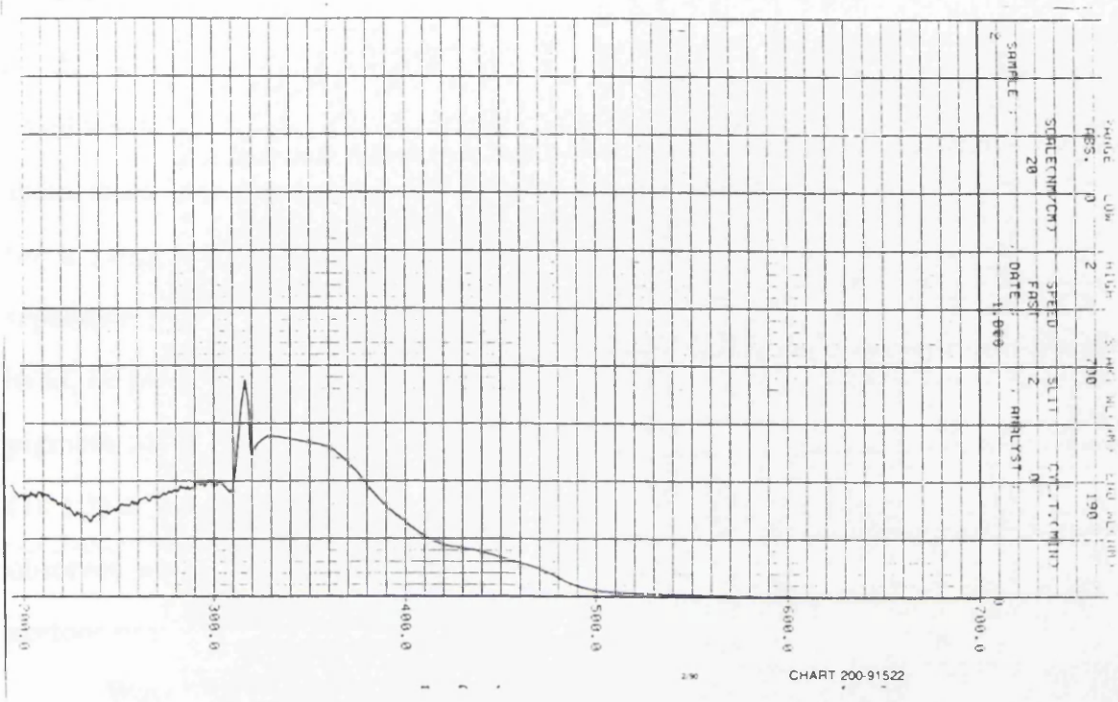

A
CHART 200-91522
CHART 200-91522
B
CHART 200-91522
CHART 200-91522

Figure 85.

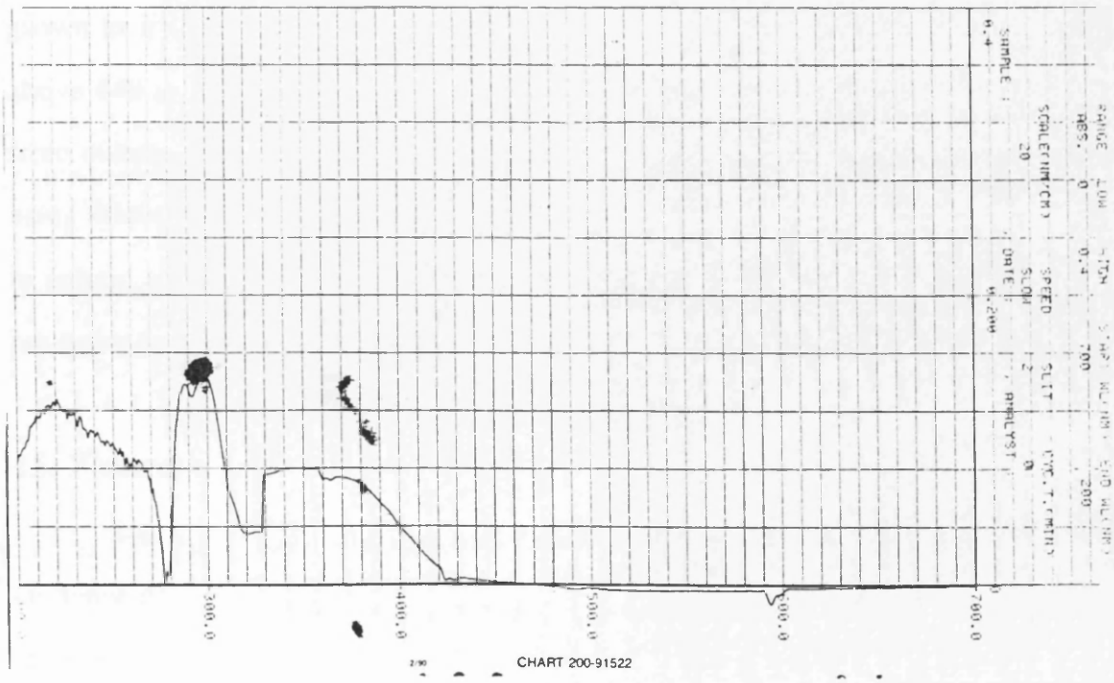
Automatic wavelength scanning of green pigment extracted from culture supernates with acetone (A) or ethanol (B).

Samples were read against acetone (A) or ethanol (B).

A



B



The purified yellow fluorescent component of the green pigment was scraped from TLC plates (after separation in BEW solvent), dissolved in distilled water and subjected to automatic wavelength scanning at very low speed. The chart showed two peaks, the highest at 360 nm and another at 440 nm; there was no absorption at 520 nm. (Fig. 86). Two peaks (at 360 and 440 nm) were also noted when wavelength scanning was carried out manually with further samples; again, there was no absorption at 520 nm (Fig. 87). A similar result was obtained for a sample of yellow fluorescent material prepared by chromatographic separation in chloroform: methanol: acetic acid: water (75:25:3:8). On the other hand, no peaks were observed with the blue fluorescent component of the green pigment after separation in BEW (Fig. 88), BAW and ethanol: water: NH_3 (18:1:1) or in chloroform: methanol: acetic acid: water (75:25:3:8). No peaks were observed when the yellow or blue fluorescent materials were dissolved in either acetone or methanol rather than water.

Wavelength scanning of culture supernate of *C. albicans* 'Outbreak' strain grown in tryptophan medium (containing pink pigment) showed no absorption above 640 nm and a peak at 520 nm (Fig. 89). Ethanol-extracted pink pigment from culture supernates and cell pellets of *C. albicans* 'Outbreak' were scanned using ethanol as a blank. The results confirmed that the pink pigment was found in culture supernate not in cell pellets (Fig. 90). There was no absorption at 640 nm and a peak was noted at 440 nm.

15. Fluorescence spectra of green and pink pigments

Since TLC of the green pigment produced blue and yellow fluorescent components when plates were viewed under UV illumination, spectrofluorimetric studies were also carried out. Four samples of yellow and blue fluorescent materials were scraped from TLC plates run in BAW and BEW solvents, and dissolved in distilled water. In a spectrofluorimeter at an excitation wavelength of 290 nm, both blue fluorescent samples showed an emission peak at 440 nm (blue

Figure 86.

Automatic wavelength scanning of the yellow fluorescent component of green pigment taken from TLC plates run in butanol: ethanol: water (4:1:1).

Samples were dissolved in, and read against, distilled water.

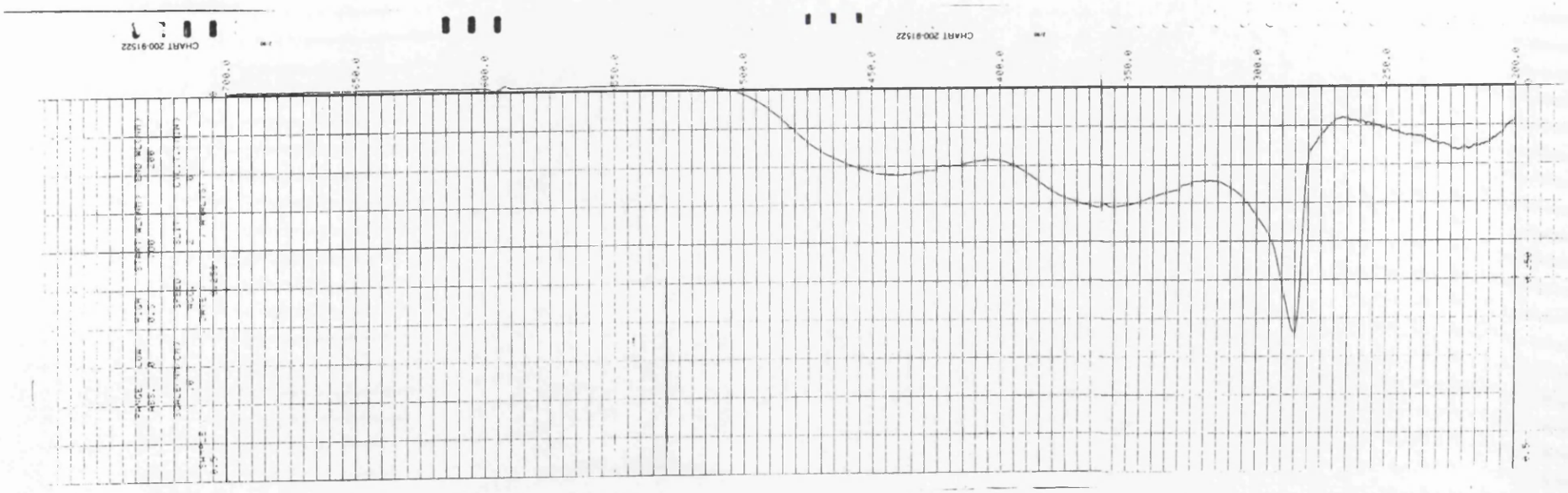


Figure 87.

Manual wavelength scanning of the yellow fluorescent component of green pigment taken from TLC plates run in butanol: ethanol: water (4:1:1).

Samples were dissolved in, and read against distilled water. The chart shows samples from two different TLC plates.

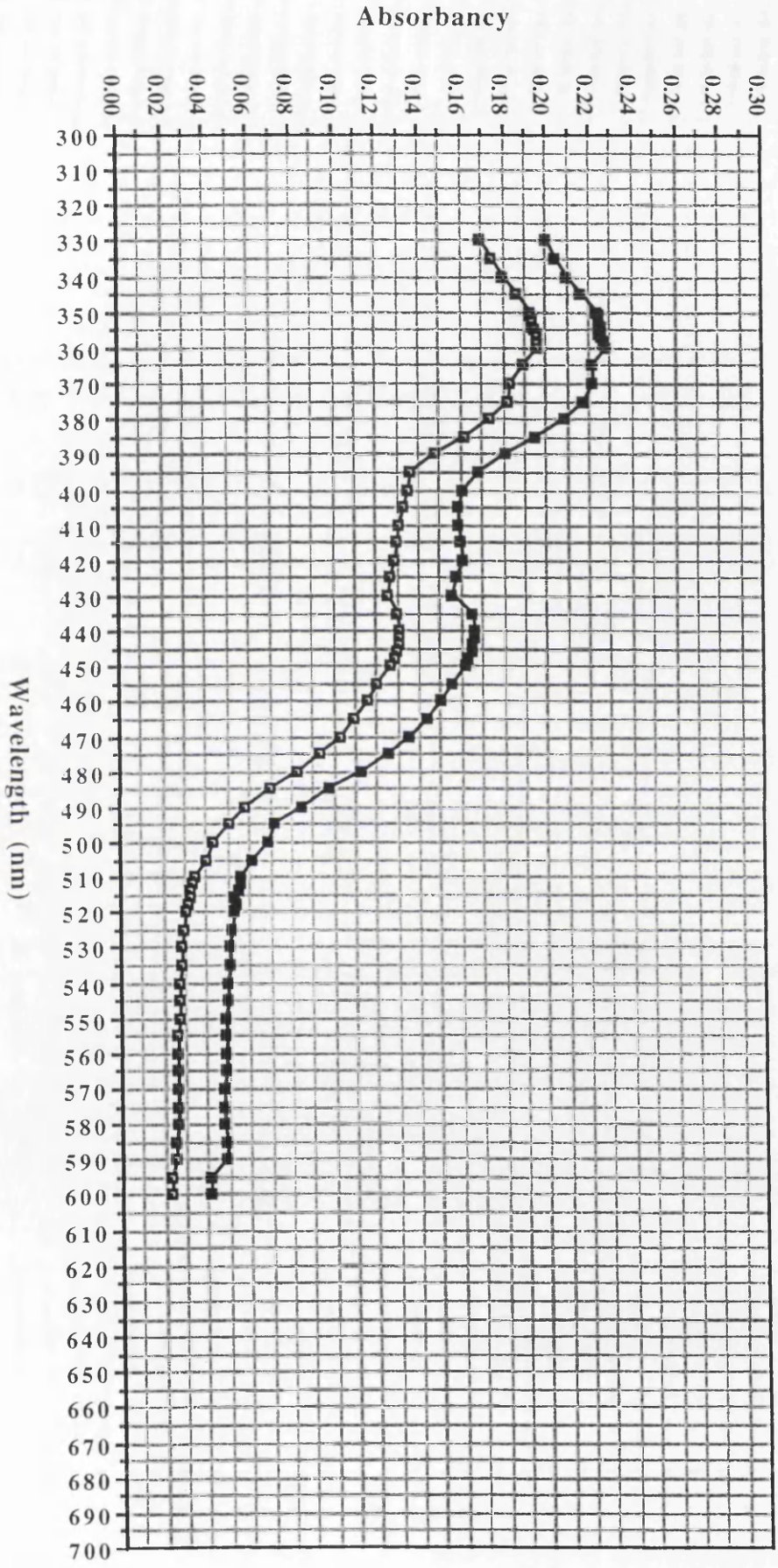

Absorbancy
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
0.22
0.24
0.26
0.28
0.30
Wavelength (nm)
300
310
320
330
340
350
360
370
380
390
400
410
420
430
440
450
460
470
480
490
500
510
520
530
540
550
560
570
580
590
600
610
620
630
640
650
660
670
680
690
700

Figure 88.

Automatic wavelength scanning of the yellow (B) and blue (A) fluorescent components of green pigment taken from TLC plates run in butanol: ethanol: water (4:1:1).

Samples were dissolved in, and read against, distilled water. Scanning was done using a Shimadzu UV-3101 PC Spectrophotometer.

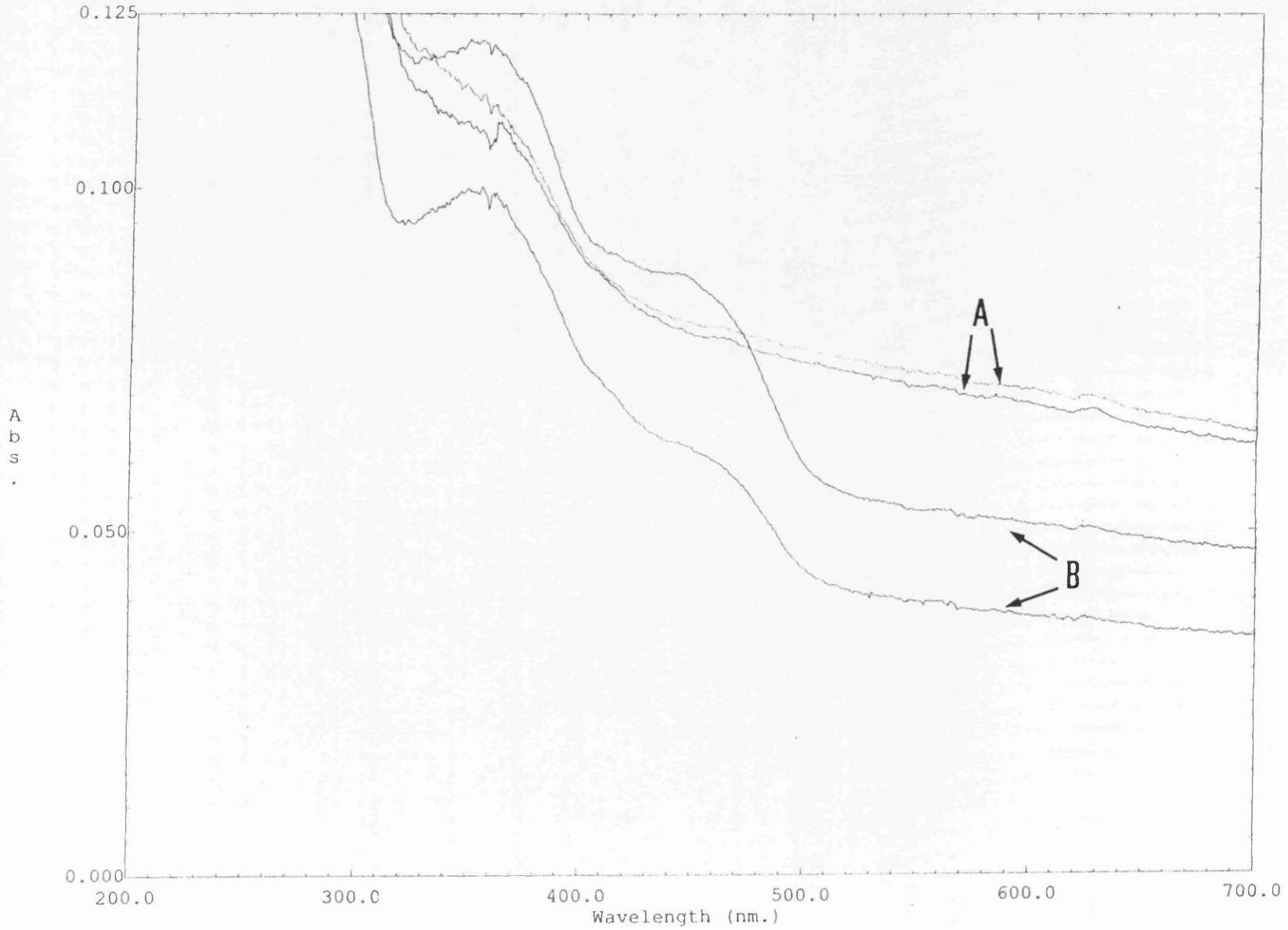

0.125
0.100
0.050
0.000
Abs.
A
B
200.0
300.0
400.0
500.0
600.0
700.0
Wavelength (nm.)

Figure 89.

Automatic wavelength scanning of pink pigment in culture supernates of tryptophan medium.

Samples were read against sterile tryptophan medium.

CHART 200-91522
2/90
RANGE LOW HIGH START NLC(NM) END NLC(NM)
HSS. 0 1 700 200
SCALE(NM/CM) SPEED SLIT CYC.T.(MIN)
20 FAST 2 0
SAMPLE) DATE) 0.500) AMPLIST)
0
200.0
300.0
400.0
500.0
600.0
700.0

Figure 90.

Automatic wavelength scanning of ethanol-extracted pink pigment from the cell pellet (A) and culture supernate (B) of *C. albicans* 'Outbreak' strain grown in tryptophan medium.

Samples were read against ethanol.

A

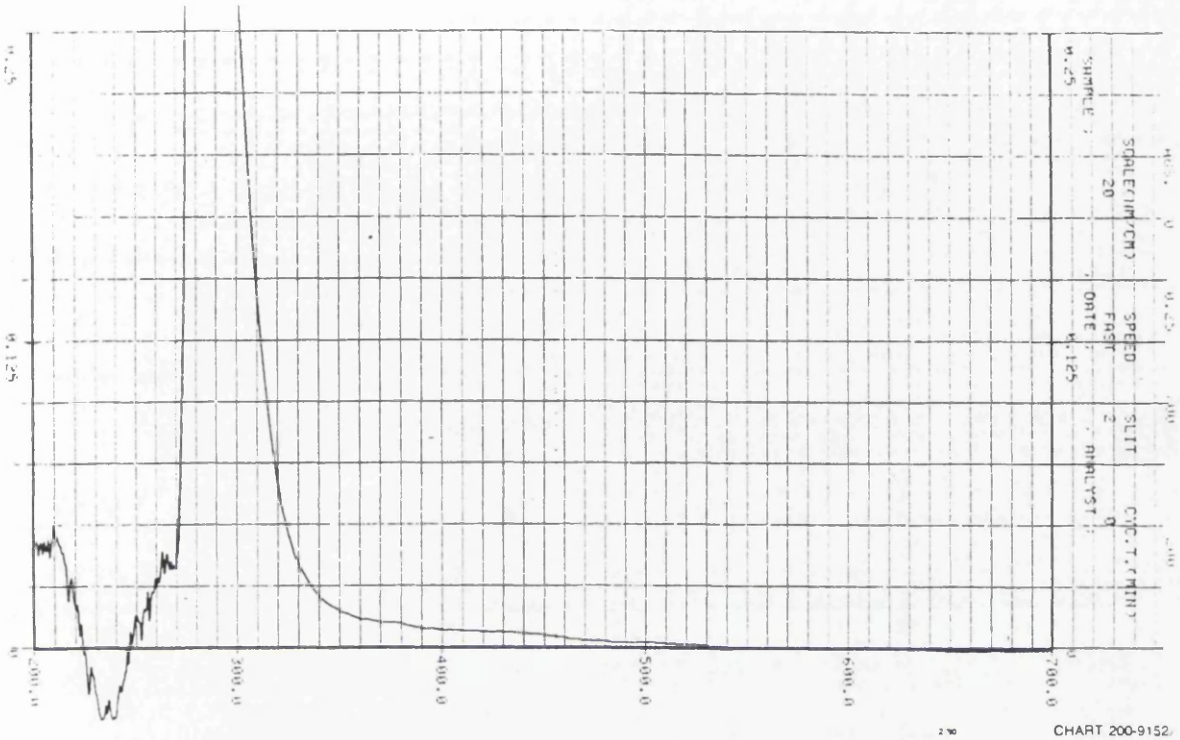


CHART 200-91522

B

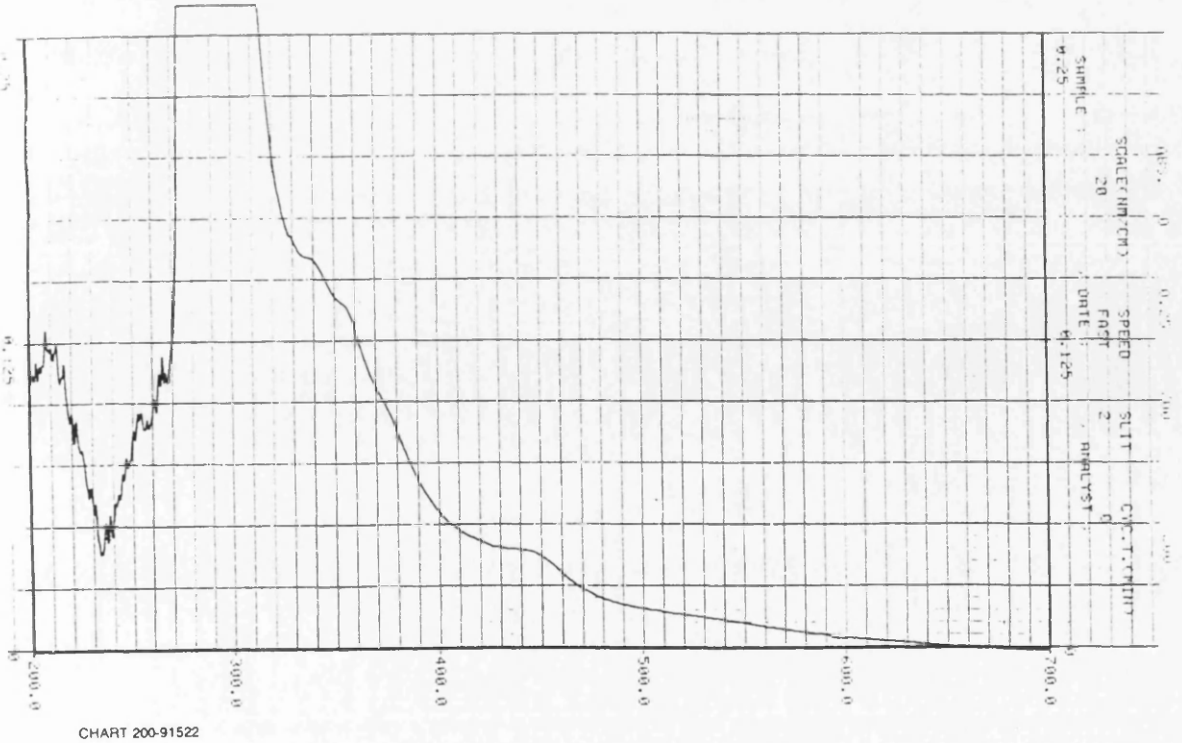


CHART 200-91522

colour area; Fig. 91 A). At an excitation wavelength of 440 nm , on the other hand, both yellow fluorescent samples, but neither blue sample, gave an emission peak at 520 nm (green colour area; Fig. 91 B). At an excitation wavelength of 370 nm, crude culture supernate of deferrated YNB containing the green pigment showed two emission peaks at 440 and 520 nm (blue and green colour areas, respectively; Fig. 92 A). No peaks were observed with culture supernates of tryptophan medium containing the pink pigment. When excitation was at 440 nm, only one peak (at 520 nm) was obtained with the green pigment ; this corresponds to the yellow fluorescent component (Fig. 92 B).

Individual chemicals which comprise YNB medium were compared with the green pigment for fluorescence. Compounds tested included vitamins (biotin, calcium panthothenate, folic acid, inositol, niacin, p-aminobenzoic acid, pyridoxine hydrochloride, riboflavin and thiamine hydrochloride) and amino acids (L-histidine monohydrochloride, LD-methionine and LD-tryptophan). Vitamins and amino acids showed no peaks similar to those of the green pigment; only small peaks at 520 nm (vitamins) and 450 nm (amino acids) were observed (Fig. 93). Excitation of the pink pigment at 370 nm showed an emission at 310 nm (blue colour area); when excitation was at 310 nm the emission was at 360 nm (also blue colour area; Fig. 94).

The results confirm that the green pigment consists of two components, one yellow (fluorescing at 520 nm) and another blue (fluorescing at 440 nm). Fluorescent spectra showed no similarity between the green and pink pigment; there was also no similarity between the green pigment and any of chemicals which comprise YNB medium.

16. Secretion of a haemolytic factor by *C. albicans*

The aim of this part of the study was to investigate possible haemolytic activity by *C. albicans* since recent reports (Manns *et al.*, 1994) showed that *C. albicans* had the ability to utilize iron derived from haemoglobin and exhibits

Figure 91.

Fluorescence spectra of the green pigment.

Chart A. Excitation wavelength of 290 nm, emission (fluorescence) wavelength of 440 nm.

Chart B. Excitation wavelength of 440 nm, emission (fluorescence) wavelength of 520 nm.

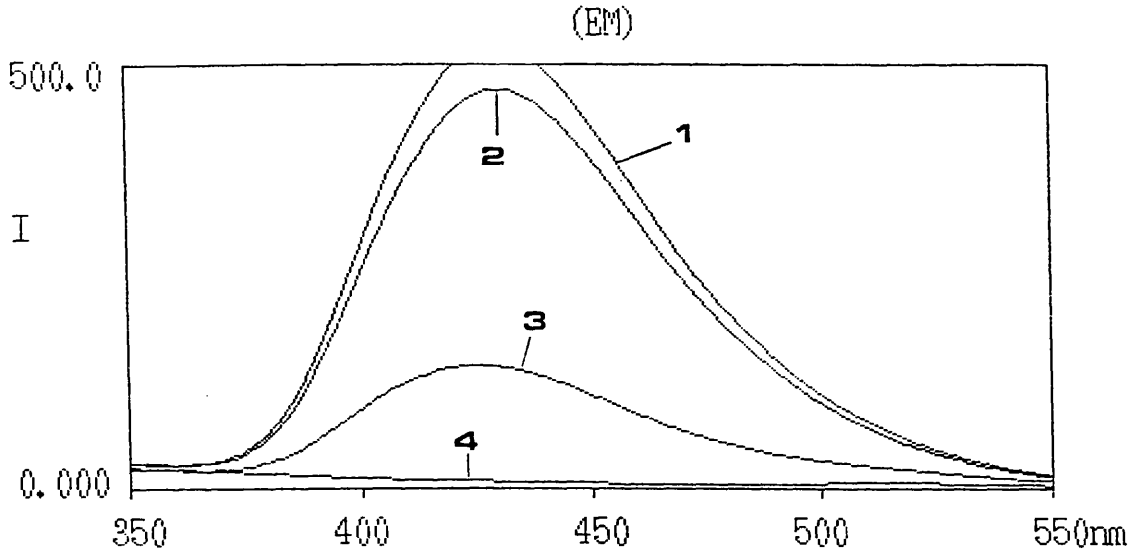
Sample 1. Blue fluorescent component of green pigment taken from TLC plates after separation in BAW (65:10:25).

Sample 2. Blue fluorescent component of green pigment taken from TLC plates after separation in BEW (4:1:1).

Sample 3. Yellow fluorescent component of green pigment taken from TLC plates after separation in BEW (4:1:1).

Sample 4. Yellow fluorescent component of green pigment taken from TLC plates after separation in BAW (65:10:25).

A.


(EM)
500.0
I
0.000
1
2
3
4
350
400
450
500
550nm

B.

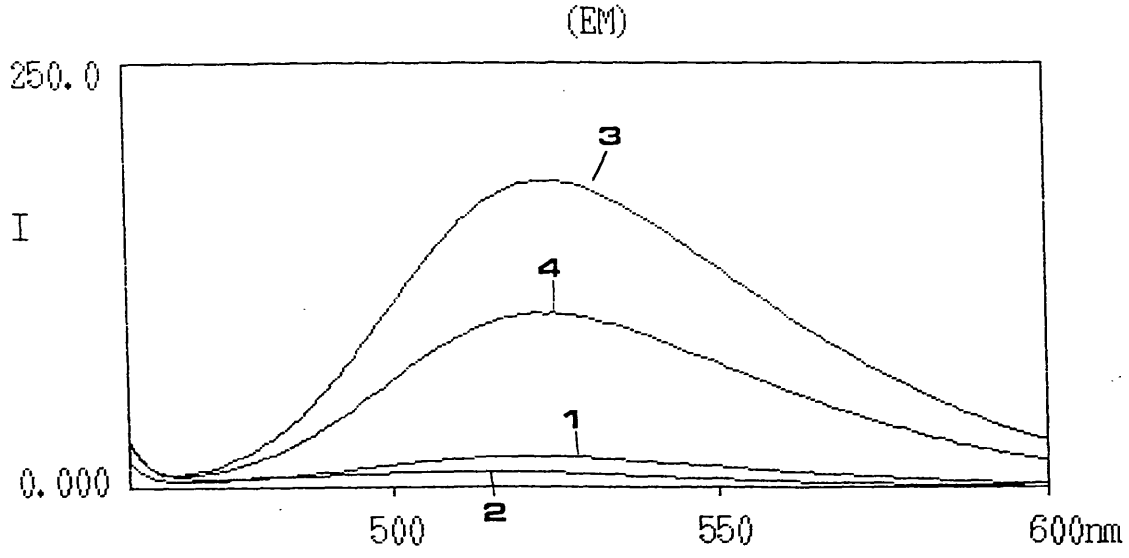

(EM)
250.0
I
0.000
1
2
3
4
500
550
600nm

Figure 92.

Fluorescence spectra of green and pink pigments and deferrated YNB

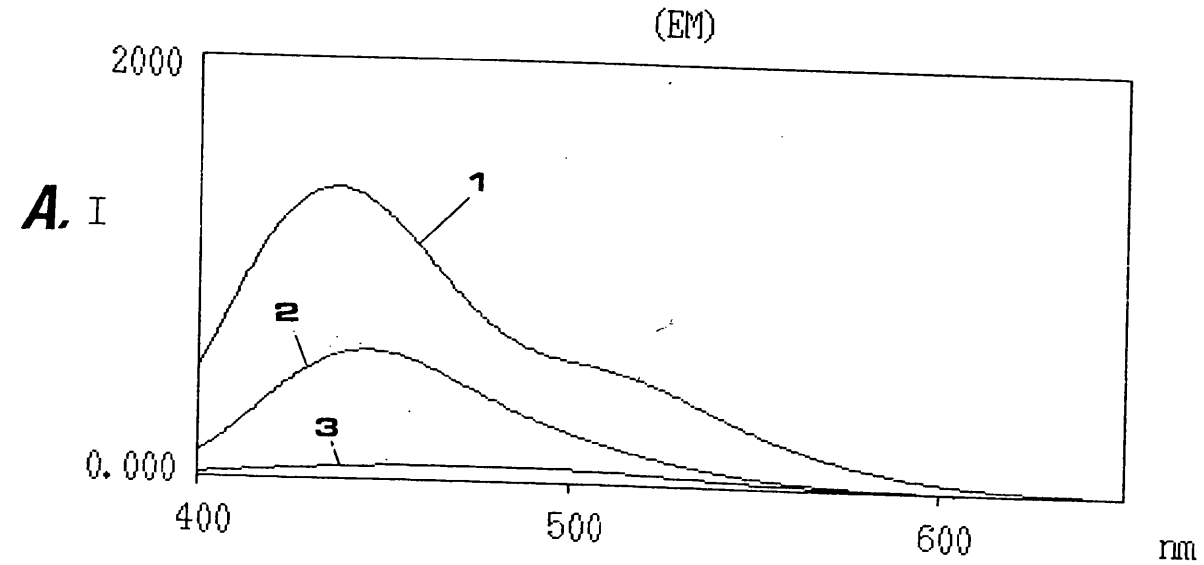
Chart A. Excitation wavelength of 370 nm, emission (fluorescence) wavelength of 440 nm.

Chart B. Excitation wavelength of 440 nm, emission (fluorescence) wavelength of 520 nm.

Sample 1. Green pigment in culture supernate of deferrated YNB medium, emission peaks at 440 and 520 nm (blue and green colour areas, respectively)

Sample 2. Sterile deferrated YNB medium.

Sample 3. Pink pigment in culture supernate of tryptophan medium.


(EM)
2000
A. I
1
2
3
0.000
400
500
600
nm

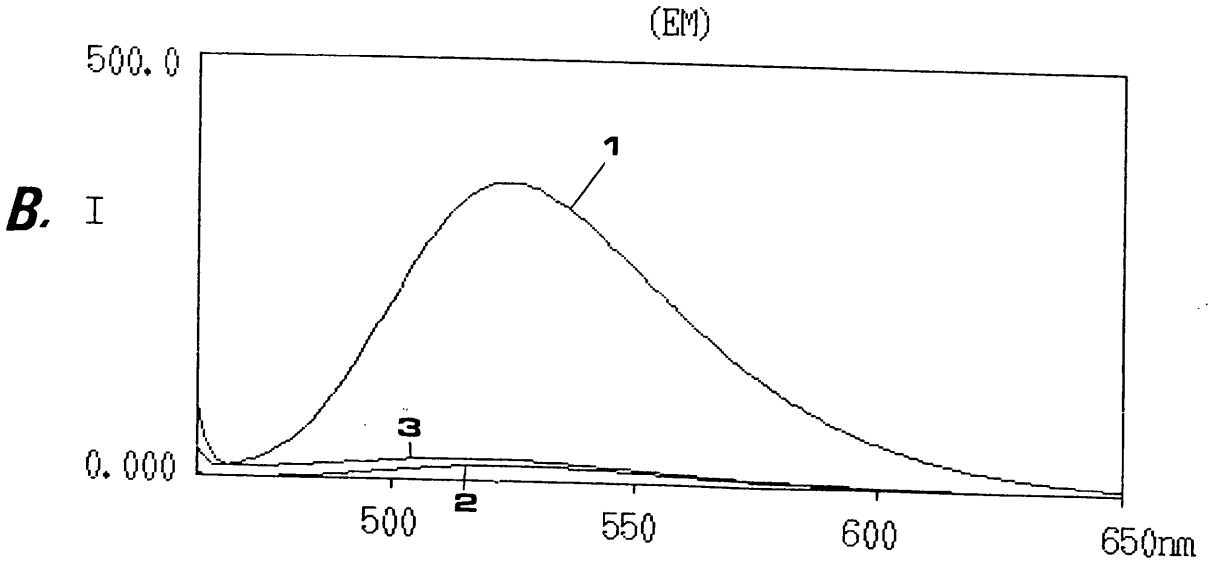

(EM)
500.0
B. I
1
3
0.000
2
500
550
600
650nm

Figure 93.

Fluorescence spectra of amino acids and vitamins in deferrated YNB media.

Chart A. Excitation wavelength of 370 nm (large scale).

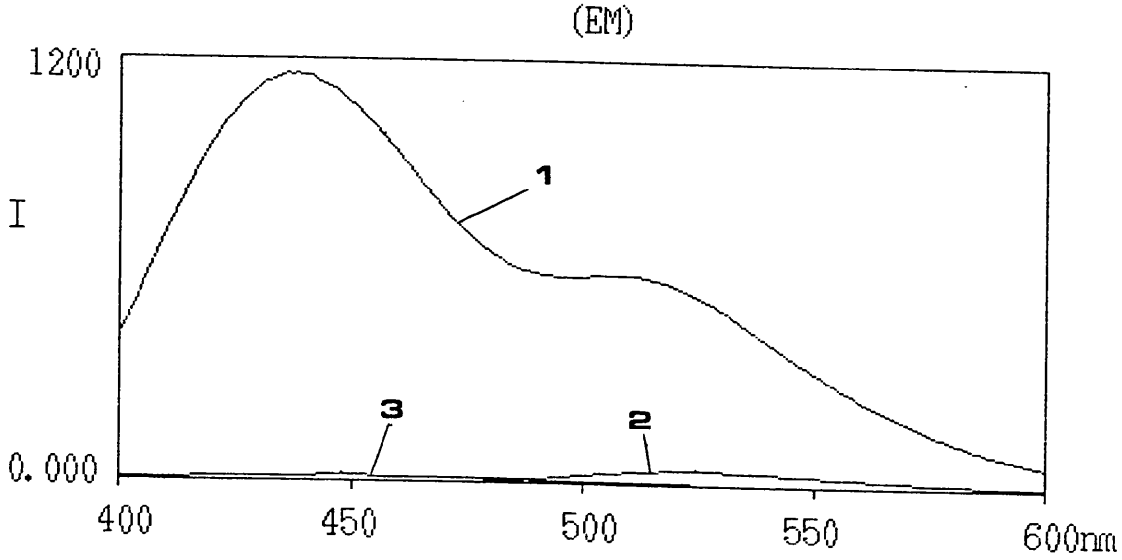
Chart B. Excitation wavelength of 370 nm (small scale).

Sample 1. Green pigment in culture supernate of deferrated YNB medium, emission peaks at 440 and 520 nm (blue and green colour areas, respectively).

Sample 2. Vitamins containing biotin, calcium pantothenate, folic acid, inositol, niacin, p-aminobenzoic acid, pyridoxine hydrochloride, riboflavin and thiamine hydrochloride.

Sample 3. Amino acids containing L-histidine monohydrochloride, LD-methionine and LD-tryptophan.

A.


(EM)
1200
I
0.000
1
2
3
400
450
500
550
600nm

B.

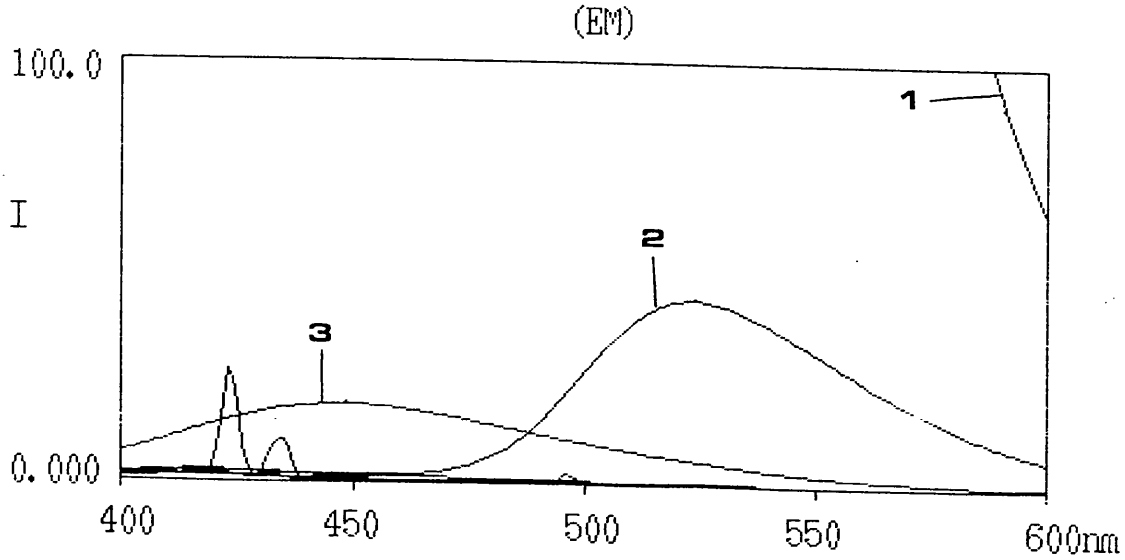

(EM)
100.0
I
0.000
1
2
3
400
450
500
550
600nm

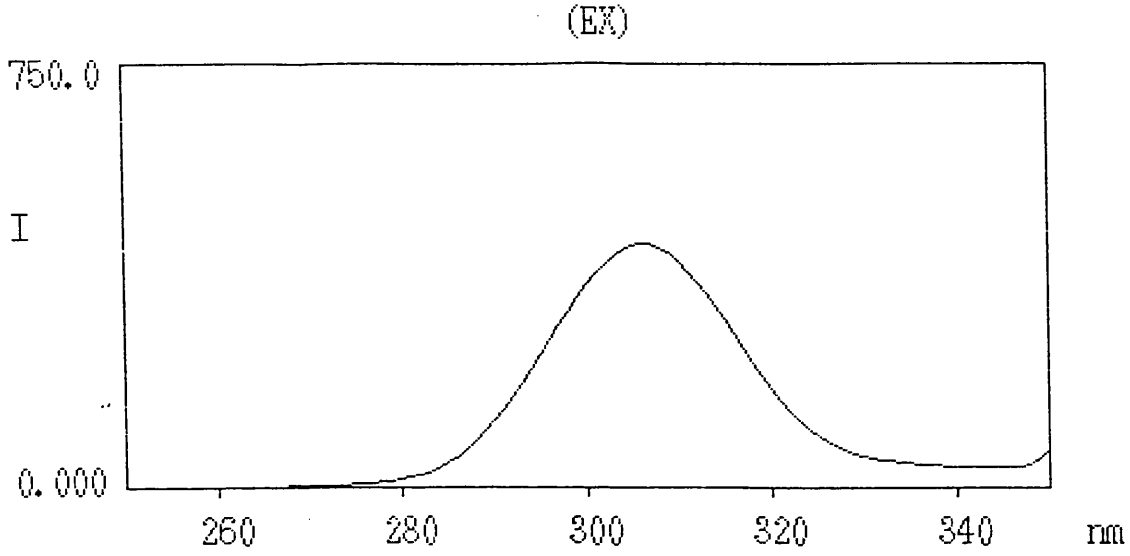
Figure 94.

Fluorescence spectra of pink pigment.

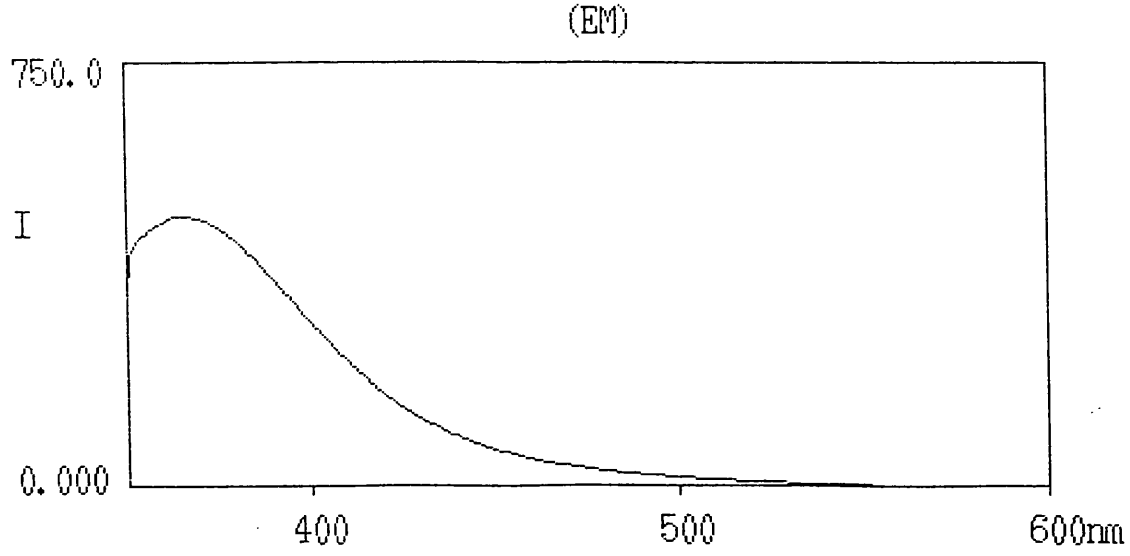
Chart A. Excitation wavelength of 370 nm, emission (fluorescence) wavelength of 310nm.

Chart B. Excitation wavelength of 310 nm, emission (fluorescence) wavelength of 360nm.

A.


(EX)
750.0
I
0.000
260
280
300
320
340
nm

B.


(EM)
750.0
I
0.000
400
500
600nm

haemolytic activity when grown on glucose-enriched blood agar at 37°C in CO_2. Twelve strains of *C. albicans* used here produced a grey zone around colonies on Sabouraud dextrose blood agar or trypticase soya blood agar when these media were enriched with 3% glucose and plates were incubated at 37°C in 5% CO_2 (Figs. 95, 96, 97 and 99). However, no grey zone was noted around colonies when plates were incubated in normal air or in media not enriched with 3% glucose (Fig. 100). Sabouraud dextrose blood agar normally contains 4% glucose so the enriched medium had a final glucose concentration of 7%.

When two strains of *C. albicans* (GDH 2346 and 'Outbreak' strain) were grown on brain heart infusion blood agar, no clear lysis was noted compared with that seen with the bacterial species, *Streptococcus pyogenes* and *Staphylococcus aureus* (Fig. 98).

In a separate series of experiments, filter paper disks saturated with a sterile solution containing either the blue or yellow fluorescent components of the green pigment were placed on Sabouraud dextrose blood agar plates enriched with 3% glucose. Six from ten plates showed some apparent haemolysis around disks containing the yellow but not the blue fluorescent component (Fig. 101). In some experiments filter paper disks saturated with sterile culture supernates (freeze-dried) of deferrated YNB medium containing 0.026 μM iron concentration showed lysis; however, disks containing culture supernates of YNB with a normal iron concentration (0.8 μM) produced no haemolysis. Similarly, no lysis occurred with culture supernates of tryptophan medium containing pink pigment.

These results are only preliminary observations; more investigations are needed to clarify the process which allows *C. albicans* to acquire iron from host erythrocytes.

Figure 95.

Growth of *C. albicans* on enriched blood agar.

C. albicans strains GRI 681, GRI 682, GDH 2023 and GDH 2346 show a dark zone around colonies streaked on Sabouraud dextrose blood agar enriched with 3% glucose (w/v). Cultures were incubated for 24 to 48 h at 37°C in 5% CO_2 .

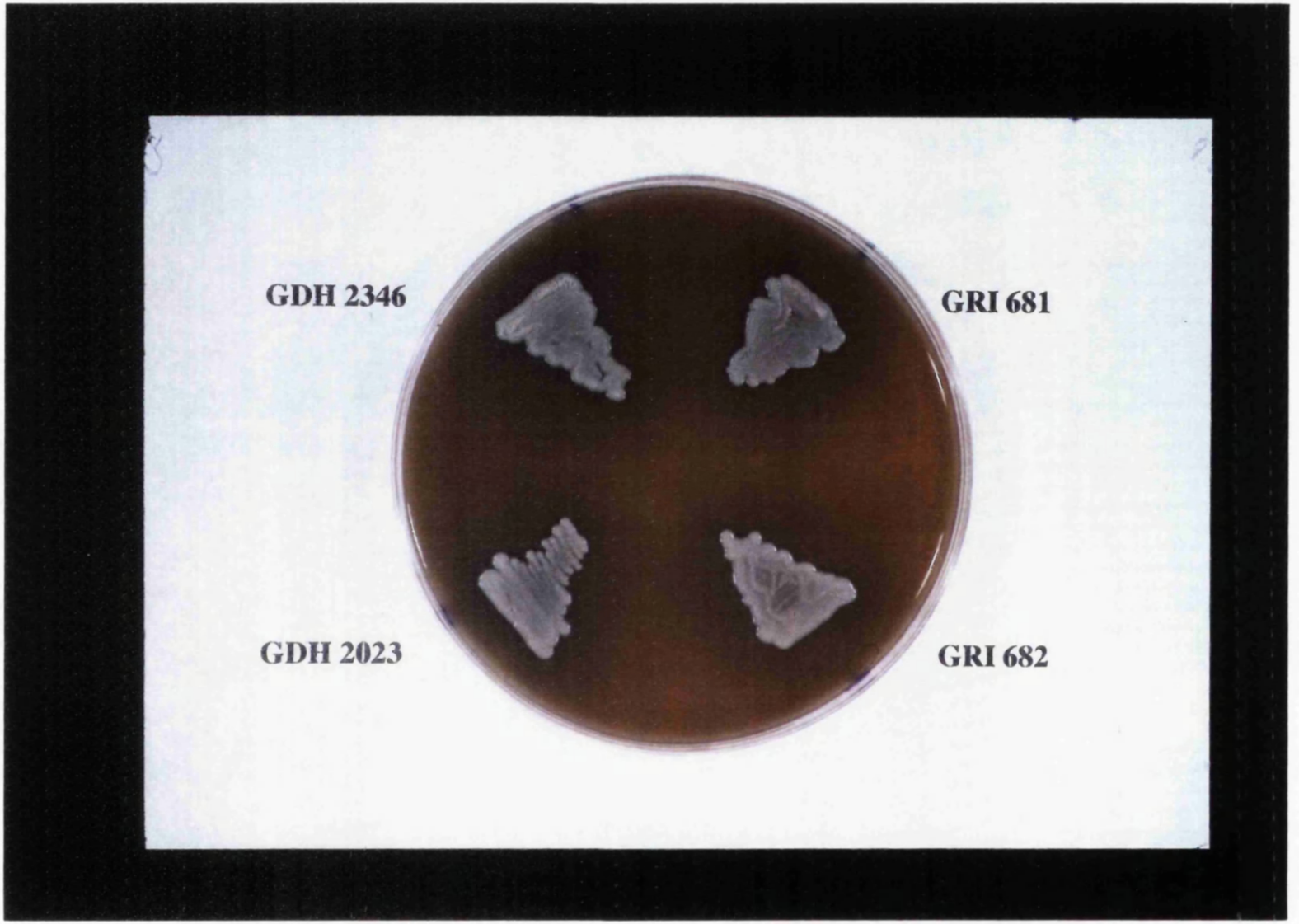

GDH 2346
GRI 681
GDH 2023
GRI 682

Figure 96.

Growth of *C. albicans* on enriched blood agar.

C. albicans strains 1001 (wild type), 1001-92'(HY^-), 1001-FR(Y^-) and 'Outbreak' show a dark zone around colonies streaked on Sabouraud dextrose blood agar enriched with 3% glucose (w/v). Cultures were incubated for 24 to 48 h at 37°C in 5% CO_2 .

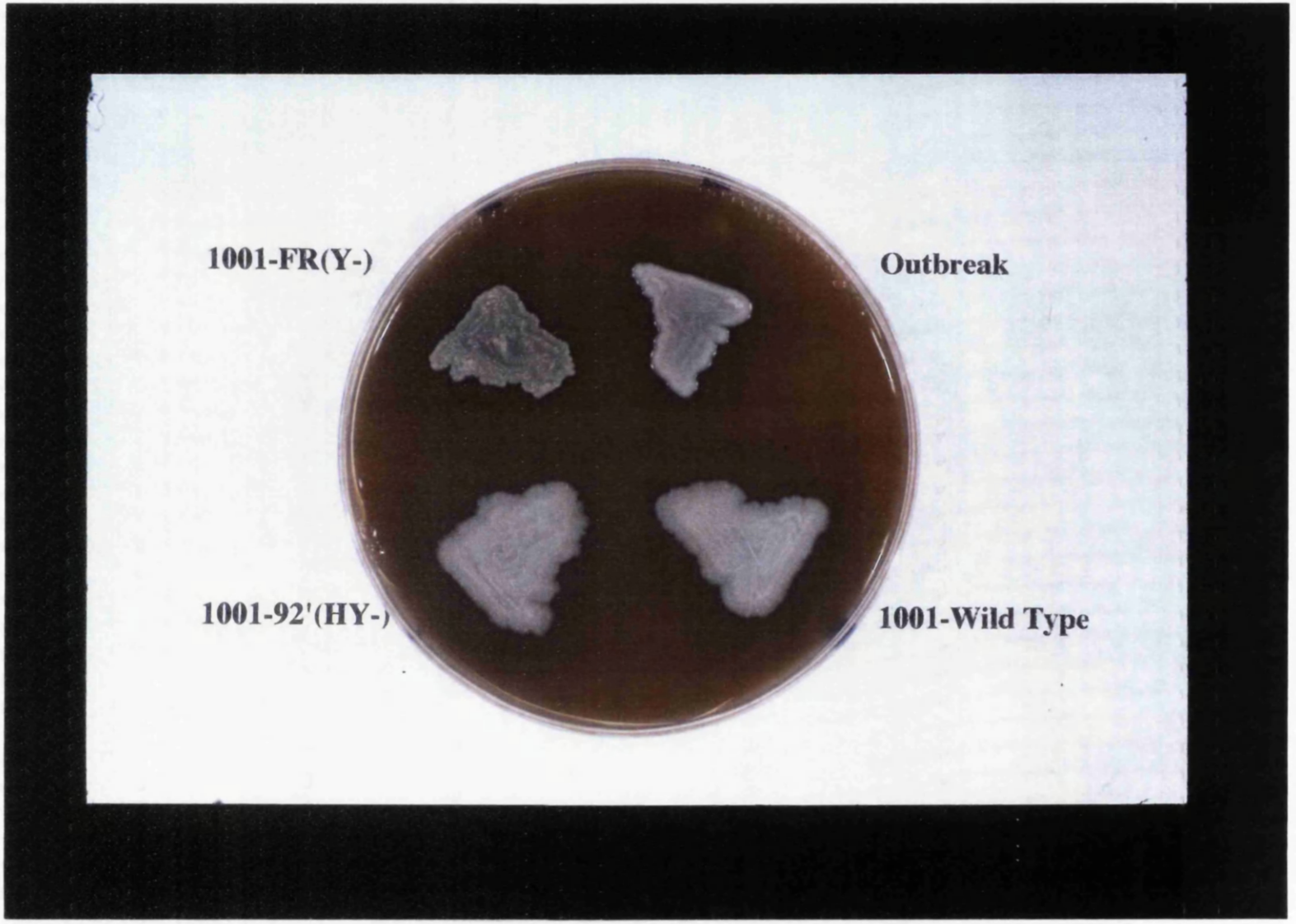

1001-FR(Y-)
Outbreak
1001-92'(HY-)
1001-Wild Type

Figure 97.

Growth of *C. albicans* on enriched blood agar.

C. albicans strains ATTC 10261, HOG 301, MEN and MM2002 show a dark zone around colonies streaked on Sabouraud dextrose blood agar enriched with 3% glucose (w/v). Cultures were incubated for 24 to 48 h at 37°C in 5% CO_2 .

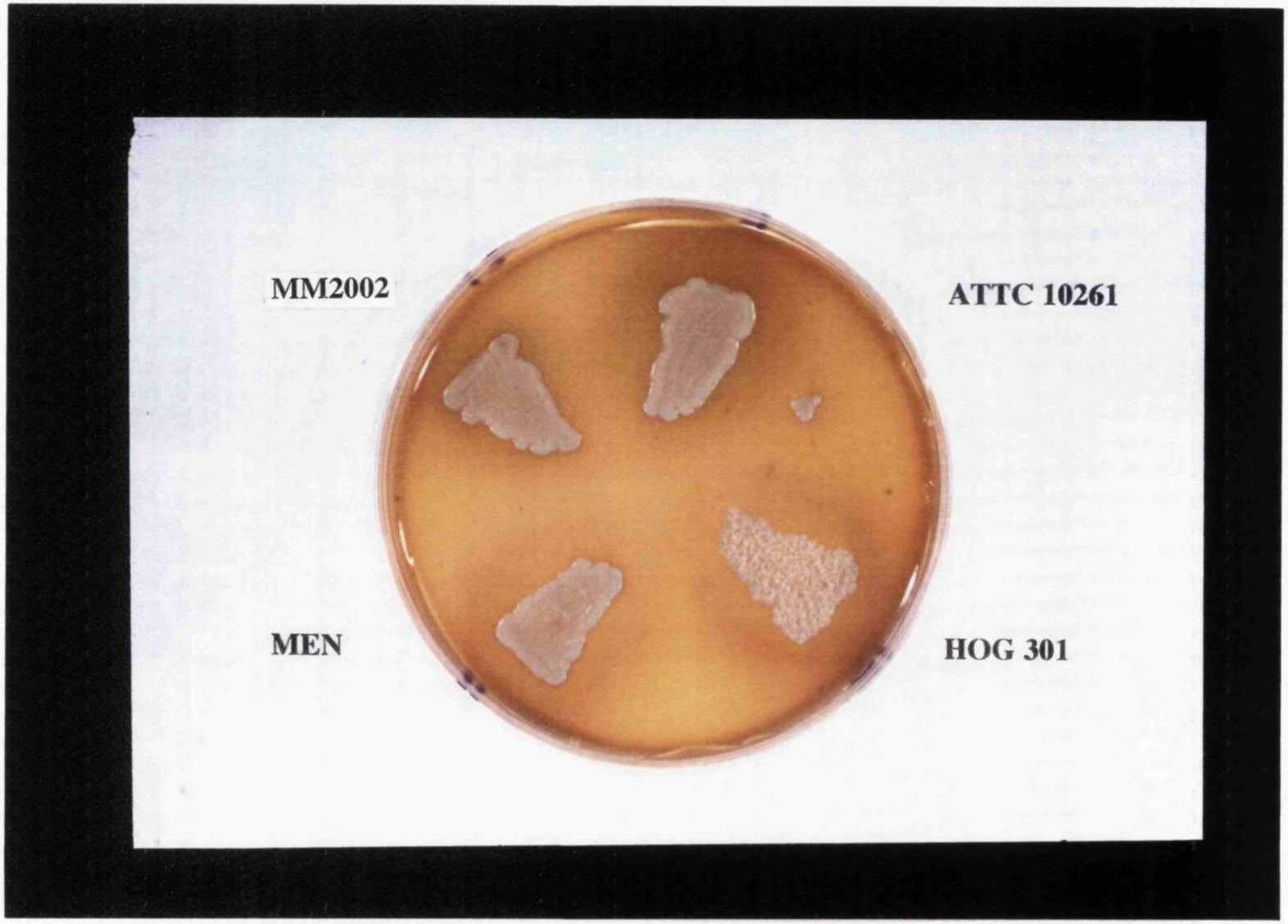

MM2002
ATTC 10261
MEN
HOG 301

Figure 98.

Growth of *C. albicans* and bacterial species on brain heart infusion blood agar incubated at 37˚C in 5% CO_2 for 48 h.

A. *C. albicans* 'Outbreak' strain

B. *Streptococcus pyogenes* NCTC 5763

C. *C. albicans* GDH 2346

D. *Staphylococcus aureus*

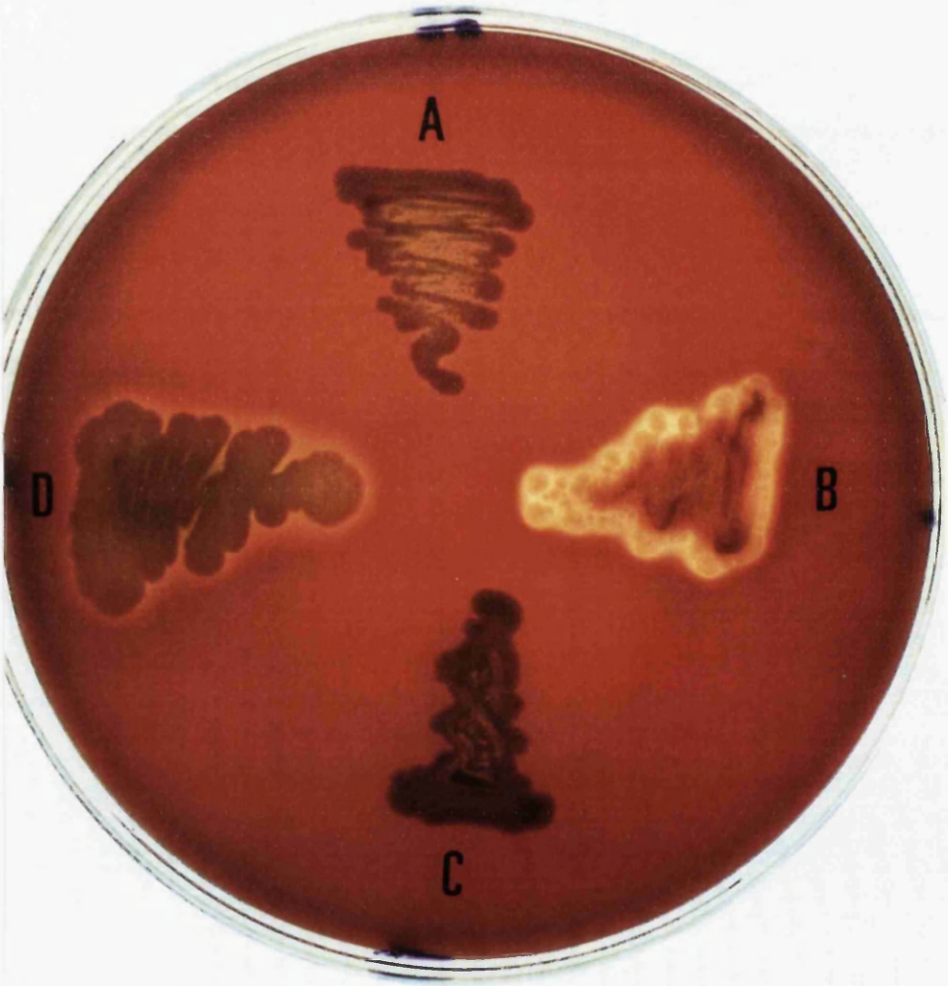

A
B
C
D

Figure 99.

Growth of *C. albicans* and bacterial species on Sabouraud dextrose blood agar enriched with 3% glucose (w/v).

Cultures were incubated for 24 to 48 h at 37°C in 5% CO_2.

A. *Staphylococcus aureus*

B. *C. albicans* 'Outbreak' strain

C. *Streptococcus pyogenes* NCTC 5763

D. *C. albicans* GDH 2346

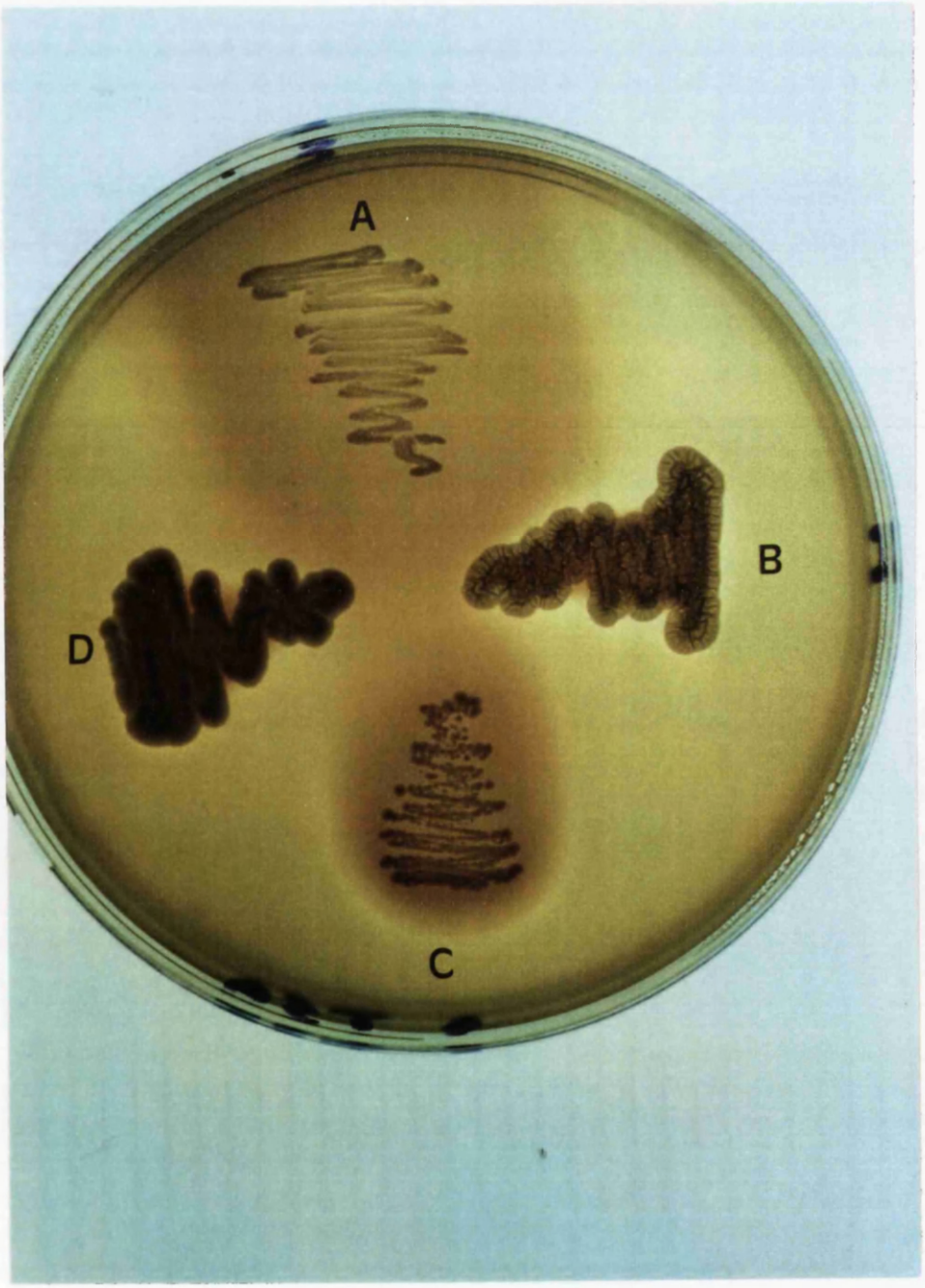

A
B
C
D

Figure 100.

Growth of *C. albicans* and bacterial species on Sabouraud dextrose blood agar incubated for 48 h at 37˚C in 5% CO_2.

The medium contained the normal glucose concentration (4%, w/v) and was not enriched by the addition of a further 3% glucose.

A. *Staphylococcus aureus*

B. *C. albicans* 'Outbreak' strain

C. *Streptococcus pyogenes* NCTC 5763

D. *C. albicans* GDH 2346

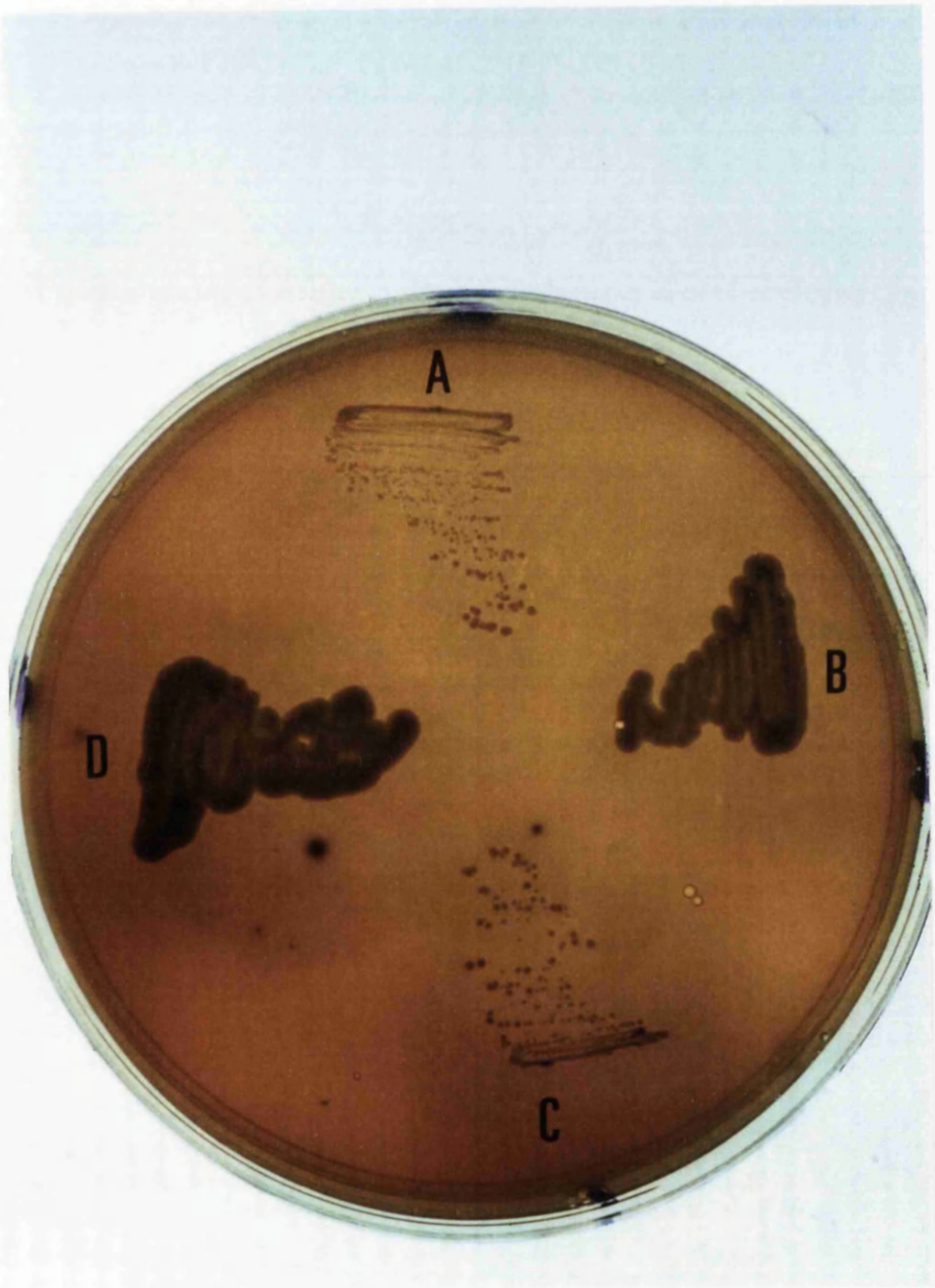

A
B
C
D

Figure 101.

Possible haemolytic effects of blue and yellow pigment components.

Sterile filter paper disks were saturated with a sterile solution containing blue (A) or yellow (B) fluorescent components of the green pigment taken from TLC plates run in BEW (4:1:1). Plates were incubated at 37°C for 24 h in 5% CO_2.

DISCUSSION

1. Production of pure yeast-form and hyphal-form cultures of *C.albicans*

Of the many *Candida* species, the ability to form true hyphal cells is virtually unique to *C.albicans*. Several studies have shown that it is possible to produce cultures of *C.albicans* consisting exclusively of yeast or hyphal forms; this should be the goal of investigators following the relative virulence, invasiveness and immunogenicity of the two forms of this organism. The yeast form is generally the easiest form to grow *in vitro*, whereas hyphal cultures almost always generate some budding cells (Odds, 1985). The aim of this part of the study was to select the best liquid medium for the production of pure yeast or hyphal cultures to be used in subsequent investigations concerning siderophore and green pigment synthesis by the two forms of *C. albicans*.

Lee *et al.* (1975) described an amino acid synthetic medium with the capacity to support growth of *C. albicans* in pure hyphal form for up to 27h when incubated at 37°C, and in pure yeast form if incubated at 25°C, in contrast with the mixture of yeasts and hyphae which always occur when this organism is cultivated on Sabouraud or other common media. Muerkoester *et al.* (1979) confirmed these results when comparing Lee's medium with other liquid media, but obtained maximal hyphal formation after 11h.

Studies with Lee's medium described here revealed that both yeasts and hyphae were present when seven strains of *C. albicans* were incubated at 37°C for 24 or 48h, and no pure hyphal cultures were noted even with the mutant strain 1001-FR, which produces only hyphae on Sabouraud dextrose agar. However only Lee's medium (compared with other media tested) gave heavy clumps consisting of a mixture of the two morphological forms. In addition, the percentage of hyphae varied depending on the strain. On the other hand, pure yeast cultures were obtained when the cells were incubated at 25°C, which corresponds with the findings of Lee *et al.* (1975). Even when cultures were incubated for as long as 15

days, and examined daily microscopically, there was no evidence of hyphal formation.

Other studies have concluded that *C. albicans* is capable of growing in either of the two morphological forms, depending upon the pH of the supporting medium. In the procedure described by Staebell and Soll (1985), Lee's synthetic medium is supplemented with arginine, and adjusted to pH 4.5 or 6.7. The results obtained in the present study confirm the observations of Staebell and Soll; a high percentage of hyphal forms was noted when the pH of the medium was 6.7 (at 37°C), but the cultures still did not consist of pure hyphae. However, at 25°C pure yeast cultures were obtained at both pH values after different incubation times, which highlights the importance of temperature as a major factor in the yeast-to-hyphal transformation.

Incubation at 40°C was less effective in supporting hyphal growth. In a comparative study by Muerkoester *et al.* (1979), incubation in neopeptone-starch broth at 40°C produced peak hyphal growth of 68% after 5h (but only 26% at 37°C); this figure was less than 20% after 7h. Results obtained here after 5, 24 and 48h showed a low percentage (1.4- 26.2%) of hyphal forms with all four strains tested, and did not remotely approach the objective of pure hyphal cultures. Nevertheless, pure yeasts were obtained when cultures were grown at 25°C in this medium.

Marichal *et al.* (1986) described a culture medium which promoted the selective formation of either yeasts or hyphae, and involved N-acetylglucosamine, the precursor of the polysaccharide, chitin. The medium, NYP (N-acetylglucosamine-yeast nitrogen base-proline), lacked glucose since it had been demonstrated that glucose promotes yeast formation. Yeast nitrogen base was added to meet nitrogen requirements, and proline was included because it had often been mentioned as a stimulant for hyphal growth (Marichal *et al.*, 1986).

In this investigation, all strains of *C. albicans* tested showed excellent growth (high optical density) and a high percentage of hyphae when incubated in

NYP medium at 37°C for 24h; pure hyphal cultures were noted with the *C.albicans* mutant 1001-FR(Y^-) at 37°C for up to 72h. These findings correspond with the results of Marichal *et al.* (>90% hyphae). However, *C.albicans* grew exclusively in the yeast form when incubated at 25°C in this medium.

Casein-yeast extract medium was supplemented with glucose (casein hydrolysate-yeast extract-glucose) by Marichal *et al.* (1986), even though glucose was removed from other media, and it became suitable for the production of pure hyphae at 37°C. However, these workers observed only one strain to form hyphae and when the temperature was changed to 25°C even this strain formed no hyphae. Experiments described here reveal that a very low percentage (9.4-13%) of hyphae was formed at 37°C by *C. albicans* GDH 2346 in this medium after 24h; the pure yeast form was obtained at 25°C.

Yeast nitrogen base medium containing 50 mM glucose as the carbon source (McCourtie and Douglas, 1981, 1985; Sweet and Douglas, 1991a, b), produced pure hyphal cultures (99.6%) of the *C. albicans* mutant 1001-FR(Y^-) at 37°C after 24, 48, or 72h, and also about 85% hyphae at 25°C. However, pure yeast cultures were consistently obtained with five other strains incubated at 25, 30, or 37°C in medium containing either 50 mM glucose or 500 mM galactose. These results are similar to those of the previous workers, although strain GRI 681 exhibited about 12% hyphae when incubated for 15 days with 50 mM glucose only. Different results were obtained with static cultures; two strains, GRI 681 and GDH 2346, revealed hyphal formation of up to 68% and 87% respectively, in both carbon sources (galactose or glucose). These findings highlight the effect of aeration on the yeast-to-hyphal transformation. However, other strains exhibited insignificant hyphal formation when incubated as static cultures. Aerated cultures produced greater growth with all strains tested in this medium. In addition, the yeast form was consistently obtained even in YNB medium containing low concentrations of salts and trace elements, such as iron, copper, zinc, magnesium, manganese, and phosphate. Several studies have shown that severe iron or zinc restriction produces

a loss of yeast-to-hyphal transformation or germ-tube-forming ability (Soll, 1985; Sweet and Douglas, 1991b).

The results obtained with glucose-glycine broth are in close agreement with those reported by Evans *et al.*(1974) and Muerkoester *et al.* (1979). Glucose glycine broth incubated at 37°C induced the hyphal form of *C. albicans* as had been demonstrated previously by these workers. In this investigation, with strain GDH 2346, hyphal formation was much higher at up to 97%. Muerkoester and his group tested two temperatures, 37° and 40°C; an insignificant percentage of hyphal forms was noted at 40°C. Here, this medium was tested at 25°C also, and the pure yeast form was observed with all strains. In addition, good growth was noted with all strains. All these findings identify this medium as the best of those examined; it produced a high percentage of hyphae at 37°C but the pure yeast form at 25°C. It was considered suitable for a comparative study between the two morphological forms of *C.albicans* .

The transition of morphologies in *C.albicans* between yeast and hyphae is an environmentally regulated process. Glucose in the medium had little effect on hyphal formation as far as our observations were concerned and no effect was noted when galactose was used as the carbon source, except for a higher cell yield. Overall, this study has demonstrated that optimal physical conditions for rapid hyphal growth are high temperature (37°C) and neutral pH; the chemical composition of the growth medium appears to be somewhat less important in the stimulation of particular morphologies. Several growth media have been used in various laboratories for the production of *C. albicans* hyphae. Serum is probably the most consistently successful medium for stimulation of *C. albicans* hyphae (Landau *et al.*, 1965); this appears to be related to the albumin fraction (Barlow *et al.*, 1974) and specifically to some low molecular weight peptides (Chattaway *et al.*, 1976). However, serum was not used in this study owing to the presence of the iron-binding protein, transferrin, which might interfere with the siderophore assay. It would also have been difficult, if not impossible, to obtain yeast-form cultures in

such a medium. In contrast to the 8-15 μm hyphae formed in glucose-glycine and neopeptone-starch broth, serum germ tubes average 6-8 μm in length (Muerkoester *et al.*, 1979).

Finally, one of the most important environmental factors affecting growth, survival and the yeast-to-hyphal transformation of *C. albicans* is temperature. The ability of *C. albicans* to form hyphae has long been established as an essential virulence factor which enables the yeast to penetrate host tissue. In general, however, the relationship between growth temperature and transition is poorly understood and needs to be further investigated.

2. Production of siderophores by yeasts and hyphae of *C. albicans*

The ability of *C. albicans* to alter the shape of its cells from yeasts to hyphae and vice versa is commonly thought to be significant in the pathogenesis of *C. albicans* infections (Odds, 1985). Moreover, one common and essential factor in all infections is the ability of the invading pathogen to multiply successfully in the tissues of the host. Since *C. albicans* needs iron to multiply (Sweet and Douglas, 1991b), it must, initially, be able to adapt to the iron-restricted environment usually found *in vivo*, where iron in body fluids exists tightly complexed to the high affinity iron-binding glycoproteins transferrin and lactoferrin. For this reason, *C. albicans* produces low molecular mass iron-chelating compounds, known as siderophores (Holzberg and Artis, 1983; Ismail *et al.*, 1985 a,b; Sweet and Douglas, 1991a).

Generally, siderophores are produced by aerobic and facultatively anaerobic organisms in response to an iron-deprived environment. Siderophore secretion by several species of non-pathogenic and pathogenic fungi has been reported (Holzberg and Artis,1983; Winkelmann and Huschka, 1987). However, siderophore production by *Candida* species has not been extensively studied. The earliest work in this laboratory on the effect of iron concentration on siderophore synthesis by *C. albicans* growing in the yeast form suggested that *C. albicans*

synthesises a siderophore of the hydroxamate type (Sweet and Douglas, 1991a). Other studies (Ismail *et al.*, 1985 b) indicated that certain isolates are capable of secreting both hydroxamate and phenolate chelators; however, the morphological form of the cells used was not identified.

The aim of this part of the project was to measure siderophore production by *C. albicans* using chemical assays to determine which morphological form produces greater amounts of siderophore. For the study of iron uptake systems, and the detection and determination of siderophores, a variety of methods have been developed. The most effective assay used so far for the detection of siderophores in liquid or solid media is the universal chemical assay (Schwyn and Neilands, 1987).

The results described here reveal that nine *C. albicans* strains (GRI 681, GRI 682, GDH 2023, GDH 2346, 1001-wild type, 1001-FR(Y^-), 1001-92'(H^-), ATTC 10261, and HOG 301) produce siderophores after growth in four media (glucose glycine broth, NYP medium, Lee's medium and YNB medium) in either the yeast or hyphal form. However, the extent of siderophore production seems to be low; this could be because all media contained normal iron concentrations since growth of *C. albicans* under iron-limitation might affect the formation of hyphae. When siderophore production was considered in relation to cell dry weight, it was found that hyphal forms of *C. albicans* synthesise substantially greater amounts of siderophore than yeast forms after incubation in glucose glycine broth (a medium giving good hyphal production); similar results were obtained with two other media (NYP medium and Lee's medium). However, pure hyphal cultures reported to be produced at 37°C in Lee's medium were not observed; instead mixtures of yeasts and hyphae were obtained. There was also a high level of interference between the chemicals which compose Lee's medium and the universal chemical assay; no such effects were evident with glucose glycine broth and NYP medium.

In the universal siderophore assay, all culture supernates of *C. albicans* tested gave a change in colour, when mixed with CAS solution, from blue to pink due to removal of iron from the dye complex. Siderophores were detected as a

decrease in absorbance at 630 nm after 1 h (Sweet and Douglas, 1991a). Strain GRI 681 (hyphal form) appeared to produce the greatest amount of siderophore compared with other strains (GRI 682, GDH 2023 and GDH 2346) after an incubation time of either 24 or 48h. However, all nine strains produced strong pink halos around the colonies after growth on YNB blue agar at 37°C for 24h, indicating siderophore production. These pink halos were obtained from both morphological forms; strains 1001-FR and HOG 301 grew in the hyphal form on YNB blue agar while other strains grew exclusively in the yeast form at 37°C.

To eliminate the possibility that high temperature was the cause of induction of siderophore synthesis, and not morphological form, two wild-type strains and their morphological mutants were grown at 37°C in four media. In each case, siderophore production by the wild type was greater than that of the mutant (s) in glucose glycine broth, indicating a possible effect of the mutation on siderophore production. However, several studies have shown that temperature may play a major role in siderophore production. For example, increase in incubation temperature from 37°C to 41°C produced a marked decrease in both the rate and quantity of siderophore production by *C. albicans* (Ismail *et al.*, 1985a). Other studies have revealed that induction can occur at higher temperatures, such as 39°C (Winkelmann and Huschka, 1987) or temperatures as low as 27°C (Holzberg and Artis, 1983).

When grown in an iron-restricted environment certain strains of *C. albicans* are capable of secreting siderophores (Sweet and Douglas, 1991a; Ismail *et al.*, 1985 a,b). In this study, the effect of iron concentration on siderophore production was investigated by growing *C. albicans* 'Outbreak' strain in medium with iron concentrations ranging from 0.026 to 0.8 μM. A concentration of 0.8 μM provides excess iron, since adding more $FeCl_3$ does not increase growth (Sweet and Douglas, 1991a). The results revealed that siderophore synthesis by *C. albicans* was induced at low iron concentrations. It was maximal at the growth-limiting iron concentration of 0.026 μM; this finding is similar but not identical to those of Sweet

and Douglas (1991a), who reported maximal siderophore production at 0.2 μM iron concentration.

3. Presence of hydroxamate-type siderophores and absence of the phenolate-type

Generally, fungi produce siderophores of the hydroxamate-type (Holzberg and Artis, 1983; Winkelmann and Huschka, 1987), and to date, the production of both classes of siderophores by yeasts has not been reported except by Ismail and her colleagues (Ismail *et al.*, 1985b). Some 40% of the *C. albicans* isolates they tested by the Arnow method produced phenolate-type siderophores in addition to hydroxamate-type compounds. In the present study, only one type of iron-binding compound, a hydroxamate-type siderophore, was produced by *C. albicans* . These results were obtained after growth of *C. albicans* strains in three liquid media as yeasts (at 25°C) or hyphae (at 37°C) for different time periods. The method used to detect hydroxamate-type siderophores was based on that described by Holzberg and Artis (1983), who demonstrated that oxidation of certain hydroxamic acids yields a material with a very strong absorption at 264 nm.

These findings coincide with earlier results obtained by Sweet and Douglas (1991a), that *C. albicans* , as well as isolates of *C. lusitaniae*, *C. glabrata* and *C. parapsilosis* synthesised only hydroxamate compounds. When absorption readings were related to cell dry weight, hydroxamate production by hyphae appeared to be greater than that of yeast-form cells after growing in glucose glycine broth. However, no significant difference in hydroxamate production was noted between the morphological forms of *C. albicans* in NYP medium and Lee's medium. Nevertheless, hydroxamate secretion appeared to be greater in Lee's medium than in NYP medium.

Phenolate-type siderophores were assayed by a method based on that described by Arnow (1937) but all nine strains produced readings of less than 0.05, which was considered negative. These results were obtained with all *C. albicans*

strains tested after growth in four media in the yeast or hyphal form for different time periods up to 7 days. In addition, no yellow colour was observed following the addition of nitrous acid to the culture supernates, and no red colour was produced when the solutions were made strongly basic, with no absorbance at 515 nm.

Comparison of hydroxamic acid production by *C. albicans* wild-type strains and morphological mutants revealed that all strains produced hydroxamate but not phenolate-type siderophores in four media tested but that there was no clear correlation between hydroxamate production and morphological form, However, siderophore production was very low in these experiments. The results further support the conclusion that *C. albicans* produces only one type of siderophore. Additional studies would be needed to confirm the absence or presence of phenolate-type siderophores using the biological assay described by Ismail *et al.* (1985b).

4. Influence of medium components on siderophore assays

A universal chemical assay (Schwyn and Neilands, 1987), which was used for the non-specific detection of siderophores in solution, was tested using desferal solutions. A strong pink colour was noted with all desferal concentrations and a very intense colour appeared at high concentrations. Results showed a positive relationship between desferal concentration and the percentage reduction in absorbance.

Chelators in the growth medium interfere with this assay and have to be avoided. Schwyn and Neilands (1987) showed that ligands with a lower iron affinity, like phosphate, citrate, or DHBA (2,3-dihydroxybenzoic acid), did interfere with the universal chemical assay but only at higher concentrations. In this study, only Lee's medium (an amino acid synthetic medium) interfered in an irreproducible manner, probably because of varying compositions of the

components. However, glucose-glycine broth and NYP medium showed no effect on the assay.

In tests with the specific hydroxamate assay (Holzberg and Artis, 1983; Emery and Neilands, 1962) and solutions containing different concentrations of desferal (the positive control), a straight-line relationship was obtained between concentration and absorbance at 264 nm. Furthermore, with three media (Lee's medium, NYP medium and glucose glycine broth) to which various concentrations of desferal had been added, similar linear relationships between absorbance at 264 nm and concentration were also obtained, indicating that there was no interference in any of these assays.

The Arnow assay (Arnow, 1937; Holzberg and Artis, 1983) was tested with solutions containing different concentrations of catechol. All catechol solutions gave a change in colour from yellow to orange-red, a more intense colour appearing at higher concentrations. There was a linear relationship between absorbance at 515 nm and catechol concentration. Without exception all three media displayed a similar, linear relationship following the addition of catechol, indicating that none of them (Lee's medium, NYP medium and glucose-glycine broth) interferes with the phenolate assay.

Siderophore and hydroxamate production was related to cell dry weight. The method used was that described by Sweet and Douglas (1991a). Higher cell dry weights were observed with Lee's medium as compared with the two other media (NYP medium and glucose glycine broth).

5. Green pigment production by *C. albicans*

Green pigment production by *C. albicans* and *C. stellatoidea* on plates of blood agar was first observed by Jones and Peck in 1940. Subsequently, McCourtie and Douglas (1985) noted the synthesis of green pigment by *C. albicans* during prolonged incubation in medium containing 500 mM galactose. More detailed work on green pigment production by *C. albicans* was reported by Sweet and Douglas in

1991. The aims of this study were first, to investigate pigment production by yeast and hyphal forms in several liquid media and on blood agar; second, to explore the effect of salts, trace elements, carbon source, and aeration on green pigment production; third, to isolate the pigment (s) using solvent extraction and compare it with another pigment (pink) produced by the same strains; fourth, to investigate the relationship between siderophore and green pigment production.

The results described here reveal that ten *C. albicans* strains (GRI 681, GRI 682, GDH 2023, GDH 2346, 'Outbreak', 1001-wild type, 1001-FR(Y^-), 1001-92'(H^-), ATTC 10261, and HOG 301) produce green pigment after growth in four media (glucose glycine broth, NYP medium, Lee's medium and YNB medium) in either the yeast or hyphal form. The amount of pigment detected varied from one strain to another. When green pigment production was considered in relation to cell dry weight, it was found that yeast forms of *C. albicans* synthesise substantially greater amounts of pigment than hyphal forms after incubation in two media (NYP medium and Lee's medium). In the case of glucose-glycine broth, only strain GDH 2023 showed greater pigment production by yeasts than by hyphae; all other strains gave the opposite result. All of the morphological mutants tested, as well as the corresponding wild-type strains, were capable of producing the green pigment. All strains inoculated on to Sabouraud dextrose blood agar showed a very dark grey-green zone around the colonies after incubation at 37°C for 24h. This result corresponds with the finding of Jones and Peck in 1940.

Green pigment production was observed in a liquid medium containing high concentrations (500 mM) of galactose and was particularly noticeable with cultures of *C. albicans* GDH 2346. This result agrees with the findings of McCourtie and Douglas (1985). Pigment production was increased sharply by extension of the incubation period and maximum readings were noted after 15 days. This result was confirmed using the 'Outbreak' strain which is a high pigment producer. Low production of green pigment was observed when a low concentration (50 mM) of glucose was used as the carbon source. Pure yeast

cultures were obtained with five strains tested on both carbon sources. Overall, these findings indicate that *C. albicans* secretes a green pigment during prolonged incubation in medium containing either 500 mM galactose or 50 mM glucose; however, the quantity of pigment produced is greater in galactose medium.

Temperature has a great influence on morphogenesis in *C. albicans*. For this reason, an investigation was made of the effect of temperature (25, 30 and 37°C) on green pigment production on different carbon sources. The findings showed that all four strains tested produce pigment at all of the temperatures tested. Statistical analysis of the data revealed only very small differences in pigment production, with an increase at 37°C for one strain. In this study, the carbon source (galactose or glucose) and incubation period had a greater influence on pigment production than did temperature.

Four strains were examined for the effect of oxygen on pigment production. By setting up static and shaking cultures for these strains, the effect of oxygen availability was studied. Cultures were grown in either high galactose or low glucose medium. After incubation for 10 days at 37°C in YNB containing 500 mM galactose or 50 mM glucose, all strains produced far more pigment in the aerated cultures than in the static cultures. This suggests that oxygen may induce pigment production to a certain extent. However, the amount of pigment produced also depended on the carbon source. With either the static or the aerated cultures, pigment production was greater in high galactose medium than in low glucose medium. Low production of pigment was observed during prolonged incubation in static cultures, and increased sharply in aerated cultures. However, for all strains, growth was better in the aerated cultures than in the static cultures. Yeast-form cells only were present in aerated cultures but a high proportion of hyphae was obtained with strains GRI 681 and GDH 2346 in static cultures.

6. Effect of various salts and trace elements on green pigment production

Several studies have indicated that the iron concentration in the growth medium has a significant effect on pigment production. Green pigment production *in vitro* has previously been shown to be regulated by the availability of iron (Sweet and Douglas, 1991a). Synthesis of a brown pigment by some strains of *C. albicans* is enhanced by iron (Chaskes and Phillips, 1974). Similarly, an iron concentration of 0.001% enhances red pigment production by *Candida pulcherrima* (Roberts, 1946). Finally, the production of riboflavin, a yellow-orange vitamin, by *Candida utilis*, *C. guilliermondii* and *C. famata* (Prescott and Dunn, 1959) was also shown to be linked to the iron concentration. In this study, *C. albicans* 'Outbreak' strain was grown at 37°C in defined liquid media supplemented with various concentrations of iron ranging from 0.026-0.8 μM, and containing 500 mM galactose or 50 mM glucose as the carbon source. On visual examination of the cultures, a distinct gradation in the amount of green pigment could be seen, with gradually decreasing amounts as the concentration of iron was increased. On examination of the culture supernates, the same pattern became much clearer. The results obtained with low glucose medium confirmed those reported by Sweet and Douglas (1991a). Surprisingly, however, high galactose medium did not induce pigment production at low iron concentrations, even after incubation for two weeks. All cultures containing galactose and low iron concentrations showed poor pigment production as compared with those containing glucose.

C. albicans grew as yeasts in 0.026-0.8μM iron-containing media with 0.026μM iron as the growth-limiting concentration. Pure yeast-form cultures were obtained with low glucose or high galactose medium.

In the light of the effect of iron limitation on green pigment production, additional experiments were carried out to see if other important salts and trace elements had similar effects. *C. albicans* was grown in defined media supplemented with various concentrations of phosphate, magnesium, manganese,

zinc and copper. Several previous studies have investigated the effect of zinc on the growth and morphology of *C. albicans*. At least two cellular characteristics were found to differ: (i) zinc-limited cells appeared more homogeneous and larger on average than zinc-excess cells, and (ii) zinc-limited cells evaginated on the average 40 min later than zinc-excess cells (Anderson and Soll, 1984). Zinc-deficient cultures consisted almost entirely of hyphae but the proportion of yeast-form cells increased with increasing concentrations of zinc, reaching a maximal level at 9 μM zinc (Yamaguchi, 1975). Zinc completely suppressed hyphal formation at 25°C (Bedell and Soll, 1979). In this study, three strains were grown in defined medium (YNB) containing 0-1μM zinc at 37°C for 5 days. Zinc limitation showed only a slight effect on green pigment production compared with iron limitation. There was little increase in pigment at concentrations of 0.1-1μM Zn compared with cultures containing the normal zinc concentration (1.39μM, the concentration of zinc in YNB powder). Good growth was noted even with media containing no added zinc which may be related to the presence of traces of zinc in other chemicals which comprise the YNB medium.

Manganese is required by yeasts at trace concentrations for optimal growth and fermentation (Parkin and Ross, 1986). In this study, manganese limitation had no effect on green pigment production. Pigment was only observed in control cultures which contained 1.79μM manganese sulfate (the concentration in YNB powder), which is not a growth-limiting concentration. Growth was most extensive in these control cultures (high-galactose) and cells were exclusively in the yeast form.

C. albicans requires magnesium for germ-tube formation. Magnesium increases the uptake and incorporation of N-acetylglucosamine, and previous studies have pointed indirectly to the involvement of Mg^{2+} ions in *C. albicans* morphogenesis (Walker *et al.*, 1984). Here, *C. albicans* was grown in defined (YNB) medium supplemented with 0-1 mM $MgSO_4$ for 5 days at 37°C. The results showed that the Mg-limited media did not induce green pigment production;

considerable amounts of pigment were apparent only in two control cultures containing a normal concentration of magnesium (2mM, the concentration in YNB powder). The results also showed that magnesium is necessary for growth; poor growth was noted in media containing no added magnesium.

Inorganic phosphate is abundant in some environments, and is the major source of phosphate for *C. albicans*. However, low phosphate concentrations limit microbial growth in many aquatic environments and this can have a significant effect on the cell-surface composition of many bacteria and fungi. Phosphate is present in nucleic acids, phospholipids, lipopolysaccharides, nucleotides such as ATP, GTP, NAD^+ and various cytoplasmic solutes (Gottschalk, 1986; Torriani-Gorini *et al.*, 1987). In this study, the effect of phosphate-limitation on pigment production was examined. Three strains of *C. albicans* were grown in defined medium supplemented with phosphate at concentrations ranging from 0-0.5 mM for 5 days at 37°C. For each strain examined under these conditions, no pigment production was seen at any of the given phosphate concentrations. A green pigment was synthesised only in YNB containing 500 mM galactose after 5 days, but not in YNB containing 50 mM glucose. These control cultures contained phosphate at a concentration of 7 mM (the concentration in YNB powder), which is not growth limiting. From these results, it can be concluded that phosphate limitation does not stimulate pigment production.

Iron and copper metabolism appear to be closely linked in *C. albicans*. A recent report suggests that *C. albicans* has an iron- and copper-regulated ferric reductase activity (Morrissey *et al.*, 1996). Iron reduction and uptake in *Saccharomyces cerevisiae* (Lesuisse *et al.*, 1987; Lesuisse and Labbe, 1989; Lesuisse *et al.*, 1990) are connected to copper reduction and the ferric-reductase activity is negatively regulated by copper. Quantitative cupric-reductase assays have indicated that *C. albicans* is capable of reducing copper and that this cupric-reductase activity is negatively regulated by both iron and copper (Morrissey *et al.*, 1996). Copper is known to affect pigmentation in the black yeast, *Aureobasidium*

pullulans , as well as the transformation of yeast-like cells into chlamydospores (Gadd and Griffiths, 1980). Since production of a green pigment by *C. albicans* is regulated by iron, the effect of copper limitation was also investigated. Three strains of *C. albicans* were grown in defined (YNB) media containing low copper concentrations (0-0.1 μM) at 37°C for 5 days. The results indicated that copper limitation does not stimulate green pigment production. However, the amount of pigment present in cultures containing low copper concentrations was equal to that of the control cultures in high-galactose YNB medium containing 0.16μM copper sulfate (the concentration in YNB powder); this was not observed when low glucose was used. Copper did not induce hyphal growth and cultures contained only yeast-form cells.

Overall, this study has demonstrated that green pigment production by strains of *C. albicans* is affected by a number of environmental and other factors including iron limitation, cell morphology, strain variation, carbon source and oxygen concentration. However, this list is not exhaustive and additional, as yet unknown parameters may be involved.

7. Production of a haemolytic factor by *C. albicans*

Several studies have shown that *C. albicans* exhibits haemolytic activity when grown on blood agar. Manns *et al.* (1994) found that *C. albicans* had the ability to utilize iron derived from haemoglobin and displayed haemolytic activity when grown on glucose-enriched blood agar at 37°C in 5% CO_2. Haemoglobin released from lysed erythrocytes could restore the transferrin-inhibited growth of *C. albicans*. In this study, twelve strains tested showed a grey zone around the colonies only when the agar was enriched with glucose and was incubated in the presence of 5% CO_2. No lysis was noted on brain heart infusion blood agar, which is a growth medium often used to demonstrate haemolytic activity by *Streptococcus pyogenes* and *Staphylococcus aureus*. In some cases, when filter paper disks saturated with solutions containing the blue or yellow fluorescent

material of the green pigment were placed on Sabouraud dextrose blood agar or Trypticase soy blood agar enriched with 3% glucose, apparent zones of haemolysis were observed around disks containing the yellow component but not around those containing the blue component. Additionally in some experiments, filter paper disks saturated with supernates from iron-limited (0.026 μM) cultures showed zones of lysis but those saturated with supernates from control (0.8 μM) cultures did not.

Overall, the results (which are only preliminary observations) showed that *C. albicans* seems to have haemolytic activity under certain conditions and that the factor responsible for this activity is secreted into the growth medium. However, further investigations are required to clarify unknown factors which affect haemolytic activity by *C. albicans*. Some factors have been studied by Manns and his group (1994). These workers have shown that: (i) no lysis occurred on plates containing sucrose, lactose, or galactose instead of glucose in the medium; (ii) no lysis occurred when cultures were grown at temperatures other than 37°C; and (iii) hyphal forms showed greater haemolytic activity than the yeast form. The findings of Manns *et al.* (1994) indicated that *C. albicans* has the ability to utilize iron derived from haemoglobin and that *C.albicans* may acquire this iron by producing a factor which can release haemoglobin by lysing erythrocytes. In preliminary observations described here, supernates from iron-limited cultures showed zones of lysis on blood agar plates but supernates from control cultures did not. However, Manns *et al.* (1994) reported no increase in haemolytic activity by cultures grown in the presence of transferrin, under conditions of iron deprivation.

8. Pink pigment production by *C. albicans*

Pigment production can be an important aid in both identification and classification of micro-organisms. Several investigators have studied pigmentation in *Candida* species. Chaskes and Phillips (1974), showed that the medically important *Candida* species produce a pink pigment when cultured on a glucose-salts-biotin medium with tryptophan as the major nitrogen source. Iron enhanced

pigment production, and Chaskes and Phillips (1974) also showed that optimal conditions for brown and pink pigment synthesis were achieved by incubating *C. albicans* in media containing tryptophan and proline (or asparagine) supplemented with iron. In this study, for a comparison with green pigment production, *C. albicans* 'Outbreak' strain was grown on tryptophan (glucose-salts-biotin) medium. The effect of adding iron and different nitrogen sources to the medium was also investigated.

The results revealed that *C. albicans* produces a pink pigment in tryptophan medium only in the presence of $FeSO_4$ (0.2 g/litre). Pigment was not observed in the absence of added iron. Pigment could be detected even after 24h incubation and giant yeast cells were observed microscopically, with a diameter two to three times that of normal yeasts. This medium did not induce hyphal formation; only yeast cells were seen. Glucose-salts-biotin medium containing proline as a nitrogen source did not induce pink pigment production, with or without added iron. These findings support the conclusion that tryptophan is required for pink pigment synthesis and that synthesis is enhanced in the presence of iron. Previous studies revealed that *Candida* species can produce indole or tryptophan derivatives which are responsible for the brown-pink colour (Schindler and Zahner, 1971; Chaskes and Phillips, 1974).

9. Comparison between green and pink pigments

Light-dependent reactions in which micro-organisms produce pigments have been described previously. Benjamin and Tamhane (1966) using *E.coli* reported that an orange-red pigment was formed in a light-dependent reaction from an indole derivative resulting from tryptophan breakdown. Zambonelli and Guerzoni (1969,1970) showed the importance of light in the formation of photorubin, an orange-red pigment. This was due to a photochemical reaction between anthranilic acid and the indole derivative, 3-indolyl-propan-1,2-diol, both of which may be excreted into the culture medium by *Saccharomyces cerevisiae*.

Indole derivatives are sensitive to physical agents such as light (Chaskes and Phillips, 1974). In this study, pink pigment production by *C. albicans* was strongly catalyzed by light. Additionally, exposure of the sterile pink supernates to ordinary laboratory light near the window for four months caused a colour change to light orange. However, exposure of the green pigment to light had an even greater effect; the pigment became colourless after just a few days. This finding agrees with observations made by Jones and Peck (1940), who found that the green pigment changed to a yellow colour on standing for a few days. The pink pigment was sensitive to alkali and on addition of sodium hydroxide it converted to a light orange, returning to pink with the addition of acid; this corresponds with the observations of Chaskes and Phillips (1974). Jones and Peck (1940) noted that the green pigment was an indicator, giving a blue-green colour in acid solution and a yellow colour in alkaline solution. However, in this study, adding acid or alkali had no effect on the green pigment.

10. Extraction and thin layer chromatography of green and pink pigments

Jones and Peck (1940) reported that the green pigment was soluble in acetone, ethyl acetate and acetic acid but insoluble in ether and methyl, ethyl or amyl alcohols. In this study, the pigment was found to be soluble in acetone, water, ethanol or acetic acid, and insoluble in amyl alcohol or ethyl acetate. Furthermore, the green pigment was found in culture supernates but not in the cells. All cell pellets became colourless after centrifugation and no pigment could be extracted with acetone. The pink pigment was soluble in water and ethanol, and was also found in culture supernates but not in cell pellets.

Both extracted pigments (green and pink) and freeze-dried culture supernates were subjected to TLC. A total of 15 different solvents were tested for pigment separation and R_f values were measured and compared. Several solvents were able to separate the green pigment into two fluorescent bands when viewed

under UV light although there was just a single visible band. The most effective solvents were butanol : ethanol : water (4:1:1) and butanol : acetic acid : water (65:10:25); both separated the green pigment into a single visible yellow band and two fluorescent bands when viewed under UV light. The latter consisted of a yellow fluorescent band with an *Rf* value the same as that of the visible yellow band, and a blue fluorescent band with a higher *Rf* value. This result was obtained when freeze-dried culture supernates were chromatographed. However, acetone-extracted green pigment separated poorly and no separation was observed with ethanol-extracted green pigment.

With most of the solvents, only two fluorescent bands were visible under UV light. Chloroform : acetic acid : water (125:73:3) was the only solvent which separated the pigment into two blue fluorescent bands and one yellow fluorescent band when viewed under UV light. Some solvents, such as phenol saturated with water, gave only a blue fluorescent band.

When freeze-dried culture supernates containing green pigment were subjected to TLC with solvents used for the separation of phospholipids, cholesteryl esters, free fatty acids and cholesterol, the results obtained showed that the pigment was not a lipid. In addition, no brown spots appeared in positions corresponding to the yellow and blue fluorescent bands of green pigment when plates were sprayed with 50% sulphuric acid then heated in an oven.

Freeze-dried culture supernates and ethanol-extracted pink pigment separated into several bands with a solvent system of butanol : ethanol : water (4:1:1); these comprised a visible pink band and three fluorescent bands (one purple and two yellow). This result corresponds with the observation of Chaskes and Phillips (1974), who recorded the same *Rf* value (0.43) for the purple fluorescent band.

Both pigments and a siderophore (desferal) were subjected to TLC using a solvent system of 5% ammonium formate plus 0.5% formic acid (Rogers, 1973). No fluorescent bands were apparent under UV light in the samples of desferal and

pink pigment but there were two fluorescent components corresponding to the green pigment. However, when plates were sprayed with $FeCl_3$, brown spots appeared only with desferal. The same results were obtained with a solvent system of butanol: ethanol: water (4:1:1). These findings indicate that there is no similarity between either pigment and siderophores. Overall, this study has demonstrated that the green pigment contains two fluorescent components (yellow and blue) and that the two pigments, pink and green, are quite different from one another.

11. Absorption spectra of the pigments

An examination of the absorption spectrum of the green pigment was carried out using pigment-containing supernates from cultures grown in low (0.026μM) and normal (0.8μM) concentrations of iron; solvent-extracted pigment (acetone and ethanol extracts), and the purified yellow and blue fluorescent pigment components were also examined. Absorption maxima were recorded at 360 nm and 440 nm, and absorption decreased to zero at a wavelength of 520 nm. The maximum (655 nm) and minimum (516 nm) absorption wavelengths reported by Jones and Peck (1940) were not observed. However, the absorption maximum was similar to that (444 nm) measured by Sweet and Douglas (1991a), who also found that the green pigment showed no absorption at 520 nm. The pink pigment-containing supernate did not absorb at 640 nm but gave a broad absorption band between 400 nm and 535 nm. Ethanol-extracted pink pigment also showed no absorption at 640 nm but a peak at 450 nm. This exactly corresponds to the absorption maximum reported by Chaskes and Phillips (1974).

Fluorescence spectra were also studied using purified components of the green pigment. When the blue fluorescent material was used, a peak at 440 nm (blue colour area) was obtained; a peak at 520 nm (green colour area) was observed when the yellow material was used. These results were confirmed using a green pigment-containing supernate. No peaks were produced by sterile YNB medium or by individual chemicals comprising YNB medium. The pink pigment showed peaks

at 310 nm and 360 nm (both blue colour area) when two excitation wavelenghts were used (370 nm and 310 nm, respectively). These results indicate that the pink pigment contains only one component. No similarities were observed between the two pigments.

Interestingly, the absorption maximum of the green pigment is close to absorption maximum of siderophores. The production of siderophores in liquid media has been investigated spectrophotometrically. Kumar and Dube (1991) found that the siderophore produced by *Pseudomonas* spp. gave a peak around 405 nm. Xu and Gross (1986) demonstrated that *P. putida* and *P. fluorescens* isolates produced an absorption maximum of approximately 400 nm, which is typical for the pyoverdine class of siderophores. Sher and Baker (1982) added $FeCl_3$ to half of the cell-free culture supernate of *P. putida*, and measured the absorption at various wavelengths (350 to 500 nm) with a spectrophotometer; they observed an absorption peak for the siderophore at 410 nm. Recently, work done by Manninen and Sandholm (1994) confirmed that siderophores showed an absorption maximum at 400 nm when *P. fluorescens* was grown without added iron.

Overall, this study has revealed that the green pigment produced by *C. albicans* consists of two chemical compounds; one has a fluorescent yellow colour and the other has a fluorescent blue colour. A combination of the two creates the green colour. The green pigment is completely different from the pink pigment. Its production is regulated by the availability of iron and maximal synthesis is attained at growth-limiting concentrations of the element. Greater amounts of siderophore are also produced under iron-limitation. Thin-layer chromatography has failed to reveal any similarity between the siderophore and green pigment produced by *C. albicans*. It is, still possible that the pigment represents a novel type of iron chelator; further work is required to clarify this interesting point.

12. Conclusions and future work

Since the levels of free iron *in vivo* are well below microbial requirements, it might be expected that possession of high-affinity iron-scavenging systems would constitute an important element in microbial virulence. The ability of *C. albicans* to form hyphae has long been proposed as an essential virulence factor which enables the yeast to penetrate host tissue. The present finding that the hyphal form produces greater amounts of siderophore than the yeast form provides additional evidence for the importance of this morphological form in pathogenicity. However, further work is required to clarify: (i) the mechanism whereby *C. albicans* normally acquires iron when growing in its human host; (ii) the chemical structure of the *Candida* chelators and their role during infection; (iii) whether iron restriction plays a part in the yeast-to-hypha transformation.

Pigment production can be an important aid in both identification and classification of microorganisms. Previous work has shown that *C. albicans* can produce a green pigment when grown in medium containing a high concentration of galactose. This study revealed that green pigment production is affected by a number of environmental and other factors including iron limitation and cell morphology, as well as carbon source. Strain variation was also evident and additional, as yet unknown parameters may be involved. The green pigment consists of two chemical compounds: one has a fluorescent yellow colour and the other has a fluorescent blue colour. A combination of the two creates the green colour. Further work is needed to establish the role of light in pigment degradation. Additional experiments are also required to distinguish between oxygen and growth rate as contributing factors to the enhanced pigment production observed in aerated cultures. Both green pigment and siderophore production are induced under iron-restricted conditions. However, thin-layer chromatography has failed to reveal any chemical similarity between the siderophore and green pigment. It is still possible that the pigment represents a novel type of iron chelator; further work is required to clarify this, and its role in fungal metabolism and pathogenicity remains to be uncovered.

With the increasing importance of *Candida albicans* as an opportunistic human pathogen, more work is clearly needed on the pathogenic mechanisms of this organism, including its ability to acquire iron, with a view to improving therapy or instituting preventative measures for *Candida* infections.

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APPENDICES

Appendix 1. Media

App. 1.1. Lee's Medium (Amino Acid Synthetic Medium).

Formulation as given by Lee *et al.* (1975).

Chemical	Weight (g)
Salts	
$(NH_4)_2SO_4$	5.0
$MgSO_4.7H_2O$	0.2
K_2HPO_4 (Anhydrous)	2.5
NaCl	5.0
Glucose	12.5
L-Amino acids	
Alanine	0.5
Leucine	1.3
Lysine	1.0
Methionine	0.1
Ornithine	0.0714
Phenylalanine	0.5
Proline	0.5
Threonine	0.5
Biotin	0.001

Distilled water to 1000 ml. Ingredients were combined and autoclaved at 110°C at 10 Ibs for 20 min. Biotin was added after autoclaving, pH is 6.8± 0.05

App. 1.2 Lee's Amino Acid Synthetic Medium plus Arginine, pH 4.5 and 6.7 (Lee's medium+Arginine).

The method was described by Staebell and Soll (1985). Lee's synthetic medium (Lee *et al.*, 1975) was supplemented with 70 µg arginine per ml. The medium was adjusted to pH 4.5 or 6.7 with HCl.

App. 1.3. Glucose Glycine Broth (GGB)

Formulation as given by Muerkoester *et al.* (1979).

Chemical	Percentage (w/v)
Glucose	1.0
Glycine	1.0
Yeast extract	0.1

The glycine-yeast extract was adjusted to pH 7.5 with solid $NaHCO_3$ before the glucose solution was added.

App. 1.4. Neopeptone Starch Broth (NSB)

Formulation as given by Muerkoester *et al.* (1979).

Chemical	Percentage (w/v)
Neopeptone	2.5
Yeast extract	0.1
Soluble starch	0.2

Before autoclaving, the neopeptone starch broth was adjusted to pH 7.5 with 10 M NaOH.

App. 1.5. Casein-Yeast Extract (CY)

Formulation as given by Marichal *et al.* (1986).

Chemical	Weight (g/l)
Casein hydrolysate	5
Yeast extract	5

App. 1.6. N-acetylglucosamine-Yeast Nitrogen Base-Proline Medium (NYP)

Formulation as given by Marichal *et al.* (1986).

Chemical	Weight (g/l)
N-acetylglucosamine	0.22
Proline	0.12
NaCl	4.5g
Yeast nitrogen base	3.35g

App. 1.7. Blue agar for detection of siderophores

Formulation of YNB agar

Chemical	Grams / 90 ml
Glucose	0.45
Yeast nitrogen base	0.60
Agar (Agar technical No. 3, Oxoid)	1.08

Formulation of blue solutions was given by Schwyn and Neilands (1987).

Chemical	Quantity/10 ml
CAS (2 mM)	5 ml
$FeCl_3$ solution (1 mM $FeCl_3$ plus 10 mM HCl)	1 ml
HDTMA (5 mM)	4 ml

5 ml of 2 mM CAS was mixed with 1 ml $FeCl_3$ solution (1 mM $FeCl_3$ and 10 mM HCl). This blue solution was slowly added to 4 ml of 5 mM hexadecyltrimethylammonium bromide (HDTMA). The resulting dark blue liquid was autoclaved. The dye solution was finally added to sterile YNB agar along the glass wall of the flask with enough agitation to achieve mixing without generation of foam.

App. 1.8. Yeast Nitrogen Base (YNB)

YNB medium was made up from individual constituents as outlined in Difco manual.

	Chemicals	**Weight/litre**
Nitrogen source	Ammonium sulfate	5 g
Amino acids	L-Histidine monohydrochloride	10 mg
	LD-Methionine	20 mg
	LD-Tryptophan	20 mg
Vitamins	Biotin	2 µg
	Calcium pantothenate (D-Pantothenic acid)	400 µg
	Folic acid	2 µg
	Inositol	2000 µg
	Niacin (Nicotinic acid)	400 µg
	P-Aminobenzoic acid	200 µg
	Pyridoxine hydrochloride	400 µg
	Riboflavin	200 µg
	Thiamine hydrochloride	400 µg
Trace elements	Boric acid	500 µg
	Copper sulfate	40 µg
	Potassium iodide	100 µg
	Ferric chloride	200 µg
	Manganese sulfate	400 µg
	Sodium molybdate	200 µg
	Zinc sulfate	400 µg
Salts	Potassium phosphate, Dibasic	1 g
	Magnesium sulfate	0.5 g
	Sodium chloride	0.1 g
	Calcium chloride	0.1 g

Final pH ± 0.2 at 25°C = 5.4

App. 1.9. Glucose-Salts-Biotin Medium (Tryptophan Medium)

Formulation as given by Chaskes and Phillips (1974).

Chemicals	Weight/litre
Biotin	20 µg
KH_2PO_4	4 g
$MgSO_4.7H_2O$	2.5 g
Glucose	10 g
$FeSO_4$	0.2 g
Proline	0.5 g
Tryptophan	1 g

The pH of the medium was adjusted to 5.5 with K_2HPO_4. The medium was sterilized by filtration (0.45 µm, filter units).

App. 1.10. Sabouraud Dextrose Agar (SDA)

Formulation as given by Difco

Chemical	Weight (g/l)
Neopeptone,Difco	10
Bacto Dextrose	20

Autoclaved for 15 minutes at 15 lbs pressure (121°C). Final pH 5.6 ± 0.2 at 25°C.

App. 1.11. Trypticase Soya Agar (TSA)

Formulation as given by Oxoid

Chemical	Weight (g/l)
Tryptone (Oxoid L42)	15.0
Soya peptone (Oxoid L44)	5.0
Sodium chloride	5.0
Agar No. 3 (Oxoid L13)	15.0

40 g were suspended in 1 litre of distilled water and brought to the boil to dissolve completely. Sterilization was by autoclaving at 121°C for 15 min. The final pH was 7.3 (approx.).

App. 1.12. Brain Heart Infusion Broth

Formulation as given by Oxoid

Chemical	Weight (g/l)
Calf brain infusion solid	12.5
Beef heart infusion solid	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Di-Sodium phosphate	2.5

37 g was added to 1 litre of distilled water, mixed well and distributed into final containers. Sterilization was by autovlaving at 121°C for 15 minutes. The final pH was 7.4 ± 0.2 .

App. 1.13. Deferrated Yeast Nitrogen Base Agar

Chemical	Quantity
Deferrated yeast nitrogen base solution (x10)	10 ml
Glucose (1 M)	2.8 ml
Agar purified (Oxoid)	1.2 g

Agar was added to 87.2 ml Chelex water (distilled deionized water after mixing with sodium-form resin), deferrated YNB stock solution and 1 M glucose (after mixing with sodium-form resin) to give 100 ml of deferrated YNB agar. Autoclaving was carried out in a pressure cooker at a pressure of 10 p.s.i. for 10 minutes.

Appendix 2. Buffers

App. 2.1. 0.03 M Citrate-buffered saline pH 5

A. 0.1 M citric acid in distilled deionized water.

B. 0.1 M sodium citrate in deistilled deionized water. To prepare 500 ml of 0.1 M citrate buffer (pH 5), 102.5 ml of solution A was added to 147.5 ml of solution B. 150 ml of 0.1 M citrate buffer was added to 350 ml of deionized distilled water to give 0.03 M citrate buffered saline pH 5.

App. 2.2. 0.15 M Phosphate-buffered saline (pH 7.2)

Chemical	Weight (g/l)
NaCl	8.00
KCl	0.20
NaH_2PO_4	0.20
Na_2HPO_4	1.15

Phosphate buffered saline tablets (Oxoid) were used. Each tablet was added to 100 ml of distilled water and the solution autoclaved at 121°C for 15 min . The pH of the subsequent solution was 7.2 .

Appendix 3. Statistical Analyses

App.3.1. Summary overall statistic analysis of variance done by Minitab of siderophore, hydroxamate and cell dry production by *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 after incubation in glucose glycine broth as yeast (25°C) or hyphal (37°C) form for 24 and 48h. High significance values in bold font.

	Statistical significance (*P*)				
Source of variation	**Dry wt.**	**A630**	**A630/mg**	**A264**	**A264/mg**
Time	0.556	**0.000**	0.120	0.544	0.617
Strain	0.919	**0.024**	**0.001**	0.634	0.661
Morphology	**0.000**	**0.000**	**0.000**	0.283	**0.000**
Experiment	**0.005**	**0.000**	**0.009**	**0.000**	**0.001**
Time*Strain	0.915	0.446	0.251	0.766	0.821
Time*Morphology	0.175	0.915	0.570	0.655	0.684
Time*Experiment	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Strain*Morphology	0.875	0.226	**0.001**	0.573	0.555
Strain*Experiment	0.811	0.453	**0.000**	0.859	**0.063**
Morphology*Experiment	**0.000**	0.461	**0.000**	0.358	**0.001**
Time*Strain*Morphology	0.818	0.497	0.229	0.426	0.181
Strain*Morphology*Experiment	0.783	0.273	**0.000**	0.203	0.002
Time*Strain*Experiment	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**

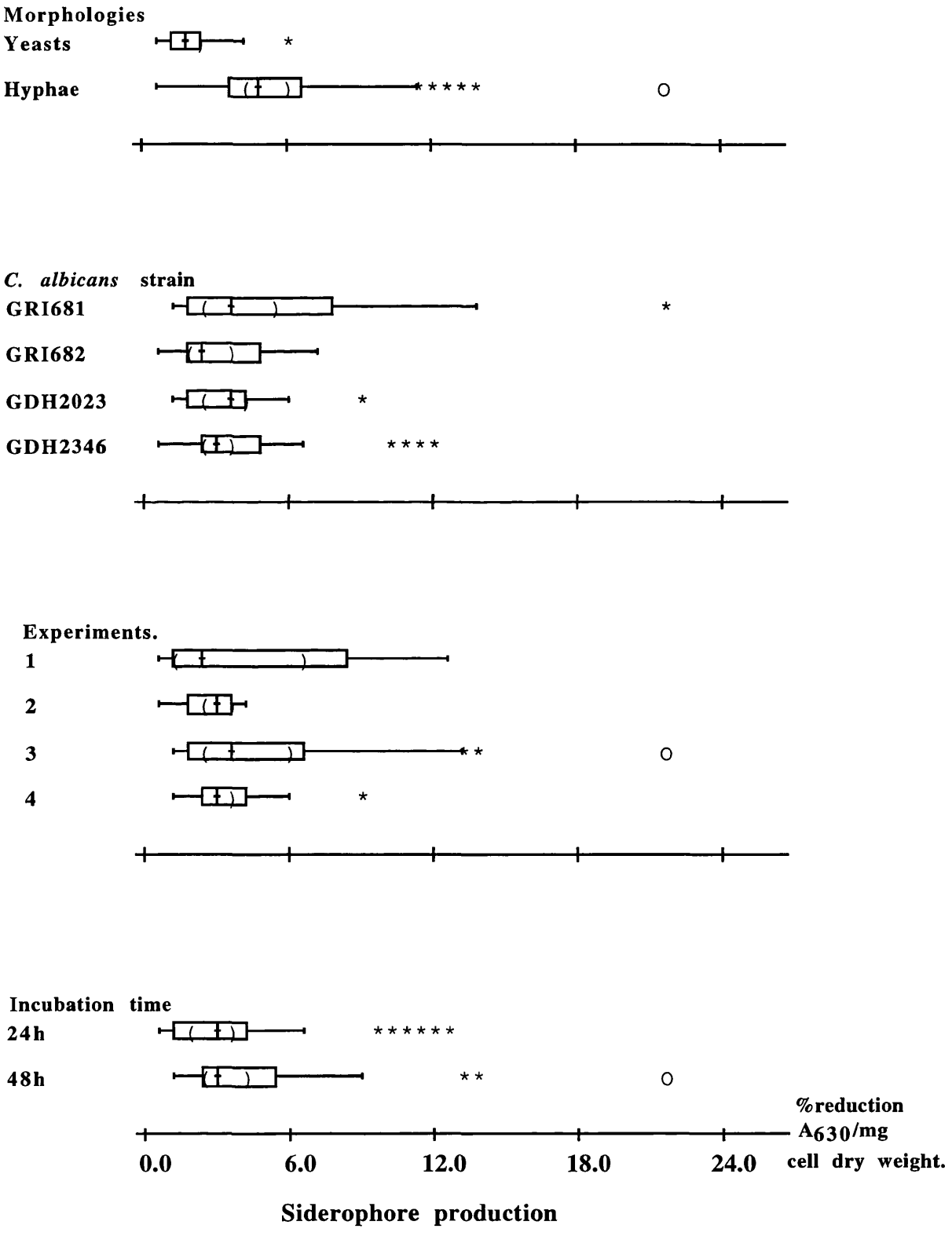
* Interaction

App.3.2. Summary overall statistic analysis of variance done by Minitab of green pigment production expressed as values of A_{444} by *C. albicans* strains GRI 681, GRI 682, GDH 2023 and GDH 2346 grown in YNB medium containing either a high galactose or low glucose as the carbon source. Cultures incubated for different period (1,2,5,10 and 15 days), at different temperatures (25°C, 30°C and 37°C) as static or shaking conditions.High significance values in bold font.

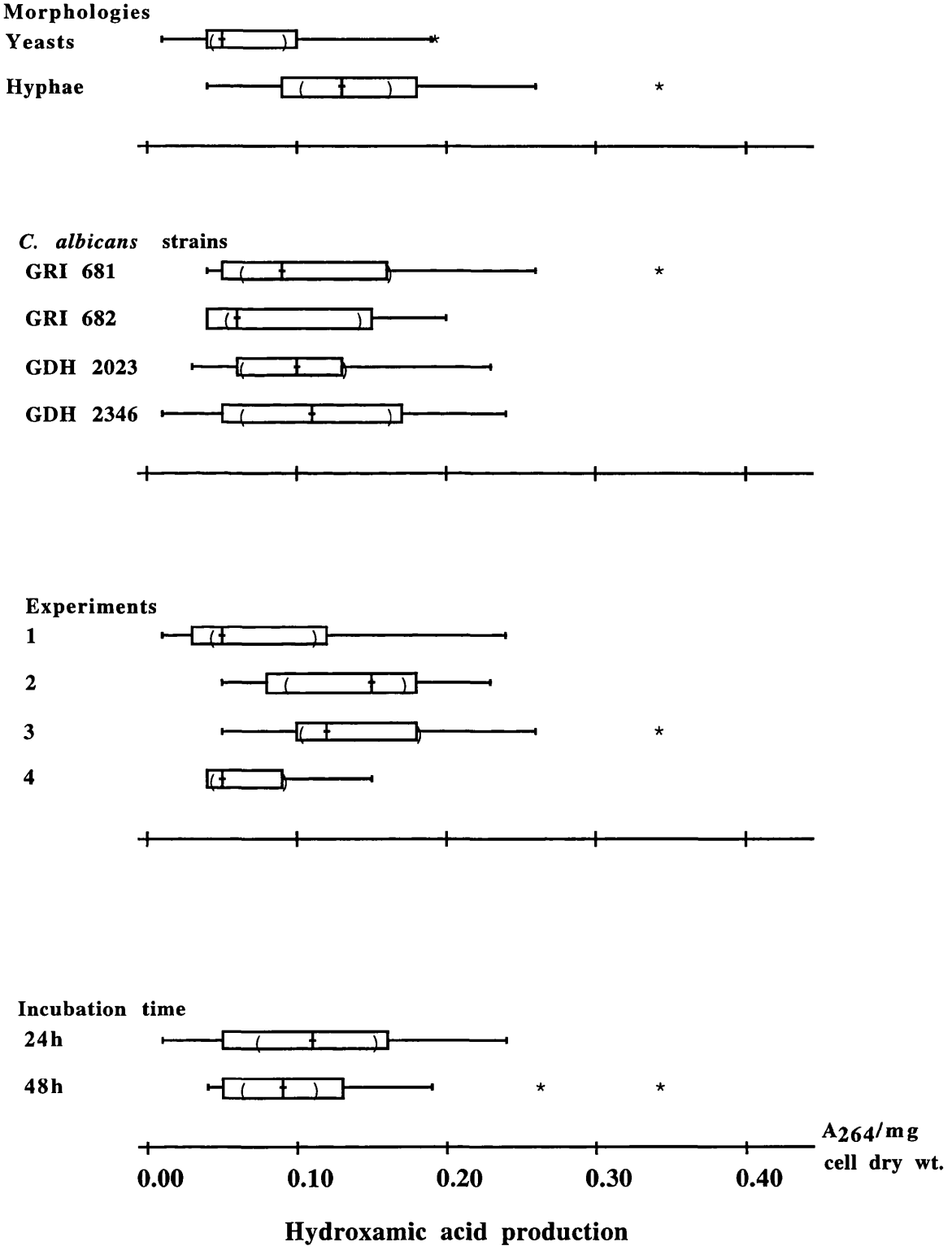
Source of variation	Incubation time	Temperature	culture conditions
Carbon	**0.000**	**0.000**	**0.000**
Strain	**0.000**	**0.003**	**0.000**
Time	**0.000**	**0.000**	**0.000**
Temperature	-	**0.000**	-
Culture conditions	-	-	**0.000**
Carbon*Strain	**0.000**	0.238	**0.016**
Carbon*Time	**0.000**	**0.000**	**0.000**
Carbon*Temperature	-	**0.000**	-
Carbon*Culture conditions	-	-	**0.000**
Strain*Time	**0.000**	0.140	**0.002**
Strain*Temperature	-	**0.000**	-
Strain*Culture conditions	-	-	**0.003**
Time*Temperature	-	**0.001**	-
Time*Culture conditions	-	-	**0.000**
Temperature*Culture conditions	-	-	-
Carbon*Strain*Time	**0.003**	0.145	**0.063**
Carbon*Strain*Temperature	-	**0.000**	-
Carbon*Strain*Culture conditions	-	-	**0.075**
Carbon*Time*Temperature	-	0.221	-
Carbon*Temperature*Culture conditions	-	-	-
Carbon*Time*Culture conditions	-	-	**0.000**
Strain*Time*Temperature	-	**0.000**	-
Strain*Time*Culture conditions	-	-	**0.000**
Carbon*Strain*Time*Temperature	-	**0.000**	-
Carbon*Strain*Time*Culture conditions	-	-	**0.012**

* Interaction

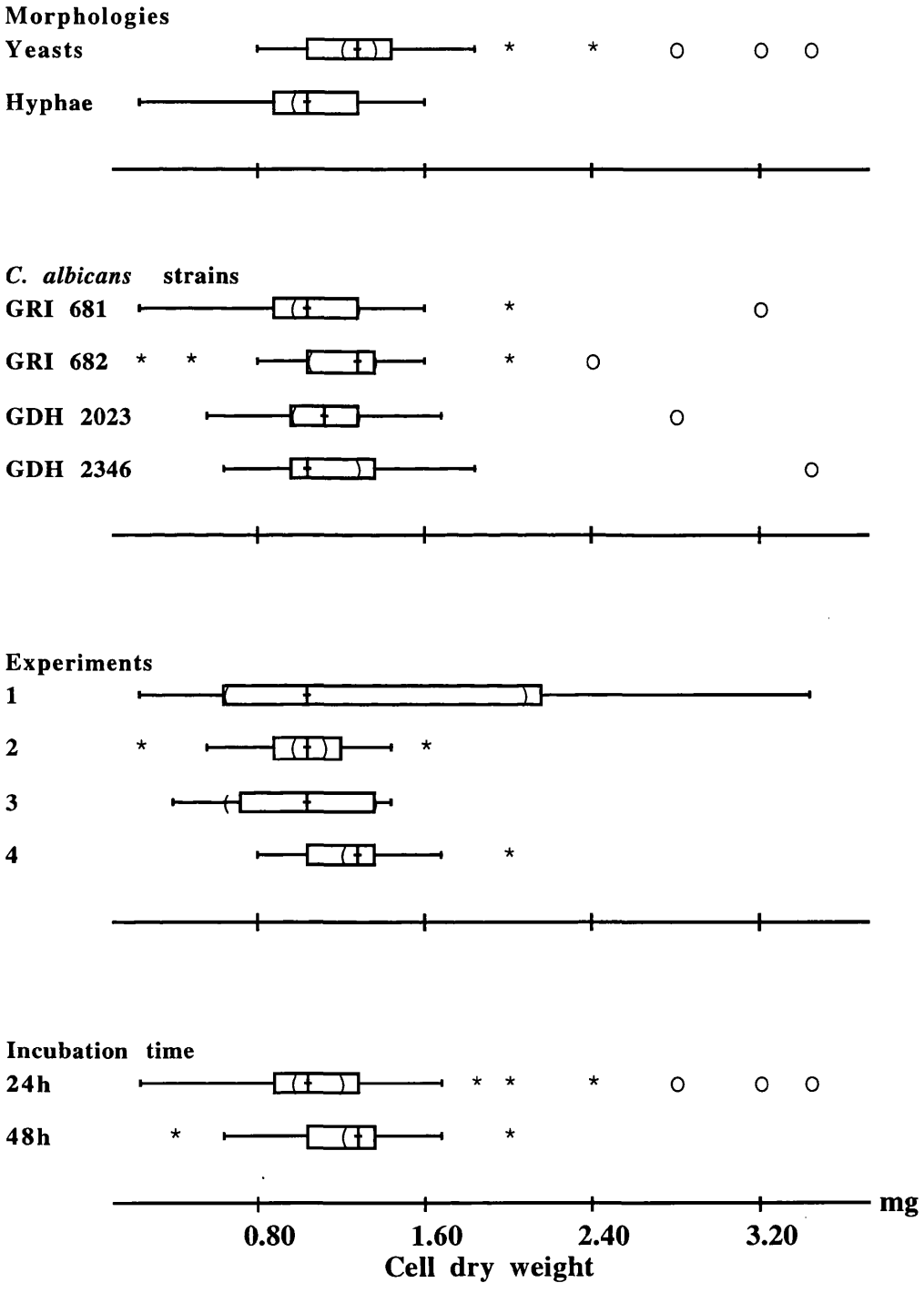
App.3.3. Minitab analysis (Boxplot) of 128 observations on siderophore production by *C. albicans* strains after incubation in glucose glycine broth at 25°C (yeasts) or 37°C (hyphae) for 24 and 48h (see results section 2.1)



App.3.4. Minitab analysis (Boxplot) of 64 observations on hydroxamate-type siderophore production by *C. albicans* strains after incubation on glucose glycine broth at 25°C (yeasts) or 37°C (hyphae) for 24 and 48h (see results section 3.1)

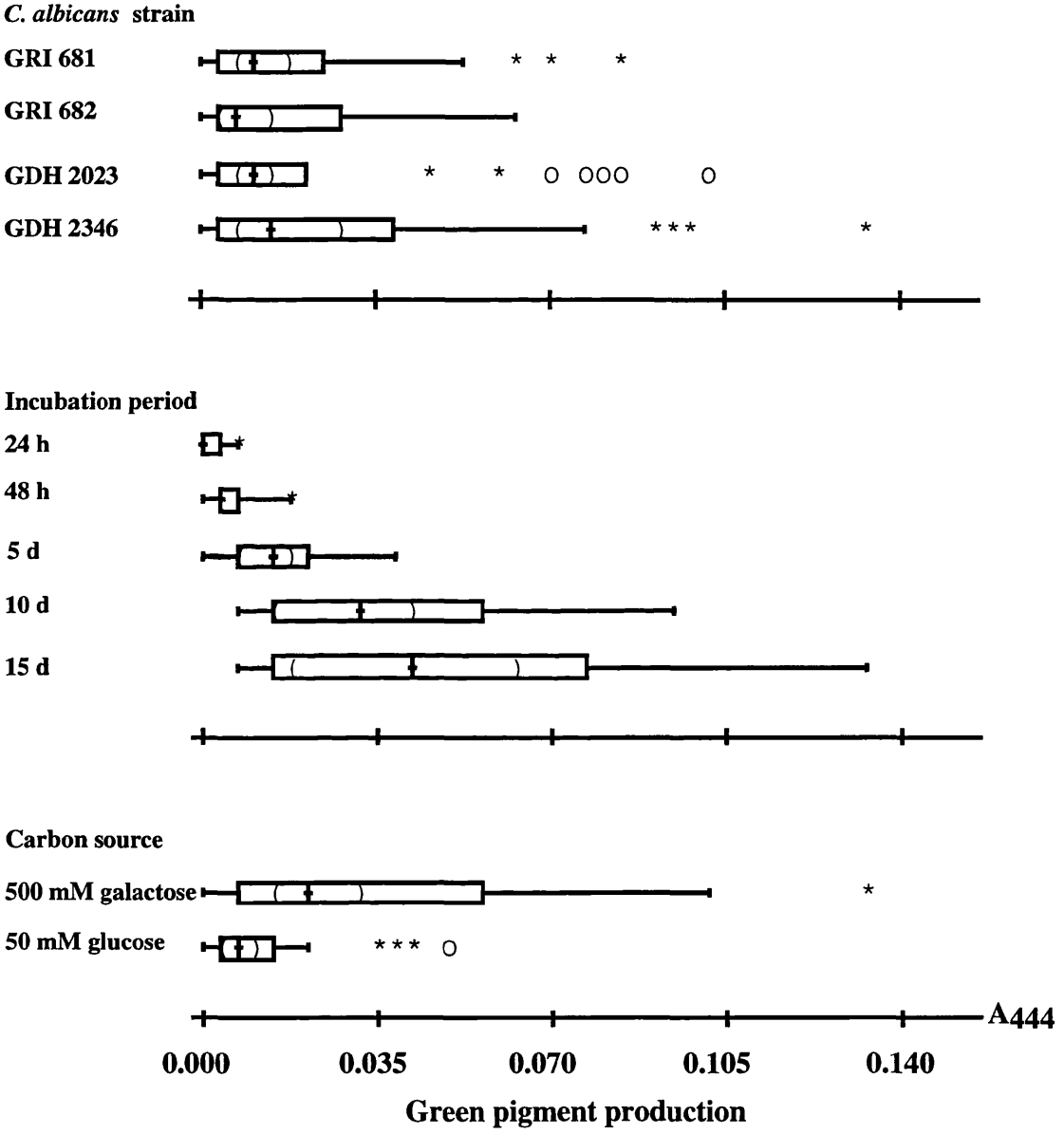


App.3.5. Minitab analysis (Boxplot) of 120 observations on cell dry weight production by *C. albicans* strains after incubation in glucose glycine broth at 25°C (yeasts) or 37°C (hyphae) for 24 and 48h (see results section 5.3).



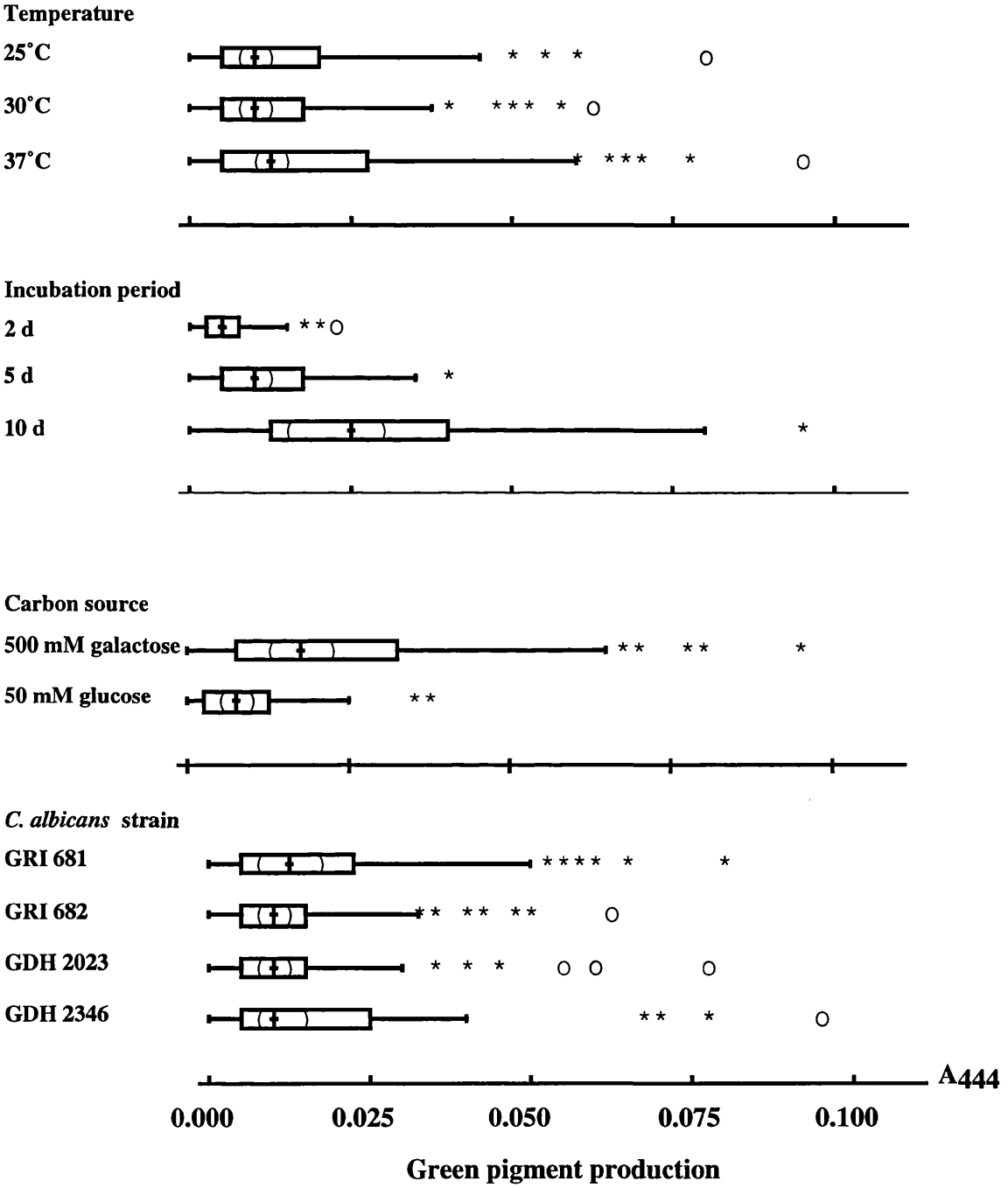
App. 3.6. Effect of carbon source and incubation time on green pigment.

Minitab analysis (Boxplot) of 192 observations on green pigment production by *C. albicans* strain GRI 681, GRI 682, GDH 2023 and GDH 2346 grown in YNB medium containing either 50 mM glucose or 500 mM galactose at different temperatures. Cultures were incubated for 2, 5 or 10 days, centriguged and A444 values of supernates determined (see results section 7.1).



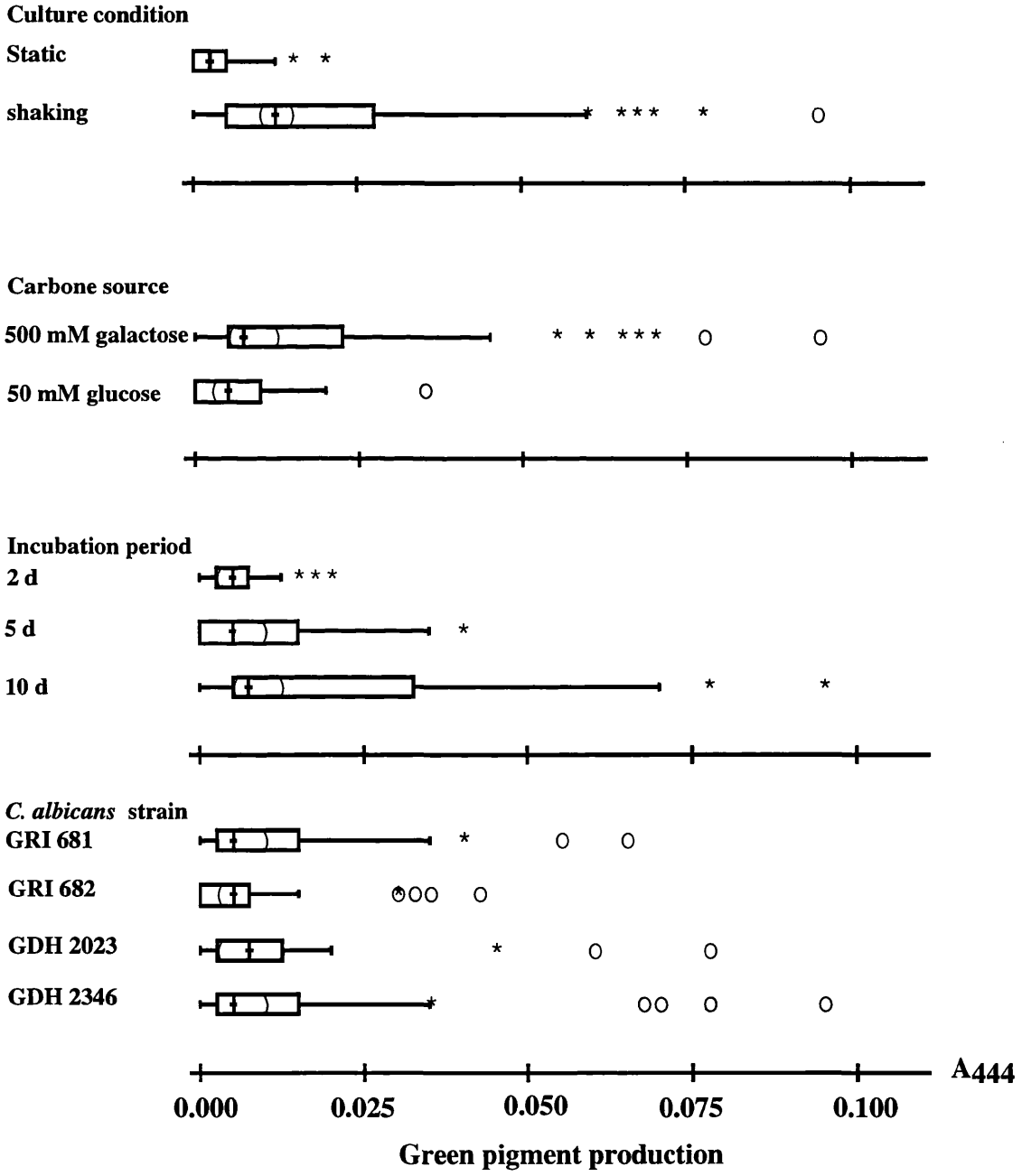
App. 3.7. Effect of temperature on green pigment.

Minitab analysis (Boxplot) of 192 observations on green pigment production by *C. albicans* strain GRI 681, GRI 682, GDH 2023 and GDH 2346 grown in YNB medium containing either 50 mM glucose or 500 mM galactose as carbon source at different temperatures. Cultures were incubated for 2, 5 or 10 days, centriguged and A444 values of supernates determined (see results section 7.2).



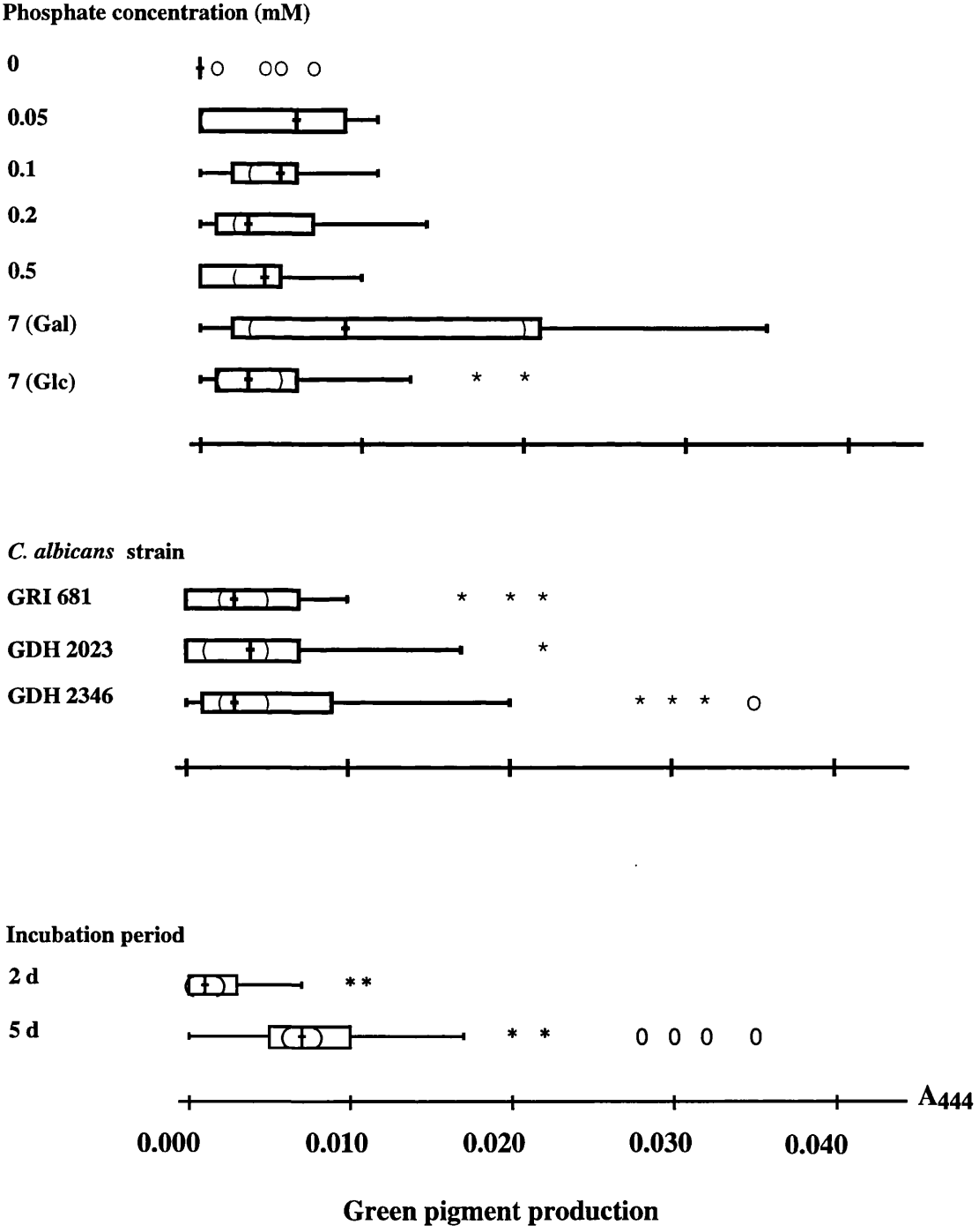
App.3.8. Effect of static and shaking culture conditions on green pigment.

Minitab analysis (Boxplot) of 192 observations on green pigment production by *C. albicans* strain GRI 681, GRI 682, GDH 2023 and GDH 2346 grown in YNB medium containing either 50 mM glucose or 500 mM galactose at 37°C for different incubation times as static and shaking cultures. Cultures were incubated for 2, 5 or 10 days, centriguged and A444 values of supernates determined (see results section 7.3).



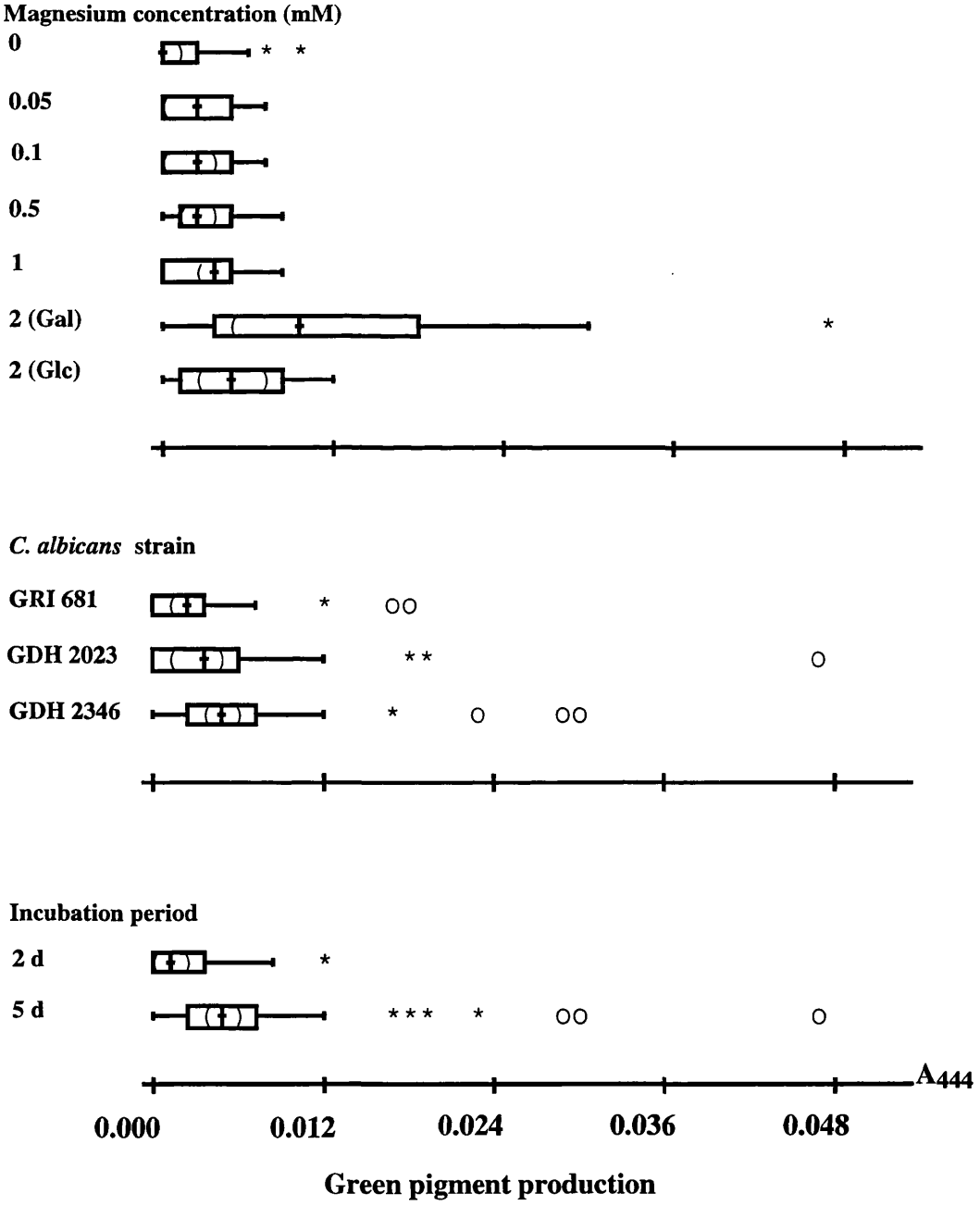
App.3.9. Minitab analysis (Boxplot) of 168 observation on green pigment production by *C. albicans* strains GRI 681, GDH 2023 and GDH 2346 grown in YNB medium containing 50 mM glucose supplemented with different concentrations of phosphate.

Cultures were incubated at 37°C for 2 or 5 days. Control cultures were grown in YNB with either 50mM glucose (Glc) or 500mM galactose (Gal) and 7mM phosphate. After incubation, cultures were centriguged and A_{444} values of supernates determined (see results section 9.1).



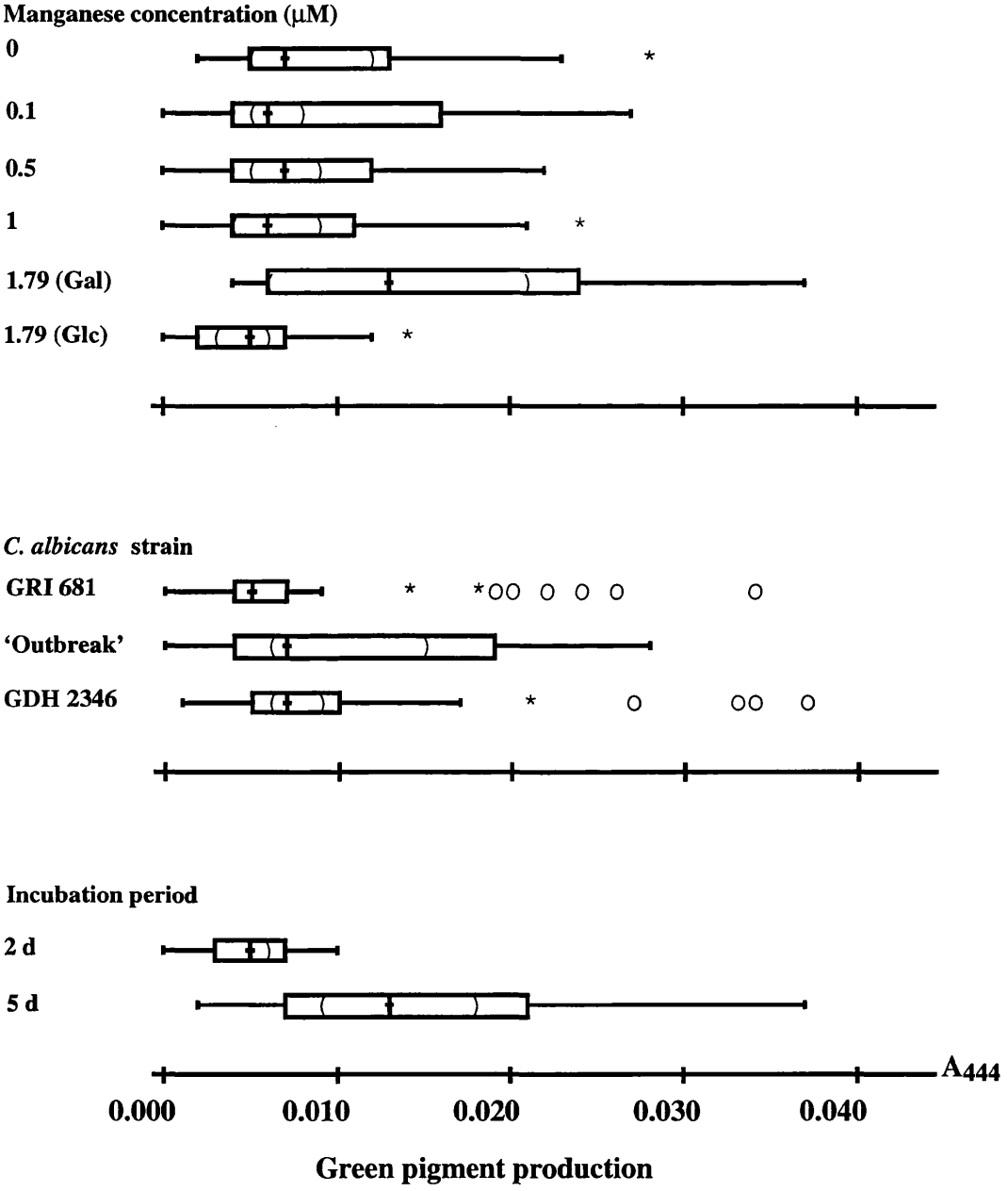
App. 3.10. Minitab analysis (Boxplot) of 168 observation on green pigment production by *C. albicans* strains GRI 681, GDH 2023 and GDH 2346 grown in YNB medium containing 50 mM glucose supplemented with different concentrations of magnesium.

Cultures were incubated at 37°C for 2 or 5 days. Control cultures were grown in YNB with either 50mM glucose (Glc) or 500mM galactose (Gal) and 2 mM magnesium. After incubation, cultures were centriguged and A444 values of supernates determined (see results section 9.2).



App.3.11. Minitab analysis (Boxplot) of 144 observation on green pigment production by *C. albicans* strains GRI 681, 'Outbreak' and GDH 2346 grown in YNB medium containing 50 mM glucose supplemented with different concentrations of manganese.

Cultures were incubated at 37°C for 2 or 5 days. Control cultures were grown in YNB with either 50mM glucose (Glc) or 500mM galactose (Gal) and1.79 μM manganese. After incubation, cultures were centriguged and A_{444} values of supernates determined (see results section 9.3).



App.3.13. Minitab analysis (Boxplot) of 144 observation on green pigment production by *C. albicans* strains GRI 681, 'Outbreak' and GDH 2346 grown in YNB medium containing 50 mM glucose supplemented with different concentrations of copper.

Cultures were incubated at 37°C for 2 or 5 days. Control cultures were grown in YNB with either 50mM glucose (Glc) or 500mM galactose (Gal) and 0.16 μM copper. After incubation, cultures were centriguged and A_{444} values of supernates determined (see results section 9.5).