

A STUDY OF KILLER YEAST ACTIVITY AGAINST
THE OPPORTUNISTIC PATHOGEN Candida albicans

THESIS
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BY

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"Directed research - whether it be against an identified biochemical target or inspired by already known activity - will always need to be supplemented by the empirical approach of drug screening; this is especially true in the case of natural product research."

- Ryley et al. (1981)

LIST OF ABBREVIATIONS

Brij 58	Polyoxyethylene 20 Cetylother
BSA	Bovine serum albumin
DMSO	Dimethylsulfoxide
EDTA	Ethylene diamine tetra acetic acid
KII fraction	An ultrafiltration fraction containing molecules with a putative molecular weight 5,000-10,000
SDA	Sabouraud dextrose agar
SLM	Sabouraud liquid medium
TCA	Trichloroacetic acid
UYEP	Ultrafiltered Yeast Extract & Peptone
YEPD	Yeast Extract-Peptone-Dextrose medium

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ABSTRACT

Based on the selective toxicity of yeast killer toxins towards other yeasts, a search was mounted for pharmacologically active proteins effective against Candida albicans and Candida glabrata, the principal aetiological agents of vaginal candidosis. Of the twenty killer yeasts screened, only Williopsis mrakii LKB169 and NCYC500 displayed strong anti-Candida activity and were selected for further study. Unfortunately both yeasts proved unstable following serial subculture on agar slants. A secretion mutant derived from LKB169 (denoted LKB169A) lacked antimicrobial activity against both C albicans and Saccharomyces cerevisiae but retained activity against C glabrata. A working hypothesis proposing the existence of multiple toxins from W mrakii was further investigated. A triplet bioassay using C albicans A72, C glabrata NCYC388 and S cerevisiae NCYC1006 was developed to differentiate toxins from W mrakii.

Based on differences in thermal decay, sensitivity to papain and specificity of action, three distinct protein toxins from W mrakii LKB169 were identified and designated KA, KG and KS. Toxin multiplicity was substantiated for strain NCYC500 also; production was characterized by an early loss of antimicrobial activity against C albicans with a concurrent reversal in the ratio of the activity against C glabrata to the activity against S cerevisiae.

In general toxins from strain NCYC500 were more stable than those from LKB169. pH was identified as an important factor in the thermostability of Williopsis toxins. For example, the KG toxin from W mrakii LKB169 retained full activity against C glabrata after boiling for 10 minutes at pH 4.0; however, at pH 4.5 biological activity decayed at 37°C.

Williopsis toxins may have therapeutic potential in vaginal candidosis where the low pH favours activity. In this regard it is encouraging that a secreted acid proteinase from C albicans did not hydrolyse the Williopsis toxins in vitro.

SECTION 1 INTRODUCTIONSECTION 1.1 YEAST KILLER TOXINS & THE KILLER PHENOMENONSECTION 1.1.1 OUTLINE OF THE KILLER PHENOMENON

The Killer Phenomenon is a specific yeast-yeast interaction discovered by Markower & Bevan (1963) and later defined by Woods & Bevan (1968). Killer yeasts are strains which secrete a specific protein or glycoprotein toxin that is lethal (Middelbeek et al., 1980c) or inhibitory (Sugisaki et al., 1983) to another yeast species but to which the killer strain itself is immune.

Killer character is widely distributed among yeast genera (see Table 1.1.1) and the killer toxins described to date are active against a variety of different yeasts often outside the genus and species of the producing strain.

TABLE 1.1.1

GENERA WHICH CONTAIN KILLER YEASTS		
GENUS	EXAMPLE	REFERENCE
<u>CANDIDA</u>	<u>albicans</u>	Bevan & Mitchell, 1979
<u>CRYPTOCOCCUS</u>	<u>laurentii</u>	Kwon-Chung & Kennedy, 1986
<u>DEBARYOMYCES</u>	<u>vanriji</u>	Young & Yagi, 1978
<u>HANSENIASPORA</u>	<u>uvarum</u>	Radler <u>et al.</u> , 1985
<u>HANSENULA</u>	<u>mrakii</u>	Ashida <u>et al.</u> , 1983
<u>KLUYVEROMYCES</u>	<u>lactis</u>	Sugiaski <u>et al.</u> , 1984
<u>PICHIA</u>	<u>kluiveri</u>	Middelbeek <u>et al.</u> , 1980c
<u>RHODOTORULA</u>	<u>glutenis</u>	Morace <u>et al.</u> , 1983
<u>SACCHAROMYCES</u>	<u>cerevisiae</u>	Woods & Bevan, 1968
<u>TORULOPSIS</u>	<u>glabrata</u>	Bussey & Skipper, 1975
<u>TRICHOSPORON</u>	<u>capitatum</u>	Morace <u>et al.</u> , 1983
<u>USTILAGO</u>	<u>maydis</u>	Kandel & Koltin, 1978
<u>WILLIOPSIS</u>	<u>saturnus</u>	Ohta <u>et al.</u> , 1984



Hansenula mrakii NCYC 500

Candida albicans UCSC 10

PLATE 1 THE KILLER PHENOMENON

The killer yeast Williopsis (Hansenula) mrakii NCYC 500¹ is inhibitory toward C. albicans at pH 4.5. The antimicrobial activity is associated with an extracellular antimicrobial substance. Each 8.5 mm well² in the 15 ml seeded agar (C. albicans, UCSC 10), contained 50 ul cell-free broth from a 24-hour culture of NCYC 500 (pH 4.5, 25°C).

Yeast Killer Toxins are a group of pharmacologically active proteins secreted by Killer Yeasts. Some toxins like those from Williopsis mrakii (Ashida et al., 1983) and Saccharomyces cerevisiae (Palfree & Bussey, 1979) are simple proteins; while those from Kluyveromyces lactis (Sugiaski et al., 1984) and Pichia kluyveri (Middelbeek et al., 1979) are glycoproteins. These yeast toxins range in size from the small 8.5 kDa toxin from Hansenula saturnus (Ohta et al., 1984) to the large K. lactis toxin consisting of a 27 kDa subunit and one greater than 80 kDa (Sugisaki et al., 1984).

These natural antimicrobial agents are not known to be active against bacteria or higher eukaryotic cells. The epithet 'toxin' may therefore mislead a reader who associates the action of toxins with mammalian targets. Young (1983) proposed that yeast killer toxin should be renamed 'Zymocide' to emphasize their highly selective toxicity. The name killer toxin is retained in this discussion with the understanding that they are not known to be toxic to mammalian cells.

In the literature, killer yeasts which are claimed to "kill" other yeasts may not in fact be lethal. To avoid this type of ambiguity the phrase 'active against' will be used since the action of a toxin may be dose-dependent or simply inhibitory.

The killer phenomenon has been the subject of numerous reviews (Pietras & Bruenn, 1976; Bevan & Mitchell, 1979; Wickner, 1979, 1981; Bussey, 1981, 1984; Tipper & Bostian, 1984; Wickner, 1985, 1986; Young, 1987). Practical applications of yeast killer toxins in medical mycology including therapeutic exploitation, have not been adequately addressed.

SECTION 1.1.2 MODE OF ACTION OF YEAST KILLER TOXINS

Presently three mechanisms of action of yeast killer toxins are recognized. Nuclease activity has been ascribed to toxins from Ustilago maydis (Levine et al., 1979). Killer toxins from Kluyveromyces lactis cause inhibition of adenylate cyclase in susceptible strains of S. cerevisiae leading to G₁ arrest in the cell cycle (Sugisaki et al., 1983). However, the best understood mechanism of action is disruption of membrane permeability. Work by Middelbeek et al. (1980b) and de la Pena et al. (1981) who studied the actions of toxins from Pichia kluyveri and S. cerevisiae respectively suggest that killer toxins of this type dissipate the electrochemical gradient across the cell membrane of sensitive yeast. In support of this mechanism, the P. kluyveri toxin was shown to produce a non-selective ion-permeable channel in phospholipid bilayers (Kagan, 1983).

Toxin action is characterized by influx of protons (de la Pena et al., 1980) leading to a reduction in intracellular pH (Middelbeek et al., 1980b). This event is linked to K⁺ leakage (de la Pena et al., 1981). Then after a lag period, the ATP pool dissipates and there is a concomitant inhibition of energy dependent processes; for example, active amino acid uptake and macromolecule synthesis (Bussey & Sherman, 1973; Middelbeek et al., 1980c). There may also be cell shrinkage (Bussey, 1974; Middelbeek et al., 1980c).

There is a certain period within which time toxin-treated yeasts can be rescued (Bussey, 1972). Middelbeek et al. (1980b) found optimal environmental conditions for rescue to be those which mimicked the intracellular concentrations of potassium ion (0.13 M) and protons (pH 6.5). Alternatively, a high extracellular calcium ion concentration can have a protective effect

(Kotani et al., 1977). Both of these findings suggest that killer toxin damages the integrity of the yeast cell membrane.

SIMILARITY OF YEAST KILLER TOXINS TO DEFENSINS

Killer toxins have been likened to bacteriocins in prokaryotes (Wood & Bevan, 1968; Bussey & Skipper, 1976; Kandel & Stern, 1979; de la Pena et al., 1980). The precedent for pore formation in membranes as a mechanism of the lethal action of such materials has been established with complement and more recently with defensins.

The defensins are cationic peptides with antimicrobial activity derived from polymorphonuclear cells and monocytes in man and other animals (Selsted et al., 1985). Polymorphonuclear leucocytes possess four main fungicidal systems: (i) Hydrogen peroxide, (ii) Myeloperoxidase, (iii) Superoxide; and (iv) Cationic proteins (Howard, 1975). The cationic proteins are an antifungal mechanism independent of the direct effects of H_2O_2 or the myeloperoxidase-mediated system. Six cysteine-rich cationic peptides that express various degrees of antibacterial, antifungal and anti-viral activity have been described in rabbit granulocytes (Selsted et al., 1985). Recently a defensin derived from cytotoxic T cells and natural killer cells called perforin 1 has been characterized (Young et al., 1986). This pore-forming protein shows remarkable structural and functional similarities to the ninth component of complement; both polymerize to form tubular lesions in lipid bilayers. The large aqueous pores are stable, non-selective for solutes and insensitive to changes in membrane potential. In addition, both show structural homology by immunological cross-reactivity.

SIMILARITY OF YEAST KILLER TOXINS TO ADP-RIBOSYLATING TOXINS

Many plant and microbial toxins share a common mechanism of action (Foster & Kinney, 1985) and it is possible that certain yeast killer toxins are similar to the ADP-ribosylating class of toxins. These latter toxins are structurally similar being disulphide-bonded heterodimers (Foster & Kinney, 1985; Lord et al., 1985); and it is worth pointing out that the K_1 toxin from S. cerevisiae shares this insulin-like structure (Bostian et al., 1984).

There are two types of ADP-ribosylating toxins. Firstly, those which covalently modify a regulatory component of adenylate cyclase. This is typical of the diphtheria toxin and the toxin from Pseudomonas aeruginosa. Kotani et al. (1977) found ADP to promote killing of a yeast strain sensitive to a sake yeast toxin and protein synthesis was required for recovery. Also the K. lactis toxin has been shown to inhibit adenylate cyclase in crude membrane preparations from sensitive yeast (Sugisaki et al., 1983); however, the regulatory component affected remains unknown. Both of these yeast toxins could be ADP-ribosylating toxins.

The second type of ADP-ribosylating toxins are those which covalently attach ADP-ribose to eukaryotic elongation factor 2, thus inhibiting protein synthesis (Foster & Kinney, 1985). Microbial toxins of this type include cholera toxin, E. coli heat labile toxin and Bordetella pertussis islet-activating protein. Also in this group are the plant toxins, abrin and ricin which are powerful inhibitors of protein synthesis. The mechanism of action of this type of toxin is well understood (Lord et al., 1985; Vitetta & Uhr, 1985). The B chain is lectin-like and promotes binding to, for example, galactose residues in the outer cell surface. Biological activity appears to

depend on cleavage of the disulfide bonds and dissociation of the A chain. The A chain is translocated into the cell and enzymatically alters ribosome function, which leads to inhibition of protein synthesis. In this way - it is claimed - one molecule of A chain in the cytosol is sufficient to kill a eukaryotic cell.

Apart from the toxins of Ustilago maydis (Levine et al., 1979) there is no evidence that killer toxins have enzymatic activity. Also it is unknown if certain killer toxins can be translocated into a yeast cell. Many of the effects of yeast killer toxins can be explained by interference of ribosome function in a streptomycin-like fashion (Tai & Davis, 1985). In low doses streptomycin is known to promote misreading of the mRNA template and the "junk protein" so produced is thought to cause membrane perturbations and loss of integrity. With ADP-ribosylation such a common mechanism of microbial toxinogenesis, it is very possible that this mode of action is also shared by certain yeast toxins.

SECTION 1.1.3 YEAST-YEAST INTERACTIONS

The hypothesis that killer character confers a selective advantage on a yeast, has not been substantiated. Much has yet to be learned of yeast-yeast interactions in mixed culture. When growing in competition, a killer yeast may produce inhibitory action or be lethal to the other yeast. A threshold concentration of toxin may be required to kill the yeast, below which, inhibitory action is produced. Alternatively, growth inhibition or retardation may be the only effect observed.

The specific effects of a killer toxin in mixed culture may be obscured by

other effects. Young & Philliskirk (1977) found that differences in growth rate and affinity for nutrients played a significant role in the rate of displacement of a brewing yeast by a killer yeast when grown together in a chemostat.

Yeasts are acidophilic organisms and when growing rapidly can markedly reduce the pH of their environments by extruding protons and organic acids. This effect can inhibit the growth of many bacteria and possibly other fungi. However, there have been a few reports of yeasts secreting specific antibacterial substances (Hipp et al., 1974; Fatichenti et al., 1983; Flegel et al., 1984). To date, most studies on yeast-yeast interactions have focused on alpha-factor and killer toxin proteins. Little is known of low-molecular weight substances or secondary metabolites with anti-yeast activity. It is possible that non-protein substances, inhibitory to other yeasts are produced under certain conditions. In fact, Flegel et al. (1984) described an naphthaquinone antibiotic produced by a black yeast which had strong antimicrobial activity against Staphylococcus aureus and had mild activity against C. albicans.

SECTION 1.1.4 STABILITY OF BIOLOGICAL ACTIVITY

In common with many pharmacologically active proteins, yeast killer toxins are notoriously unstable. Even under the most rigorously controlled conditions of temperature, pH and flow dynamics, the toxin may lose its activity. In particular, killer toxins are sensitive to mechanical agitation and surface inactivation (Woods & Bevan, 1968; Kotani et al., 1977; Middelbeek et al., 1979). An additional problem is strong binding to extraneous macromolecules (Bussey, 1981), membrane filters (Shimizu et al., 1985) and

certain chromatographic materials (Woods & Bevan, 1968; Pfeiffer & Radler, 1982). Above all with limited knowledge of physico-chemical properties, empirical development of a purification scheme is the norm.

In general killer toxins are thought to be unstable in alkaline pH (Woods & Bevan, 1968; Young & Yagiu, 1978) and are most stable within a narrow pH range close to their pI (Palfree & Bussey, 1979). However, the toxin from Candida (Torulopsis) glabrata (Bussey & Skipper, 1975) was found to be stable over a relatively wide pH range (3-7). Likewise the toxin from Cryptococcus laurentii is stable between pH 2.3-6.3 (Middlebeek et al., 1980b).

Yeast killer toxins are thought to be irreversibly inactivated by heating (Young & Yagiu, 1978) but are more stable in agar than in liquid broth (Woods & Bevan, 1968). Thermotolerant killer toxins which have been described are listed in Table 1.1.2 and the physico-chemical properties of the latter two toxins are outlined below.

TABLE 1.1.2 THERMOSTABLE YEAST TOXINS

Killer toxin from:	Stable at:	References
<u>Pichia kluyveri</u>	40°C	Middelbeek <u>et al.</u> , 1979
<u>S. cerevisiae</u>	40°C	Pfeiffer & Radler, 1984
<u>Cryptococcus laurentii</u>	45°C	Middelbeek <u>et al.</u> , 1980d
<u>W. saturnus</u>	60°C	Ohta <u>et al.</u> , 1984
<u>W. mrakii</u>	100°C	Ashida <u>et al.</u> , 1983

Ashida et al. (1983) purified and characterized a toxin from W. mrakii LKB

169. The toxin fraction designated "KII" was found to be a single polypeptide of molecular weight 8,900 daltons which was very stable to heat (for example boiling for 3 minutes at pH 4.0), and remained active over a wide pH range (4-11 at 25°C). The mode of action of this toxin was found to be disruption of cell membrane permeability.

Ohta et al. 1984 described the production, purification and characterization of a killer toxin from W. saturnus IFO 0117 which they called HYI. A remarkable feature of this toxin was its ease of production and purification, which in addition to conventional chromatography involved concentration in vacuo at 40°C and cold crystallization. HYI was found to be a single polypeptide and was estimated to have a molecular weight of 8,500 by SDS-polyacrylamide gel electrophoresis. From amino acid analysis the molecular weight was determined to be 8291 and no carbohydrate residues were detected. HYI was stable in the pH range 5-8 following incubation at 30°C for 24 hours and showed no loss of activity after incubation at 60°C for 1 hour (pH 5.0).

SECTION 1.1.5 EFFECT OF PROTEASES ON KILLER YEASTS

The killer toxins of S. cerevisiae were reported to be papain sensitive (Woods & Bevan, 1968) providing evidence for their proteinaceous nature. Young & Yagiu (1978) characterized killer toxins from different strains by differential proteolysis with papain, pepsin and pronase. Table 1.1.3 lists proteolytic enzymes which have been used to characterize killer toxins.

A strain of S. cerevisiae with a chromosomal mutation (ski 5) was found to be a super killer because it lacked a cell surface protease. This mutant

produced 10-fold more toxin than the parent strain. In addition, specific inhibition of this protease in the parent strain by phenylmethylsulfonyl fluoride potentiated toxin production (Wickner, 1985). Thus proteinases appear to reduce the level of active toxin produced by the S. cerevisiae killer strain.

TABLE 1.1.3 PROTEOLYTIC ENZYMES WHICH HAVE BEEN USED TO DIGEST KILLER TOXINS

<u>PROTEINASE</u>	<u>REFERENCE</u>
<u>Aspergillus niger</u> acid proteinase	Kotani <u>et al.</u> , 1977
<u>Bacillus subtilis</u> neutral proteinase	Kotani <u>et al.</u> , 1977
Papain	Woods & Bevan, 1968
Pepsin	Young & Yagiu, 1978
Pronase	Bussey, 1972
Trypsin	Gunge & Sakaguchi, 1981
V8 Protease from <u>Staphylococcus</u>	Bussey, 1981

SECTION 1.1.6 GENETIC BASIS

The killer systems in S. cerevisiae or Ustilago maydis are determined by double-stranded RNA genomes encapsidated in intracellular non-infective virus-like particles (Wickner, 1985). The killer system in Kluyveromyces lactis is determined by a pair of linear DNA plasmids (Gunge et al., 1981). The genetic basis of the other killer yeasts appears to remain unknown but may in several cases be associated with chromosomal genes. For example, Ohta et al. (1984) were unable to detect RNA or DNA plasmids in W. saturnus and concluded that the killer toxin genes were most likely nuclear.

SECTION 1.1.7 SCREENING FOR PHARMACOLOGICALLY ACTIVE FERMENTATION PRODUCTS

The most expedient and practical method for screening for antimicrobial

activity from killer yeasts is the streak plate assay first used by Woods and Bevan (1968). In addition it is necessary to have a bioassay which quantifies the amount of biologically active protein. This bioassay must be sensitive, accurate and reproducible. Such an assay is a prerequisite for monitoring the yields achieved in fermentation in order to optimize production, and also to follow the progress of purification (Hamill, 1982; Brewer and Sassenfeld, 1985).

An agar diffusion bioassay is a physicochemical technique that utilizes a sensitive microorganism as an indicator strain (Hewitt, 1977). The choice of indicator strain is of prime importance for detection of biologically active protein (Middelbeek et al., 1980a).

Screening for pharmacologically active fermentation products has been reviewed (Fleming et al., 1982; Hamill, 1982; Omura, 1986). Screening for killer yeast activity has been based on the "seeded agar phenotype test" of Woods & Bevan (1968); Philliskirk & Young (1975); Stumm et al. (1977); Kandel & Stern (1979); Middelbeek et al. (1980a), and Polonelli et al. (1983). All these studies with the exception of Kandel & Stern (1979) used a complex medium containing 0.003% (w/v) methylene blue. The methylene blue was included as a "specific stain for dead yeast cells" (Woods & Bevan, 1968).

SECTION 1.1.8 DOWNSTREAM PROCESSING OF YEAST KILLER TOXIN

Downstream processing consists of the primary recovery of a fermentation product and the workup stages to interface fermentation with high resolution chromatography techniques. It has been described as "the major bottleneck constraining" the widespread commercialization of genetic engineering (Rosen

et al., 1983).

Since there is a relatively high initial purity of the desired product, purification of a secreted yeast protein shows favourable economics; estimated to be 10% the cost of a non-secreted product (Pugsley, 1985; Smith et al., 1985). Provided that the secreted protein is stable in solution, then handling is comparatively easy - following removal of cells, all that may be required is a clarification filtration and concentration by ultrafiltration (Hedman, 1983). The objective is to reduce the volume of liquid to be processed since killer toxin is an extremely dilute product. Ultrafiltration is the most universally applied technique in killer toxin workup. In a diafiltration mode, the product can be adjusted to a suitable pH and ionic strength for the first chromatography stage.

SECTION 1.2 CANDIDA AND CANDIDOSIS

SECTION 1.2.1 OVERVIEW

C. albicans is an obligate associate of man and animals (Odds, 1979; Shepherd et al., 1985) and this yeast is frequently encountered as a harmless commensal in the gastrointestinal tract. Recently, however, there is controversy over the presence of C. albicans in the vagina (Carroll et al., 1973). C. albicans may not, in fact, be a commensal in the healthy vagina (Ryley, 1986).

At least eight species of Candida (see Table 1.2.1), as well as a Rhodotorula spp. can cause candidosis but C. albicans is the most frequent human pathogen. Since Candida spp. are opportunistic pathogens, the aetiology of candidosis is usually of endogenous origin, due to a local or systemic increase in susceptibility to infection. Every part of the body can be affected but by far the most common disorders are superficial lesions on mucosal surfaces.

A marked rise in the incidence of vaginal candidosis has been noticed in recent years (Hurley & deLouvois, 1979). Recurrent and even chronic infections are common. Most cases respond to standard therapies (e.g., Nystatin (cream or pessary), 5 days-2 weeks); however, there is significant chemotherapeutic failure. Part of the problem is that chemotherapy does not tackle the underlying predisposing factors.

TABLE 1.2.1 PRINCIPAL AETIOLOGICAL AGENTS OF CANDIDOSIS*

C. albicans¹
C. tropicalis

C. parapsilosis

C. glabrata²
C. kefyr³
C. krusei

C. guilliermondii

* Odds, 1979; Names given as in Kreger-van Rij (1984).

1. C. steallatoidea and C. albicans are now considered to be the same species, since the type strains show significant DNA base sequence complementarity (Meyer, 1979).

2. Candida - Torulopsis controversy: The genera Candida and Torulopsis differ by the presence and absence of pseudohyphae and the amalgamation of Torulopsis into the genus Candida has not been universally accepted (McGinnis & Schnell, 1985). Therefore, Candida glabrata and Torulopsis glabrata are used synonymously.

3. The major synonym of C. kefyr is C. pseudotropicalis.

Numerous predisposing factors have been identified for the various forms of candidosis; for example, there is a high incidence of mucosal candidosis in diabetes mellitus and during the third trimester of pregnancy. Iatrogenic factors such as aggressive corticosteroid, cytostatic or broad spectrum antibiotic therapy can predispose patients to both mucosal and systemic candidosis. Patients with autoimmune deficiency syndrome (AIDS) are susceptible to certain opportunistic fungi of normally low pathogenicity. Candidosis is the most common mycosis in these patients and is usually limited to the oropharyngeal region, the esophageal and gastrointestinal mucosa. The presence of esophageal candidosis is of diagnostic importance as a marker for AIDS, since it is indicative of the underlying cellular immunodeficiency (Chandler, 1985).

More serious is disseminated candidosis which can be a complication of cancer chemotherapy, organ transplantation or prolonged intravenous feeding and which may be the cause of death. Defective host resistance may significantly contribute to chemotherapeutic failure and chronicity in disseminated candidosis.

SECTION 1.2.2. BRIEF OUTLINE OF PATHOGENESIS OF C. ALBICANS WITH SPECIAL REFERENCE TO SECRETED ACID PROTEINASE.

Production of hydrolytic enzymes by a pathogenic organism is considered an important determinant of virulence (Chattaway et al., 1971). C. albicans is known to secrete a number of hydrolytic enzymes, for example: extracellular phospholipase A, lysophospholipase (Price et al., 1982; Barrett-Bee et al., 1985) and proteinase (Staib, 1965).

Sodek & Hoffman (1970) described the production and simple assay of an acid proteinase from C. albicans. This proteinase, which is induced by growth on simple defined medium in which bovine serum albumen is the sole nitrogen source, was found to be an extracellular protein of 40,000 daltons and had a pH optimum of 3.2 (Chattaway et al., 1971).

Odds (1979) discussed the possible role of an inducible protease in the pathogenesis of candidosis. This secreted enzyme has potential to inflict damage to host tissue and therefore should be considered a contributing factor in the process of invasion.

The multifactorial nature of microbial pathogenicity has been outlined by Smith (1985) and is applicable to C. albicans. Most Candida infections start on mucosal surfaces. Therefore to be pathogenic C. albicans must:

- (i) colonise the mucosal surfaces of the respiratory, alimentary or urogenital tract;
- (ii) enter the host by penetrating these surfaces;
- (iii) multiply in the environment of the host tissues;

resist or interfere with host defense mechanisms that try to remove or destroy them; and

(v) cause damage to the tissues of the host (after Smith, 1985).

A determinant of pathogenicity such as the secreted acid proteinase can potentiate the virulence of the yeast.

Direct evidence for secretion of proteinase in vivo was reported by MacDonald & Odds (1980) who demonstrated the presence of proteinase in the vicinity of invading yeast cells using an immunohistochemical technique. It was proposed that immunological detection of acid proteinase in serum could be of diagnostic and prognostic value since it was indicative of active disease by a proteolytic strain of C. albicans. The purified proteinase from C. albicans was further characterized by MacDonald & Odds (1980) and shown to be a carboxyl proteinase since it was selectively inhibited by pepstatin A.

Ruchel et al. (1982) found marked strain variation in protease production and the degree of induction was strongly dependent on cultural conditions. Two distinct proteases were characterized in detail. The first one, described as a "partially proteolytic enzyme", was capable of digesting immunoglobulin A. the second, a totally proteolytic enzyme, was found to digest IgG as well as IgA. both enzymes were shown to digest the two major proteinase inhibitors present in human plasma, α_1 -antitrypsin (an inhibitor of trypsin enzymes), and α_2 -macroglobulin (an inhibitor of a range of proteolytic enzymes).

Based on the role of secreted IgA in mucosal immunity and the demonstrated ability of carboxylproteinases from C. albicans to digest this immunoglobulin, Ruchel et al. (1982) went on to propose that secreted proteases play an

important role in the persistence of C. albicans on mucus membranes.

It is therefore reasonable to assume that this determinant of virulence is especially important in Candida vaginitis where the low pH of the vagina (Ryley, 1986; Schnell, 1982) is conducive to acid protease action.

A role for acid proteinase from C. albicans in the pathogenesis of oral candidosis has been proposed by Samaranayake et al. (1984) since the oral cavity can provide niches that have a low pH.

Hattori et al. (1984) described an keratinolytic proteinase produced by C. albicans. This enzyme could only be induced with yeast growth on an insoluble fraction of human stratum corneum as sole nitrogen source. Although keratin powder was incapable of inducing this enzyme, secreted protease was nevertheless capable of hydrolysing the resilient protein with a pH optimum of 4.0. Since activity is selectively inhibited by pepstatin A, it is classified as a carboxylproteinase. The authors postulated that induction of keratinase in C. albicans when growing in the stratum corneum was important for the pathoetiological progression of paronychia (nail-fold infections due to Candida) to Candida onychomycosis (true-nail infection).

MacDonald (1984) examined the relationship between ability to secrete protease and the virulence of pathogenic Candida species. Three of the most prevalent clinical species namely: C. albicans, C. tropicalis, and C. parapsilosis could secrete protease in vitro, whilst C. glabrata was not found to produce protease (3 strains tested). In addition, MacDonald (1984) found that C. krusei did not produce extracellular proteinase activity. It was

noteworthy that C. kefyr and C. guilliermondii did not secrete protease but could degrade bovine serum albumen by a cell surface protease. Thus the ability to secrete protease in vitro is clearly a property restricted to the most virulent Candida species and this has been corroborated by Schonborn et al. (1985).

Kwon-Chung et al. (1985) found a good correlation between the degree of virulence of C. albicans and the level of proteinase produced. A protease deficient mutant was obtained by nitrous acid mutagenesis from a proteolytic strain of C. albicans and its virulence was tested in mice together with a spontaneous revertant which had about 44% proteinase activity of the parent strain. The prognosis for infection with the revertant was marginally better than the parent strain. For example, at day 20 of the experimental murine candidosis the relative mortality with the parent, the revertant and the mutant were 90%, 80% and 0% respectively. At day 30 the mortality figures were 100%, 90% and 30%. Histology showed that the proteinase producing strains proliferated in the kidney; however, the proteinase-deficient mutant was cleared from the kidney by day 20.

In conclusion, the inducible carboxyl proteinases (E.C.3.4.23) in C. albicans play an important role in the pathogenesis of this organism. Ghannoum and Elteen (1986) found proteinase production to correlate with both adherence and pathogenicity. In particular, stains of C. albicans which adhered most strongly to buccal epithelial cells had the highest relative proteinase activities and were the most pathogenic in a mouse virulence test. Secreted proteinases potentiate the virulence of this yeast by increasing adherence (Ghannoun & Elteen, 1986), interfering with host defense

mechanisms (Ruchel et al., 1982) and causing tissue damage (MacDonald & Odds, 1980). Secreted acid proteases from Candida may be more relevant in pathogenesis than bacterial proteases (Ruchel, 1986), since no potent endogenous inhibitor has been found which can interfere with these Candida IgA proteases (Plaut, 1983). However, they may be only one type of the many determinants of pathogenicity since a protease-deficient strain of C. albicans can produce disease by reason of other virulence factors such as adherence (Ryley, 1986), yeast-to-hyphal transition in host tissue (Odds, 1979; Kimura & Pearsall, 1980; Sobel et al., 1984) or phospholipase activity (Barrett-Bee et al., 1985). Also the aetiology of candidosis is often of endogenous origin and due to a local or systemic increase in susceptibility to these opportunistic pathogens (Odds, 1979; Ryley, 1986; Shepherd et al., 1985).

SECTION 1.3 SEARCH AND DISCOVERY OF NEW ANTIFUNGAL AGENTS

SECTION 1.3.1 OVERVIEW

The classical approach to antibiotic discovery has shown little success in yielding clinically useful antifungal drugs (Smith, 1985). In the search for new antimycotics, pharmaceutical companies show a strong emphasis on the synthetic approach to drug discovery (Ellames, 1982; Ryley et al., 1981). However, it is important to remember that the cornerstone of antifungal chemotherapy is still amphotericin B - a polyene antibiotic produced by a strain of Streptomyces nodosus under defined and regulated conditions (Hoeprich, 1978). In addition, actinomycetes have been shown to elaborate triazoles (Imamura et al., 1985) - the chemical class of compounds which many pharmaceutical companies are presently developing for orally-active broad-spectrum antifungal drugs (Borgers, 1985).

As microorganisms can often make organic rearrangements in molecules which the synthetic chemist may find impossible to do (Omura, 1986), screening of natural products is important to inspire the medicinal chemist with the discovery of novel active compounds (Ryley et al., 1981).

SECTION 1.3.2 RATIONAL DRUG DESIGN

There are two broad areas of rational drug design which are applicable to antifungal drug discovery. The first approach is to inhibit or circumvent a determinant of microbial pathogenicity; thus, by reducing the virulence of the fungal pathogen the host's own immune system is better able to eradicate the infection (Smith, 1985).

The second approach is based on the selection of a macromolecular structure or a biochemical pathway unique to the pathogen and not shared by

the host. Destruction or inhibition of this target is expected to have a detrimental effect, at best lethal but at least there should be growth inhibition. Selective toxicity and lethality of action are the primary considerations in choice of target (Fleming et al., 1982).

A number of potential drug targets have been identified which have lethal actions against fungi and show good selectivity of action. These include the inhibitors of cell wall synthesis, membrane sterol biosynthesis, nucleic acid synthesis and nuclear division (Ryley and Rathmell, 1984). Inhibition of cell wall synthesis and fungal dimorphism are outlined below.

SECTION 1.3.3 INHIBITORS OF CELL WALL SYNTHESIS

The major difference between the eukaryotic fungal pathogens and their host is the fungal cell wall. This complex structure (reviewed by Fleet, 1985) is a prime target for antifungal agents (Kerridge, 1978; Selitrennikoff, 1984). However, no clinically useful antimycotic agent acts by interfering with cell wall synthesis (Kerridge, 1985) though several work in vitro. Indeed, recently an inhibitor of chitin synthesis (Nikkomycin X) was shown to be synergistic with an inhibitor of beta-glucan synthesis (Papulacandin B) in C. albicans (Hector & Braun, 1986) presenting the possibility of using lower doses. The therapeutic potential of the currently available inhibitors of the fungal cell wall [see Table 1.4.1] is limited by toxicity and poor pharmacokinetics (Ryley et al., 1981); however, the cell wall remains an experimentally attractive target. Since cell wall synthesis is brought about by enzymes present in the cell membrane it follows that many agents that interfere with cell wall synthesis may be acting indirectly through a component of the cell membrane (Kerridge, 1980).

TABLE 1.3.1

INHIBITORS OF FUNGAL CELL WALL SYNTHESIS

INHIBITOR	TARGET	REFERENCE
ACULECIN A	β -(1-3) glucan synthetase	Duran <u>et al.</u> , 1984; Yamaguchi <u>et al.</u> , 1985
2-deoxy-D-glucose	glucan & mannan synthesis	Ryley & Rathmell, 1984
Echinocandin	β -(1-3) glucan synthetase	Sawistowska-Schroder <u>et al.</u> , 1984
NIKKOMYCINS	Chitin synthetase	Hector & Braun, 1986; Ryley <u>et al.</u> , 1981
PAPULACANDINS	β -(1-3) glucan synthetase	Duran <u>et al.</u> , 1984; Hector & Braun, 1986
POLYOXAN D	Chitin synthetase	Kerridge, 1978; Ryley <u>et al.</u> , 1981

SECTION 1.3.4 INHIBITION OF DIMORPHISM

Yeast-to-mycelial transition in C. albicans has been implicated in pathogenesis (reviewed by Odds, 1985a). Inhibition of this dimorphic change would be expected to reduce the virulence of this organism. Although this is an experimentally attractive target for drug research, no specific inhibitors are known.

Recently, however, some success was found with the dimorphic fungus Histoplasma capsulatum (Medoff et al., 1986). Permanent and irreversible inhibition of dimorphism was obtained with p-chloromercuriphenylsulfonic acid (PCMS). Unfortunately, PCMS would not make a useful therapeutic agent. However, it is expected to lead to the development of a vaccine against this fungal pathogen since PCMS-treated fungus was avirulent in mice but stimulated resistance to infection by a pathogenic strain of H. capsulatum.

Rational drug design provides scope for developing chemotherapeutic agents with a high therapeutic index. In this type of target directed research it is possible to use defined mode-of-action screens. The frequency of discovery of new substances may be less than in the classical screening but the in vivo success rate is expected to be much higher (Fleming et al., 1982). This is the "rationally empirical" approach to screening (Ryley & Rathmell, 1984).

SECTION 1.4 INTERACTION OF KILLER YEASTS WITH PATHOGENIC YEASTSSECTION 1.4.1 KILLER YEASTS KNOWN TO BE ACTIVE AGAINST MEDICALLY-IMPORTANT YEASTS

Bussey & Skipper (1976) were the first to demonstrate that a killer yeast (Saccharomyces cerevisiae K₁₂) could kill a pathogenic yeast (Candida glabrata). Although the killer factor was described as a specific fungal antibiotic which killed C. glabrata by causing membrane damage, it apparently had no effect against the medically-important yeasts C. albicans or Cryptococcus neoformans. When Stumm *et al.* (1977) screened for killer-sensitive relationships between yeast strains belonging to at least 9 genera they found that Pichia and Hansenula strains killed yeasts of the genus Candida. In addition, Cryptococcus and Candida strains were found to kill yeasts of the genus Cryptococcus. However, in this study, which established important intergeneric killer-sensitive relationships, no attempt was made to identify species within a genus.

In the first detailed medical mycology study of killer yeasts, Kandel and Stern (1979) showed that Cryptococcus and Candida species were killed by Saccharomyces killer yeasts. Furthermore, certain Candida species were found to kill other Candida species. Kandel & Stern (1979) found the following medically-important yeasts to be sensitive to a variety of yeast killer toxins: C. glabrata, C. guilliermondii, C. krusei, C. parasilosis, C. tropicalis, and Cry. neoformans. Notably, none of the 120 strains of C. albicans tested were identified as killer or sensitive strains. A similar study was conducted by Middelbeek *et al.* (1980a) who screened for killer-sensitive properties among isolates of the opportunistic human pathogens of the genera Candida, Cryptococcus and Torulopsis. A high

incidence of sensitivity to killer toxins was observed within the genus Candida and Torulopsis. For example, approximately 80% of clinical isolates of C. albicans were found to be sensitive to killer yeasts - a finding contrasting with the results of Kandel & Stern (1979). In particular, Middelbeek et al. (1980a) identified Hansenula sp 1034 and Pichia sp. 1035 as killer yeasts with a broad spectrum of action against Candida species. In addition the list of aetiologic agents of candidosis which were sensitive to killer toxins was extended with the inclusion of C. kefir and C. stellatoidea (= C. albicans).

By cluster analysis*, Lehmann et al (1987) found a "typical" C. albicans was most probably sensitive to the following killer yeasts:

Debaryomyces vanriji NCYC 577 (MCO 57)**
Pichia anomala NCYC 434 (MCO 85)
Williopsis mrakii NCYC 500 (MCO 82)
Kluyveromyces drosophilorum NCYC 575 (MCO 87)
Hansenula sp. stumm 1034 (MCO 171)
Pichia anomala CBX 5759 (MCO 174)
Williopsis californica Ahearn WC40 (MCO 176)
Pichia canadensis Ahearn WC41 (MCO 177)
Williopsis dimennae Ahearn WC44 (MCO 178)
Williopsis mrakii Ahearn WC51 (MCO 179)

Other medically-important yeasts were included in this study. For example, Cr. neoformans was typically killed by Debaromyces vanriji NCYC 577 and C. glabrata isolates were sensitive to Pichia membranaefaciens NCYC 333.

* IBM software package

** MCO = Medical College of Ohio accession No.

Finally some of the antimicrobial activities of two purified killer toxins have been reported in the literature. The physico-chemical properties of these heat stable toxins are outlined in Section 1.1.4.

The first toxin called "KII" was purified from cell-free supernatant of Williopsis mrakii LKB 169 (Ashida et al., 1983). Among the sensitive yeasts demonstrated by agar diffusion bioassay were three etiological agents of candidosis; namely, Candida glabrata, C. krusei, and C. tropicalis. Activity against C. albicans was not reported.

The second killer toxin, called "HYI" is a product of Williopsis saturnus IFO 0117 (Ohta et al., 1984). The minimum inhibitory concentration (MIC, ug/ml) of HYI was determined for various yeasts by the agar dilution method. This was made possible by the availability of a large amount of this heat-stable toxin. Two medically-important yeasts were sensitive to HYI, namely C. krusei with an MIC of 0.8 ug/ml and C. pseudotropicalis (= C. kefir) with an MIC of 0.2 ug/ml. Activity of HYI against C. albicans was not reported.

SECTION 1.4.2 KILLER SYSTEM BIOTYPING

Recent studies of killer yeast activity against medically-important yeasts has concentrated on their potential for biotyping within a species. The many systems of biotyping medically-important fungi has been reviewed by Odds (1985b). Killer typing is analogous to the biotyping system for Gram-negative bacteria based on bacteriocin susceptibility.

Polonelli et al. (1983) proposed this procedure of "killer" biotyping to differentiate clinical isolates of C. albicans based on susceptibility of the strains to 9 selected killer yeasts (8 Hansenula spp., 1 Pichia spp.). this system has the potential to differentiate up to 2^9 (512) biotypes of C. albicans.

TABLE 1.4.1 (FROM POLONELLI et al., 1983)

Triplet groupings and activity codes for killer strains

Activity of the first triplet			Activity of the second triplet			Activity of the third triplet					
A	B	C	Activity Code	D	E	F	Activity Code	G	H	I	Activity Code
+	+	+	1	+	+	+	1	+	+	+	1
+	+	-	2	+	+	-	2	+	+	-	2
+	-	+	3	+	-	+	3	+	-	+	3
-	+	+	4	-	+	+	4	-	+	+	4
+	-	-	5	+	-	-	5	+	-	-	5
-	+	-	6	-	+	-	6	-	+	-	6
-	-	+	7	-	-	+	7	-	-	+	7
-	-	-	8	-	-	-	8	-	-	-	8

- A = Hansenula sp. Stumm 1034
 B = Pichia sp. Stumm 1035
 C = H. anomala UM
 D = H. anomala CBX 5759
 E = H. anomala Ahearn UN 866
 F = H. californica Ahearn WC 40
 G = H. canadensis Ahearn WC 41
 H = H. dimennae Ahearn WC 44

All of 100 clinical isolates tested were sensitive to at least one of the 9 selected yeasts and by this method 25 biotypes of C. albicans were identified. The most frequently encountered biotype was "111" which is a strain sensitive to all 9 killer yeasts (see Table 1.4.1). 52% of the C. albicans strains tested were found to be biotype 111. Polonelli et al. (1983) proposed that the killer system could be an effective screening method that could easily be applied to epidemiological investigations of infections due to C. albicans.

This killer system biotyping was extended by Morace et al. (1984) to Cryptococcus neoformans and pathogenic yeasts other than C. albicans. High sensitivity to the activities of 25 killer yeasts was reported. The highest activity was observed in species of the genera Pichia and Hansenula. No pathogenic yeasts tested as sensitive strains proved to be resistant to all of the killer strains, although C. glabrata showed the lowest sensitivities. Polonelli & Morace (1983) asserted that killer biotyping is a valid epidemiological marker in tracing the origins of yeast nosocomial infections (hospital acquired) and in general epidemiological surveillance.

Caprilli et al. (1985) extended this line of analysis and showed that the resolution of the killer system could be improved by using a larger number of killer yeasts. Using 21 killer strains grouped in triplets they showed that it was possible to differentiate 27 biotypes among the 100 strains of C. albicans studied. In contrast, only 7 biotypes were identified by the method of Polonelli et al. (1983). Once again the most prevalent isolate was biotype 111 (78%) and Caprilli et al. (1985) resolved this fraction into 13 biotypes. However, 48% of the total isolates remained identical with type 111 1111 being sensitive to all 21 killer strains. The system employed by Polonelli et al.

(1983) and Caprilli et al. (1985) both show this bias which may reflect their choice of killer yeasts.

Recently, Lehmann et al. (1987) amplified the killer system further by using 30 killer yeasts. In addition, this data was supplemented with sensitivity to 3 antifungal agents to yield a score of 11 digits. With this approach, 80 strains of C. albicans were resolved into 33 biotypes with the most prevalent biotype accounting for 12.5% of isolates. This low degree of bias is remarkable; however, the method was labour intensive.

Since this method also incorporated the 9 yeasts of Polonelli et al. (1983), the 3 studies are directly comparable. It is noteworthy that the most prevalent biotypes found by Polonelli et al. (1983) and Caprilli et al. (1985) only accounted for one isolate (1.25%) in the report of Lehmann et al. (1987). This discrepancy may be due to loss of potency by a single killer strain (P. anomala, MCO 173). Lehmann et al. (1987) identified 6 biotypes from 80 isolates of C. albicans using the original 9 killer yeasts. 70% of these isolates were of one biotype.

This recent study differs from the previous two by the use of a number of killer yeasts which had been shown to produce clearly different killer toxins. The choice of killer yeasts proved to be more discriminating. Lehmann et al. (1987) drew attention to the possibility that many of the killer yeasts employed by Polonelli et al. (1983) and Caprilli et al. (1985) may produce different quantities of the same toxin.

In fact, Polonelli et al. (1985) found purified toxin preparations to give more reliable results than those obtained using live cultures of killer

yeasts. Undoubtedly the use of purified killer toxins which are biochemically characterized will greatly increase the reproducibility and objectivity of this biotyping system. Two killer toxins with potential in this area were reviewed in Section 1.1.4.

SECTION 1.5 AIMS AND OBJECTIVES

OVERVIEW

Yeast killer toxins may have therapeutic potential in yeast infections of animals. Since the therapeutically useful antifungal drugs are few in number and are inherently toxic, the search for new antimycotics is justified. To this end, the actions of yeast killer toxins against C. albicans are of particular interest since this is the most prevalent opportunistic pathogen in man.

Today killer yeasts are known which are active against the principal aetiological agents of candidosis and other yeast pathogens like Cryptococcus neoformans (see Section 1.4.1). C. albicans has been shown to be sensitive to numerous killer toxins particularly those from the genera Hansenula and Pichia (Middelbeek et al., 1980a; Polonelli et al., 1983).

Classically killer toxins are thought to be lethal to yeasts alone and are not known to be toxic to mammalian cells. In view of their highly selective toxicity and lethality of action, these natural antimicrobial agents can be considered specific fungal antibiotics (Bussey & Skipper, 1976). Therefore if a screening program resulted in a potent anti-C. albicans toxin, it would be expected to have a high therapeutic index.

The pharmacological basis of this novel therapeutics is thought to be the interaction of the yeast toxin with putative receptors on the surface of this robust pathogen. Selected killer toxins may be used as molecular probes to toxin recognition elements to aid in the elucidation of the receptor-mediated mechanism of action and to identify potential sites for drug action (Bussey, 1981).

To have stable, biotransportable non-peptide analogs of bioactive peptides is desirable (Farmer & Ariens, 1982). Low molecular weight analogs of the protein toxins which retain activity could be synthesized. In theory, chemical modification may lead to increased stability, increased potency and a broader spectrum of action.

SPECIFIC AIMS AND OBJECTIVES

1. Screen killer yeasts for antimicrobial activity against Candida albicans.
2. Development of a quantitative bioassay.
3. Optimize production of killer toxin from selected yeasts.
4. Concentration and purification of toxin.
5. Study the pharmacology of yeast killer toxin:
 - (i) Spectrum of antimicrobial activity
 - (ii) Biochemical and physico-chemical nature
 - (iii) Effect of toxin on morphogenesis of C. albicans
 - (iv) Mode of action
6. Examine the possible degradation of toxin by the secreted acid proteinase from C. albicans.
7. Evaluation of the therapeutic potential of yeast killer toxins in vitro for the treatment of yeast infections in humans.

SECTION 2 MATERIALS AND METHODS

SECTION 2.1 ORGANISMS

SECTION 2.1.1 YEAST CULTURES

Known killer yeasts which were examined for antimicrobial activity against C. albicans are enumerated in Table 2.1.

A number of yeast strains, which are not known to be killer yeasts were screened for antimicrobial activity against C. albicans. These yeasts - none of which were found to have anti-C. albicans activity - are listed in Table 2.2.

C. albicans strains employed in this study are listed in Table 2.3.

Clinical isolates were confirmed to be C. albicans by the following criteria:

(i) germ-tube formation in rabbit serum at 37°C (Koneman et al., 1978);

and

(ii) chlamyospore formation in Czapek Dox agar with 1% (v/v) Tween 80 at 25°C (Dawson, 1962).

Finally, Saccharomyces cerevisiae NCYC 1006 was one of a number of indicator organisms used to detect killer activity (Young & Yagiu, 1978).

TABLE 2.1 KILLER YEASTS USED IN THIS STUDY

KILLER TYPE ¹	YEAST*	STRAIN	OBTAINED FROM	REFERENCE TO KILLER ACTIVITY
K ₄	<u>Candida glabrata</u>	NCYC 388	NCYC	1
	<u>Candida glabrata</u>	UCSC 0	Dr. L. Polonelli	2
K ₅	<u>Debaryomyces vanriji</u>	NCYC 577	NCYC	1
K ₆	<u>Kluyveromyces marxianus</u> ^a	NCYC 587	NCYC	1
	<u>Kluyveromyces lactis</u> ^b	MAYNOOTH 161	Prof. P. Whittaker	-
	<u>Kluyveromyces lactis</u> ^b	NRRL Y-1140	USDA	3
	<u>Pichia anomala</u> ^c	NRRL Y-366	USDA	-
K ₅	<u>Pichia anomala</u> ^c	NRRL Y-2153	USDA	1
K ₈	<u>Pichia anomala</u> ^c	NRRL Y-2153-4	USDA	1
K ₇	<u>Pichia membranaefaciens</u>	NCYC 333	NCYC	1
K ₅	<u>Pichia subpelliculosa</u> ^c	NCYC 16	NCYC	1
K ₂	<u>Saccharomyces cerevisiae</u>	NCYC 1001	NCYC	1
	<u>Saccharomyces cerevisiae</u>	c.d.c. 4	Prof. P. Whittaker	-
	<u>Saccharomyces cerevisiae</u>	MAYNOOTH 347	Prof. P. Whittaker	-
	<u>Saccharomyces cerevisiae</u>	NCYC 923	NCYC	-
K ₉	<u>Williopsis mrakii</u> ^d	NCYC 500	NCYC	1
	<u>Williopsis mrakii</u> ^d	LKB 169	Dr. K. Kodama	4
	<u>Williopsis saturnus</u> ^d	IFO 0117	IFO	5
	<u>Williopsis saturnus</u> ^d	NCYC 22	NCYC	-
	<u>Williopsis saturnus</u> ^d	NCYC 23	NCYC	-

* Taxonomy as in Kreger van Rij (1984) except where noted below:

a = K. marxianus var. marxianus

b = K. marxianus var. lactis. The trivial name K. lactis is preferred. Work by Sidenberg & Lachance (1986) support the separation of K. lactis from K. marxianus.

c The genus of these strains, as named in Kreger van Rij (1984) is Hansenula. They have been transferred to Pichia (Kurtzman 1984).

d The genus of these strains, as named in Kreger van Rij (1984) is Hansenula. Hansenula and Pichia species with saturn shaped spores have been transferred to Williopsis (Barnett et al., 1983; Kurtzman, 1984).

References: 1, Young & Yagiu, 1978; 2, Polonelli, et al., 1983; 3, Sugisaki et al., 1984; 4, Ashida et al., 1983; 5, Ohta et al., 1984.

TABLE 2.2 OTHER YEASTS SCREENED FOR KILLER ACTIVITY AGAINST C. ALBICANS

<u>Candida kefir</u>	NCYC 744
<u>Kluyveromyces marxianus</u>	NCYC 100
<u>Kluyveromyces marxianus</u>	CBS 5795
<u>Kluyveromyces marxianus</u>	NRRL Y-665
<u>Kluyveromyces marxianus</u>	NRRL Y-1109
<u>Phaffia rhodozyma</u>	NCYC 874
<u>Pichia canadensis</u> ^d	NRRL Y-1888
<u>Williopsis saturnus</u> ^e	NRRL Y-1
<u>Williopsis saturnus</u>	NRRL Y-12
<u>Williopsis saturnus</u>	NRRL Y-838
<u>Williopsis saturnus</u>	NRRL Y-1304
<u>Schizosaccharomyces pombe</u>	972 h ⁻

d, e - as in footnotes to Table 2.1

TABLE 2.3 CANDIDA ALBICANS STRAINS USED IN THIS STUDY

STRAIN	OBTAINED FROM:	REFERENCE
ATCC 10261	ATCC	Gopal <u>et al.</u> , 1982
NCYC 854	NCYC	Quoted in the British Pharmacopoeia for Sterility Testing
CA 2	Prof. M. G. Shepherd	Chattaway <u>et al.</u> , 1981
A 72	Prof. M. G. Shepherd	Torosantucci & Cassone, 1983
HOG 301	Prof. M. G. Shepherd	A non-blastospore, growth deficient mutant
UCSC 10	Dr. L. Polonelli	Polonelli <u>et al.</u> , 1983
#12 A	Dr. D. Reen	Clinical isolate from sputum
#80 A	Dr. D. Reen	Clinical isolate from gastric aspirate

SECTION 2.1.2 SOURCE OF YEAST CULTURESCULTURE COLLECTIONS:

A.T.C.C.	American Type Culture Collection (Rockville, Maryland, U.S.A.)
C.B.S.	Centraalbureau voor Schimmel cultures (Baarn, The Netherlands)
I.F.O.	Institute for Fermentation (Osaka, Japan)
N.C.Y.C.	National Collection of Yeast Cultures (Norwich, U.K.)
U.S.D.A.	Midwest Area Northern Regional Research Center (Peoria, Illinois, U.S.A.)

PRIVATE CULTURE COLLECTIONS:

Dr. K. Kodama	Laboratory of Kodama Brewing Co., Ltd., Akita Prefecture, Japan
Dr. L. Polonelli	Istituto di Microbiologia, Universita Cattolica del Sacro Cuore, Rome, Italy
Dr. D. Reen	Research Center, Our Ladys Hospital for Sick Children, Dublin, Ireland
Prof. M. G. Shepherd	School of Dentistry, University of Otago, New Zealand
Prof. P. Whittaker	Department of Biology, St. Patricks College, Maynooth, Ireland

SECTION 2.1.3 MAINTENANCE OF STRAINS

Yeast cultures were maintained by serial subculture on Sabouraud Dextrose Agar slopes. Following transfer, which was made monthly or bimonthly, slopes were incubated (48 h, 30°C) and stored at 4°C.

SECTION 2.2 GENERAL MYCOLOGICAL METHODSSECTION 2.2.1 PREPARATION OF INOCULUM

Starter cultures were grown on Sabouraud Dextrose Agar (SDA) slopes for 16-20 hours at 30°C. Growth was suspended in sterile distilled water and a direct cell count was made using an improved Neubauer haemocytometer. A minimum of 200 cells were counted. For W. mrakii an excellent correlation was found between the direct cell count, made with microscopy and the colony count made on SDA.

SECTION 2.2.2 DRY WEIGHT DETERMINATION

Disposable glass culture tubes (16x75mm, CORNING) were used for dry weight determination (mg dry weight/ml). The tare weight was taken after drying in a dessicator. 10 ml culture broth was sedimented at 1000 x g for 10 minutes., The supernatant was aspirated off and the pellet was washed once with 10 ml distilled water. The tubes were dried (105°C, 24 h) and finally cooled to constant weight in a dessicator.

SECTION 2.2.3 TOXIN PRODUCTION

Batch cultivation of W. mrakii was in cotton plugged 250 or 1000 ml Erlenmyer flasks containing 100 or 400 ml nutrient broth respectively. Flasks were incubated at 25°C in a reciprocating water bath (65 rev./min).

SECTION 2.2.4 STORAGE OF TOXIN SOLUTIONS

Toxin containing solutions (e.g., cell-free culture broth) were generally stored at 4°C (or at -20°C, in small quantities).

SECTION 2.3 COMPOSITION OF CULTURE MEDIA

SECTION 2.3.1 SCREENING AND BIOASSAY MEDIUM

FORUMLA: SABOURAUD LIQUID MEDIUM (OXOID, CM 147) 0.3% (w/v)

AGAR TECHNICAL (OXOID, L 13) 2% (w/v)

METHYLENE BLUE (SIGMA, CERTIFIED) 0.003% (w/v)

0.1 M Na₂HPO₄ - CITRIC ACID BUFFER pH 4.5

The agar and SLM were suspended in the buffer. Methylene blue was added as a 0.3% (w/v) aqueous solution. The medium was brought to the boil to dissolve completely, and autocalved (115°C, 15 min.).

- MODIFICATIONS: (i) Media at pH 7.0 was supplemented with methylene blue (approx. 0.1% w/v) to obtain an adequate blue color.
- (ii) Addition of 0.001% (w/v) cycloheximide (Sigma) before autoclaving made this medium selective for growth of C. albicans.
- (iii) 10% (w/v) glycerol was included in the bioassay medium when C. albicans was the indicator organism.

SECTION 2.3.2 MINIMAL MEDIUM

The chemically defined Wickerham medium (Barnett et al., 1983) was used with the following modifications:

- (i) Carbon source : Glucose, 2.0% (w/v)
- (ii) Nitrogen source : Ammonium chloride, 0.5% w/v
- (iii) The medium was buffered with 0.1M Na_2HPO_4 - citric acid buffer, pH 4.5
- (iv) The following were omitted: L-asparagine, amino acids and $\text{NH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$

This medium was sterilized by filtration (0.45 um pore size).

SECTION 2.3.3. UYEPD

(Ultra-filtered Yeast Extract - Peptone-Dextrose Medium)

Preparation of UYEP

UYEP was prepared as a 20-fold concentrate. 250 ml YEP containing 50g yeast extract (Oxoid, L21) and 50g mycological peptone (Oxoid, L 40) was ultra-filtered through a Diaflo YM 30 membrane (Amicon) until 50 ml remained. 200 ml of permeate was sterilized by autoclaving (121°C/15 min) and stored at room temperature.

UYEPD medium

To 50 ml UYEP was added 20g glucose and the volume was made up to 1 liter with 0.1M Na_2HPO_4 -citric acid buffer. The medium was autoclaved (121°C, 15 min).

SECTION 2.3.4 SKIM MILK AGAR

SKIM MILK POWDER (OXOID, L 31) 1% (w/v)

SABOURAUD LIQUID MEDIUM (OXOID, CM 147) 0.3% (w/v)

AGAR TECHNICAL (OXOID, L 13) 2% (w/v)

0.1 M Na_2HPO_4 -CITRIC ACID BUFFER, pH 4.0

SECTION 2.3.5 PROTEASE INDUCTION MEDIUM

The medium of MacDonald & Odds (1980) was used to induce proteinase production by C. albidans; this was modified by the inclusion of the non-ionic detergent Brij 58 at 0.001% (sterilized by filtration) which facilitated protein secretion by the yeasts.

SECTION 2.4 SCREENING METHOD

SECTION 2.4.1 SEEDED METHYLENE BLUE-AGAR TECHNIQUE

Screening for antimicrobial activity against C.albicans was based on the streak-plate method of Stumm et al. (1977) using a novel nutrient medium (see Section 3.1.1). 15 ml aliquots of this medium (pH 4.5 or pH 7.0) were autoclaved in screw-cap vials. The molten agar was cooled to 55°C and Candida yeasts were seeded at a density of 5×10^4 cell/ml and poured into 9 cm petri dishes. When set, each plate was streaked with three different killer yeasts. Plates were incubated at either 25°C or 37°C and scored after 72 hours. Screening assays were determined in triplicate.

2.4.2 NOMENCLATURE FOR SCORING KILLER ACTIVITY

Since the killer phenomenon is a yeast-yeast interaction, it depends as much on relative growth of the two yeasts, as it does on environmental conditions. A positive result indicative of killing is seen as a zone of clearing; there may, in addition be a border of blue staining colonies which classically are presumed dead (Woods & Bevan, 1968). Based on the magnitude of the observed inhibition zone, an arbitrary score of killer activity was used to record the result of the screening and, the presence or absence of blue-staining colonies was recorded (Table 2.4).

The appearance of blue-staining colonies is desirable since it may be indicative of lethal action by the killer toxin. However, attention is drawn to a frequent observation that apparently healthy colonies of C. albicans absorb the blue dye. One must therefore be cautious when interpreting this feature in C. albicans strains. This is corroborated by a recent study which concluded that dye tests do not necessarily differentiate living Histoplasma capsulatum yeast cells from dead cells (Kwon-Chung & Tewari, 1987). Although

the methylene blue test correlates well with the viability of S. cerevisiae as determined by the plate count (Young & Philliskirk, 1977), it may be necessary to use a different technique for detecting the viability of different yeast species (Trevors et al., 1983). In view of this, the presence of blue staining colonies immediately beneath the streak, alone, cannot be considered sufficient evidence to identify a killer-sensitive relationship, since it occurs in the area of maximum nutrient competition.

TABLE 2.4 ARBITRARY SCORE OF KILLER ACTIVITY

-	No killer activity
+	Trace activity
++	Weak activity
+++	Moderate activity
++++	Good activity
+++++	Excellent activity
+/-	Ambiguous
n.d.	Not determined
()	Parenthesis indicates "fuzzy" zone of growth suppression
n.g.	No growth
0	Absence of blue-staining colonies
1	Blue-staining colonies <u>beneath streak only</u>
2	Border of blue-staining colonies present

SECTION 2.5 AGAR DIFFUSION BIOASSAY

2.5.1 PETRI DISH BIOASSAY PROCEDURE

Methylene blue agar medium (Section 2.3.1) was brought to the boil and equilibrated at 55°C (water bath). 15 ml aliquots of the molten agar were dispensed into thin-walled glass 35 ml vials. The vials were autoclaved (110 lbs/in²; 15 min), then were equilibrated at 55°C. Oxidation of the methylene blue dye was facilitated by shaking once. The selected indicator strain (see Section 2.5.3) which had been prepared as in Section 2.2.1, was added as a 50-200 ul suspension to give a seeding density of 5×10^4 cell/ml. After mixing by inversion (15 times) the contents were poured immediately into a 9 cm dish (Sterilin, plastic disposable, single vented) and bubbles were popped with the flame of a Bunsen burner. After the agar had gelled, 3-6 wells were cut with 8.5 mm diameter, sterile cork borer. The plugs were removed by aspiration. Sealing the bottom of the well with agar as recommended by Holt (1975) was found to be unnecessary.

For an assay, 50 ul test solution was added to each well, making sure to wet the walls of the well. Following this the petri dishes were incubated (25°C, 2d) during which time the yeasts within the agar grew to form a cloudy background. The diameter of zone inhibition surrounding a well was measured to the nearest 0.5 mm with dividers. Three measurements were taken (at 60° angles) for each zone (Shadomy & Espinel-Ingroff, 1984). Test solutions were assayed in duplicate or in triplicate.

SECTION 2.5.2 DIFFERENTIAL BIOASSAY

Based on the relative specificity of killer toxins for yeast species, the following test organisms were used to differentiate toxins in a mixture:

- (i) C. albicans A 72 (KA bioassay),
- (ii) C. glabrata NCYC 388 (KG bioassay), and
- (iii) S. cerevisiae NCYC 1006 (KS bioassay).

The growth medium of the KA bioassay contained 10% (v/v) glycerol. S. cerevisiae NCYC 1006 - the indicator strain in the KS bioassay - was given a heat shock prior to use: following growth at 25°C for 2 days, the starter culture was incubated at 37°C for 2 hours. These heat-shocked yeasts were then used to seed the agar used in the KS bioassay. This analytical technique is depicted in Plate 4.

SECTION 2.5.3 LARGE PLATE BIOASSAY

Glass mast assay dishes (Medical Supply Company, Dublin) with dimensions 25x25x2 cm and containing 300 ml agar medium (Section 2.3.1) were used to analyse large numbers of samples. Each plate contained 36, 64 or 100 wells of 5 or 8.5 mm diameter. The volume of the test solutions was either 100 or 200 ul.



PLATE 2 ULTRAFILTRATION APPARATUS (COLD ROOM 4°C)

The ultrafiltration cell (Amicon, Model 8400) on a magnetic stirring box, received approximately 70 p.s.i. positive pressure from oxygen-free nitrogen. The permeate which passes through the 76 mm ultrafiltration membrane is collected in a flask; the retentate is concentrated and remains in the cell.

SECTION 2.6 FILTRATION

SECTION 2.6.1 MEMBRANE FILTRATION

The following membranes were used:

- (i) GN-6 Metrical membranes, 0.45 um pore size/47 mm diameter, made of mixed esters of cellulose. (Gelman #60512).

- (ii) FlowPore D26, sterile disposable syringe filters, 0.2 um pore size (Medical Supply Company, Dublin).

SECTION 2.6.2 ULTRAFILTRATION

Ultrafiltration was performed in a cold room (4°C) with an Amicon Model 8400 cell. Plate 2 depicts this ultrafiltration set-up.

Ultrafiltration membranes used: Diaflo YM5, YM10 and YM30 (Amicon Ltd, Glos., England).

SECTION 2.6.3 PREPARATION OF THE KII FRACTION

Twenty-four hour culture broth (UYEPD, pH 4.5) from W. mrakii LKB 169 was adjusted to pH 4.0. this was ultrafiltered at 4°C through a YM 10 membrane and the YM 10 permeate was diafiltered on a YM 5 membrane. The YM 5 retentate which results is the KII fraction containing molecules with putative molecular weight 5,000-10,000.

SECTION 2.7 METHODOLOGY FOR CANDIDA PROTEASES

SECTION 2.7.1 SOLID SCREENING ASSAY

A solid medium containing skim milk powder (1% w/v; OXOID, L31) and Sabouraud Liquid Medium (OXOID, CM 147) was used to screen C. albicans strains for secretion of acid protease. The agar (2%, w/v) was autoclaved with the medium (pH 4.0, 0.1 M Na₂HPO₄ - Citric acid buffer) giving a soft agar gel, which was firm enough to streak. This technique gave satisfactory results which were easily visualized after seven days' incubation at 25°C (see Table 3.5.1).

Undoubtedly, the milk agar protease screening medium would be improved by use of a minimal medium so that the milk proteins are the sole nitrogen source; in this regard, Ruchel et al. (1982) found that the sensitivity for protease detection can be improved by inclusion of yeast extract in BSA agar, or by use of Yeast Carbon Base supplemented with vitamins.

SECTION 2.7.2 EFFECT OF ACID PROTEINASE ON TOXINS

Cell-free broth from 24-hour culture (UYEPD, pH 4.5) of either W. mrakii LKB 169 or NCYC 500 was incubated at 37°C in the presence of the following proteinase:

- (i) Acid proteinase from C. albicans NCYC 854 (0.036 ug L-Tyrosine equivalents/min/ug protein)*
- (ii) Acid proteinase from Aspergillus saitoi (Sigma Chemical Co., #P2143; 0.15 mg/ml)*
- (iii) Papain (0.09 mg/ml; 15 mM L-cysteine/2 mM EDTA)* (Biocon, Cork).

* working concentration

0.2 ml enzyme solution (in 0.1 M Na_2HPO_4 -citric acid buffer, pH 4.5) was added to 2 ml toxin solution (preincubated at 37°C) in a capped Biju bottle. With time, residual activity was assayed by the "triplet bioassay" (Section 2.5.2).

Pepstatin A (Sigma Chemical Co.) was dissolved in DMSO and used at 20 ug/ml (1% v/v final concentration DMSO).

SECTION 2.8 BIOCHEMICAL ASSAYS

SECTION 2.8.1 PROTEIN ASSAY

Protein was determined by the Bradford Method (Bradford, 1976) using Bio-Rad protein assay Kit-II (Alfa Analytical, England) with BSA fraction V as standard.

SECTION 2.8.2 ACID PROTEINASE ASSAY

Quantitative assay of acid proteinase activity was based on hydrolysis of acid denatured haemoglobin (30 min., 37°C, pH 3.2). Absorption at 275 nm of the trichloroacetic acid (TCA) soluble material was related to a tyrosine standard curve.

Preparation of substrate

An aqueous solution of bovine haemoglobin (5% w/v), was adjusted to pH 1.7 with 1N HCl and with continuous stirring (room temperature). After 10 minutes the pH was adjusted to pH 3.2 by the addition of 0.2 M Na_2HPO_4 and the volume was adjusted to 2% (w/v) haemoglobin. The substrate was refrigerated and used within 5 days.

Procedure

1 ml enzyme preparation was incubated (37°C) with 5 ml haemoglobin substrate (pH 3.2) in a disposable culture tube (16x75 mm, Corning; AGB Dublin). Addition of 5 ml ice cold TCA (5%, w/v) terminated the reaction. Following refrigeration for 1 hour, the tubes were sedimented at 1000xg/10 min and A_{275} of the supernatant was determined and compared to a standard curve for tyrosine (BDH, Chromatographic grade, range 25-100 ug L-tyrosine).

SECTION 2.9 SOURCE OF CHEMICALS

In general, Pharmaceutical grade chemicals were used; Reidel-de Haen, West Germany. Fine biochemicals were from Sigma (London) LTD or B.D.H. (England). Microbiological growth media and Nystatin susceptibility discs were from Oxoid Limited (England). Ketoconazole was a gift from Janssen Pharmaceutica (Belgium).

SECTION 3.1 SCREENING FOR KILLER YEAST ACTIVITY AGAINST Candida albicansSECTION 3.1.1. DEVELOPMENT OF A KILLER YEAST SCREENING ASSAYChoice of medium

Screening for killer yeast activity against Candida albicans was based on the streak-plate assay (Stumm et al., 1977). The growth medium contained 0.003% (w/v) methylene blue which for Saccharomyces cerevisiae is reported to differentiate dead colonies by selectively staining them blue (Woods & Bevan, 1968). Plate 3 depicts this killer yeast screening assay which detects antimicrobial activity against C. albicans.

Rapid overgrowth of the seeded C. albicans made YEPD (Yeast Extract-Peptone-Dextrose) medium unsuitable for screening. This was due to the background 'lawn' becoming confluent before the streaked killer yeast had time to develop. Polonelli et al. (1983) successfully used Difco Sabouraud agar in their screening procedure with C. albicans. However, in this study, the methylene blue dye was found to be incompatible with Sabouraud dextrose agar from Oxoid. The source of variability was found to be the type of peptone in the medium, regardless of the pH or autoclaving regime used. Specifically, the basic dye formed an unacceptable green complex with certain peptones, and this was found to be particularly severe with mycological peptone (Oxoid, L40), a component of Sabouraud dextrose agar (Oxoid).

In searching for an alternative peptone source, Tryptone (OXOID) was found to be compatible with methylene blue and led to the introduction of Sabouraud Liquid Medium (SLM) as the basis of the test medium. A slower growth of C. albicans was supported in SLM.



PLATE 3

Screening technique - to detect antimicrobial activity against Candida albicans.

The test organism (C. albicans UCSC10) was seeded in a modified Sabouraud medium (5×10^4 cells/ml)¹ containing 0.003% methylene blue (pH 4.5). The killer yeast (W. mrakii NCYC500)² was streaked on the surface and incubated for 2 days at 25°C.

Choice of screening conditions

Polonelli et al. (1983) identified temperature and pH as the most important factors affecting the killer phenomenon in the streak-plate assay. Since the objective of this screening program was to identify toxin-producing strains with therapeutic potential, it was expedient to examine activity under physiological conditions. For a therapeutic agent it is desirable to have activity at "physiological pH and temperature" - i.e., that of human plasma (37°C and pH 7.4). However, due to colour instability of the methylene blue dye at this pH, neutral pH was chosen instead.

Most yeast infections occur on mucous membranes and are considered superficial in nature (Odds, 1979). Of particular clinical interest today is the development of new therapies for *Candida vaginitis*. The pH range of the vagina is between 4 and 5 (Schnell, 1982). Therefore to screen for potential therapeutic agents for vaginal candidosis, the conditions of pH 4.5 and 37°C are appropriate. In fact, the majority of killer yeast examined to date are active at pH 4.5 (Stumm et al., 1977; Middelbeek et al., 1980a; Polonelli et al., 1983).

Most yeasts have optimum growth temperatures well below 37°C. When growth and production of killer toxin are the primary considerations, an incubation temperature of 25°C is the best compromise. Although this temperature favours toxin stability it may not be physiologically relevant if a toxin were to be used therapeutically.

The following four sets of conditions were examined in killer yeast screening assays: a) 25°C/pH 4.5; b) 25°C/pH 7.0; c) 37°C/pH 4.5; and d) 37°C/pH 7.0. These are further discussed in Section 3.1.2.

TABLE 3.1.1 EFFECT OF ASSAY AT 25°C AND pH 4.5 ON THE DETECTION OF KILLER ACTIVITY AGAINST CANDIDA SPECIES

SENSITIVE STRAINS TESTED											
KILLER STRAINS TESTED	Ca1	Ca2	Ca3	Ca4	Ca5	Ca6	Ca7	Ca8	Cg1	Cg2	Ck1
<i>C. glabrata</i> NCYC 388	-	-	-	-	-	-	n.d.	n.d.	-	-	-
	0	0	0	0	0	0			0	0	0
<i>C. glabrata</i> NCSC 0	-	-	-	+	-	-	n.d.	n.d.	-	-	-
	0	0	0	2	0	0			0	0	0
<i>D. vanrii</i> NCYC 577	+	+	+	++	+	++++	n.d.	n.d.	+++	+++	-
	0	0	0	0	2	2			0	2	0
<i>K. lactis</i> NRRL Y-1140	n.d.	-	-	-	n.d.	+/-	-	-	(+++)	n.d.	n.d.
		0	0	0		0	0	0	0		
<i>K. lactis</i> 161	-	-	-	-	-	-	n.d.	n.d.	+/-	+/-	-
	0	0	0	0	0	0					0
<i>K. marxianus</i> NCYC 587	-	-	-	-	-	-	n.d.	n.d.	-	-	-
	0	0	0	0	0	0			0	0	0
<i>P. anomala</i> NRRL Y-2153	n.d.	(+++)	+/-	(++++)	n.d.	(++)	(+++)	(+++)	++++	n.d.	n.d.
		0	1	0		1	0	0	2		
<i>P. anomala</i> NRRL Y-2153-4	n.d.	(++)	(++)	(++++)	n.d.	(++)	(+++)	(+++)	++++	n.d.	n.d.
		0	1	0		1	0	0	2		
<i>P. membranaefaciens</i> NCYC 333	+	-	+	-	+	+	n.d.	n.d.	++++	++	+++
	0	0	0	0	0	0			2	0	2
<i>P. subpelliculosa</i> NCYC 16	-	-	-	-	-	-	n.d.	n.d.	-	-	-
	0	0	0	0	0	0			0	0	0
<i>S. cerevisiae</i> NCYC 1001	-	-	-	-	-	-	n.d.	n.d.	-	-	-
	0	0	0	0	0	0			0	0	0
<i>S. cerevisiae</i> 347	-	-	-	-	-	-	n.d.	n.d.	+++	-	-
	0	0	0	0	0	0			0	0	0
<i>S. cerevisiae</i> cdc 4	-	-	-	-	-	-	n.d.	n.d.	++	-	-
	0	0	0	0	0	0			0	0	0
<i>W. mrakii</i> NCYC 500	++++	++++	++++	++++	+++	++++	n.d.	n.d.	++++	+	-
	2	2	2	2	2	2			2	0	0
<i>W. mrakii</i> LKB 169	n.d.	++	+++	+++	n.d.	+++	(+)	(+)	++++	n.d.	n.d.
		2	2	2		2	2	2	2		
<i>W. saturnus</i> IFO 0117	n.d.	+/-	+/-	+	n.d.	+/-	-	+/-	++++	n.d.	n.d.
		1	1	2		1	0	1	2		
<i>W. saturnus</i> NCYC 22	+	-	-	+/-	+	+	n.d.	n.d.	++++	+/-	+++
	0	0	0	2	0	2			2	2	2

Ca1, *C. albicans* NCYC 854; Ca2, *C. albicans* ATCC 10261; Ca3, *C. albicans* CA2; Ca4, *C. albicans* A72; Ca5, *C. albicans* HOG301; Ca6, *C. albicans* UCSC 10; Ca7, *C. albicans* 12A; Ca8, *C. albicans* 80A; Cg1, *C. glabrata* NCYC 388; Cg2, *C. glabrata* UCSC0; Cfl, *C. kefir* NCYC 744.

Arbitrary score:

- No killer activity
- + Trace activity
- ++ Weak activity
- +++ Moderate activity
- ++++ Good activity
- +++++ Excellent activity

- +/- Ambiguous
- n.d. Not determined
- () Parenthesis indicates "fuzzy" zone of growth
Suppression
- n.g. No growth
- 0 Absence of blue-staining colonies
- 1 Blue-staining colonies beneath streak only
- 2 Border of blue-staining colonies present

SECTION 3.1.2. RESULTS OF SCREENINGSECTION 3.1.2.1 TEMPERATURE AND pH EFFECTS

a) 25°C/pH 4.5

The results are summarized in Table 3.1.1. It is apparent from the results that at pH 4.5 and 25°C the Candida species are particularly sensitive to killer yeasts from the genera Debaryomyces, Pichia and Williopsis. Williopsis mrakii NCYC 500 showed the strongest killer activity against all strains of C. albicans tested. Debaryomyces vanriji showed good activity against C. albicans, particularly against strain UCSC 10. The degree of differential susceptibility of C. albicans strains to these two killer yeasts may not simply reflect a difference in quantity of toxin secreted since C. albicans UCSC 10 was equally sensitive. This suggests the Debaryomyces toxin is quite distinct from that of Williopsis. On the other hand, the activity of C. glabrata UCSC 0 against one strain of C. albicans may reflect a low level of toxin secretion, since strain A72 was the most sensitive of the C. albicans strains used. The killer yeast C. glabrata NCYC 388 was itself sensitive to other killer yeasts.

Two fresh clinical isolates used in this study (#12 A, #80 A) were much less sensitive to the killer phenomenon than the C. albicans strains maintained in the laboratory. However, both yeasts were found to be markedly more sensitive to cell-free broth of the LKB 169 killer when tested by the agar diffusion bioassay. The basis of this "resistance" is unknown, but it may be associated with the secretion of an acid proteinase by the fresh clinical isolate.

Both strains of Pichia anomala produced a similar pattern of activity against the Candida species (Table 3.1.1); although NRRL Y-2153 is a K₅ killer

type and NRRL Y-2153-4 is a K_8 killer type, according to the classification of Young & Yagiu (1978). The activity of the P. anomala killers was difficult to interpret in this assay due to the appearance of broad "fuzzy" zones of growth suppression together with delayed staining. This is quite distinct from the typical killing pattern produced by W. mrakii (see plate 3). The unusual antimicrobial activity of the Pichia killers may reflect a late production of toxin or a delay in the response to this toxin. Alternatively, it may be a dose dependent phenomenon.

W. saturnus IFO 0117 produced a poor response against C. albicans. In particular, it exhibited only weak activity against strain A72. Since the toxin from IFO 0117 - characterized by Ohta et al., 1984 - has many advantageous properties (see Sections 1.1.4 and 1.4.1), early elimination from the screening program was resisted. In an attempt to overcome the major disadvantages of the streak-plate assay, cell-free broth (pH 4.5) of 24-hour cultures (25°C) was tested by the agar diffusion assay. Seven strains of W. saturnus were tested by this "growth-independent" screening method for activity against six strains of C. albicans. No killer-activity was detected; however, by this new approach, P. anomala NRRL Y-366 was found to exhibit weak activity when tested against C. albicans (data not shown).

W. mrakii LKB 169 has been reported to be active against three aetiological agents of candidosis: C. glabrata, C. krusei, and C. tropicalis (Ashida et al., 1983). However, activity against C. albicans has not been documented. Here, LKB 169 was shown to be moderately active against C. albicans. In view of the particular merits of this yeast (see introduction) it was selected for further investigation together with W. mrakii NCYC 500.

TABLE 3.1.2 EFFECT OF ASSAY AT 25°C AND pH 7.0 ON THE DETECTION OF KILLER ACTIVITY AGAINST CANDIDA SPECIES

	Ca1	Ca2	Ca3	Ca4	Ca5	Ca6	Gg1	Gg2	Ck1
<u>C. glabrata</u> NCYC 388	- 0	- 0	- 0	- 0	n.g.	- 0	- 0	- 0	- 0
<u>C. glabrata</u> UCSC 0	- 0	- 0	- 0	- 0	n.g.	- 0	- 0	- 0	- 0
<u>D. vanriji</u> NCYC 577	- 0	- 0	- 0	- 0	n.g.	- 0	- 0	- 0	- 0
<u>K. lactis</u> 161	- 0	- 0	- 0	- 0	n.g.	- 0	- 0	- 0	- 0
<u>K. marxianus</u> NCYC 587	- 0	- 0	- 0	- 0	n.g.	- 0	- 0	- 0	- 0
<u>P. membranaefaciens</u> NCYC 333	- 0	- 0	- 0	- 0	n.g.	- 0	- 0	- 0	- 0
<u>P. subpelliculosa</u> NCYC 16	+ 0	- 0	- 0	- 0	n.g.	- 0	- 0	- 0	- 0
<u>S. cerevisiae</u> NCYC 1001	- 0	+/- 0	- 0	- 0	n.g.	- 0	- 0	- 0	- 0
<u>S. cerevisiae</u> 347	- 0	- 0	- 0	- 0	n.g.	- 0	- 0	- 0	- 0
<u>S. cerevisiae</u> cdc 4	- 0	+/- 0	- 0	- 0	n.g.	- 0	- 0	- 0	- 0
<u>W. mrakii</u> NCYC 500	- 0	++++ 0	- 0	- 0	n.g.	- 0	- 0	- 0	- 0
<u>W. saturnus</u> NCYC 22	- 0	++++ 0	- 0	- 0	n.g.	- 0	+ 2	- 0	- 0

Explanation of abbreviations as in Table 3.1.1

b) 25°C/pH 7.0

The results are summarized in Table 3.1.2. At neutral pH, W. mrakii NCYC 500 and W. saturnus NCYC 22 showed good activity against one strain of C. albicans (ATCC 10261). It is noteworthy, that W. saturnus NCYC 22 was not active against this yeast at pH 4.5. No other yeasts (Table 3.1.2) showed killer activity under these screening conditions. C. albicans strain HOG 301 did not grow at pH 7.0 since it is a growth deficient mutant.

These results suggest that a biologically active toxin was produced by the Williopsis killer yeasts but most Candida strains were insensitive when tested in the streak plate assay. The basis of this "conditional killing" may be due to: (i) production of a Candida protease which effectively neutralizes toxin; or (ii) non-expression of a putative toxin receptor at pH 7.0 in the majority of C. albicans strains.

TABLE 3.1.3 EFFECT OF ASSAY AT 37°C AND pH 4.5 ON THE DETECTION OF KILLER ACTIVITY AGAINST CANDIDA SPECIES

	Ca1	Ca2	Ca3	Ca4	Ca5	Ca6	Cg1	Cg2	Ck1
<u>C. glabrata</u> NCYC 388	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0
<u>C. glabrata</u> UCSC 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0
<u>D. vanriji</u> NCYC 577	+++ 0	- 0	- 0	+ 0	+ 2	++ 0	+++ 0	++ 0	- 0
<u>K. lactis</u> 161	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0
<u>K. marxianus</u> NCYC 587	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0
<u>P. membranaefaciens</u> NCYC 333	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0	-/+ 0
<u>P. subpelliculosa</u> NCYC 16	+ 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0
<u>S. cerevisiae</u> NCYC 1001	- 0	+/- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0
<u>S. cerevisiae</u> 347	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0
<u>S. cerevisiae</u> cdc 4	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0
<u>W. mrakii</u> NCYC 500	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0
<u>W. saturnus</u> NCYC 22	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0

Explanation of abbreviations as in Table 3.1.1

c) 37°C/pH 4.5

The results are summarized in Table 3.1.3. Under these conditions, D. vanriji NCYC 577 produced biologically active toxin and was found to be active against 4 of the C. albicans strains tested. This suggests that toxin from this killer yeast could have therapeutic potential in Candida vaginitis.

W. mrakii NCYC 500 did not grow well at 37°C and showed no activity against the Candida strains. Since this yeast is known to be a strong killer when grown at lower temperature (see Table 3.1.1), it can be inferred that toxin production is dependent on growth. In addition it may be unstable at 37°C. This does not exclude the possibility that cell-free toxin from W. mrakii NCYC 500 may be active against C. albicans at 37°C.

d) 37°C/pH 7.0

The results are summarized in Table 3.1.4. When incubated at 37°C and neutral pH, W. mrakii NCYC 500 and W. saturnus NCYC 22 showed weak activity against C. albicans ATCC 10261 and trace activity against C. albicans NCYC 854.

At 37°C, both killer yeasts grew better at pH 7.0 than they did at pH 4.5; however, growth was significantly depressed. C. albicans strains UCSC 10 and HOG 301 did not grow at 37°C in neutral pH. In general all Candida strains grew less well at 37°C than at 25°C.

The present conditions are very close to the physiological pH and temperature of human plasma. Therefore, it is possible that toxin from W. mrakii NCYC 500 and W. saturnus NCYC 22 would be active against C. albicans in vivo if administered parenterally.

TABLE 3.1.4 EFFECT OF ASSAY AT 37°C AND pH 7.0 ON THE DETECTION OF KILLER ACTIVITY AGAINST CANDIDA SPECIES

	Ca1	Ca2	Ca3	Ca4	Ca5	Ca6	Cg1	Cg2	Ck1
<u>C. glabrata</u> NCYC 388	- 0	- 0	- 0	+ 0	n.g.	n.g.	- 0	- 0	- 0
<u>C. glabrata</u> UCSC 0	- 0	- 0	- 0	- 0	n.g.	n.g.	- 0	- 0	- 0
<u>D. vanriji</u> NCYC 577	- 0	- 0	- 0	- 0	n.g.	n.g.	- 0	- 0	- 0
<u>K. lactis</u> 161	- 0	- 0	- 0	- 0	n.g.	n.g.	- 0	- 0	- 0
<u>K. marxianus</u> NCYC 587	- 0	- 0	- 0	+/- 0	n.g.	n.g.	- 0	- 0	- 0
<u>P. membranaefaciens</u> NCYC 333	- 0	- 0	- 0	- 0	n.g.	n.g.	- 0	- 0	- 0
<u>P. subpelliculosa</u> NCYC 16	+ 0	- 0	- 0	+ 0	n.g.	n.g.	- 0	- 0	- 0
<u>S. cerevisiae</u> NCYC 1001	- 0	- 0	- 0	- 0	n.g.	n.g.	- 0	- 0	- 0
<u>S. cerevisiae</u> 347	- 0	- 0	- 0	- 0	n.g.	n.g.	- 0	- 0	- 0
<u>S. cerevisiae</u> cdc 4	- 0	- 0	- 0	- 0	n.g.	n.g.	- 0	- 0	- 0
<u>W. mrakii</u> NCYC 500	+ 0	++ 0	- 0	- 0	n.g.	n.g.	- 0	- 0	- 0
<u>W. saturnus</u> NCYC 22	+ 0	++ 0	- 0	- 0	n.g.	n.g.	- 0	- 0	- 0

Explanation of abbreviations as in Table 3.1.1

TABLE 3.1.5 VARIABILITY OF THE KILLER PHENOMENON DUE TO ENVIRONMENTAL CONDITIONS

Target Yeast	Killer Yeast Used			
	<u>D. vanriji</u> NCYC 577	<u>W. mrakii</u> NCYC 500	<u>W. saturnus</u> NCYC 22	
<u>C. albicans</u> ^a	CONDITIONS OF ASSAY			
	25°C, pH 4.5	++++ ^a	++++ ^b	- ^b
	25°C, pH 7.0	- ^a	++++ ^b	++++ ^b
	37°C, pH 4.5	++ ^a	- ^b	- ^b
	37°C, pH 7.0	n.g. ^a	++ ^b	++ ^b
<u>C. glabrata</u> ^c	CONDITIONS OF ASSAY			
	25°C, pH 4.5	+++	+++++	+++++
	25°C, pH 7.0	+++	-	-
	37°C, pH 4.5	-	-	+
	37°C, pH 7.0	-	-	-
	^a <u>C. albicans</u> UCSC 10			
	^b <u>C. albicans</u> ATCC 10201			
	^c <u>C. glabrata</u> NCYC 388			

Explanation of abbreviations as in Table 3.1.1

SECTION 3.1.2.2 VARIABILITY OF THE KILLER PHENOMENON

Table 3.1.5 illustrates the variability of the killer phenomenon due to incubation temperature and pH. This figure is a compilation of data from tables 3.1.1 and 3.1.4 inclusive.

C. albicans was sensitive to killer toxin under the 4 sets of environmental conditions tested. W. mrakii showed the broadest range of activity. D. vanriji NCYC 577 was only active against C. albicans at pH 4.5 and W. saturnus NCYC 22 was only active at neutral pH. C. glabrata was not shown to be "killed" at pH 7.0 (37°C) in this screen, but it was sensitive to killer toxin at pH 4.5 (37°C).

If one assumes there is only one toxin type secreted by each killer yeast then the pattern of toxin activity shown by D. vanriji against C. glabrata is apparently independent of pH. However, the same killer when used with C. albicans does not show evidence of pH independent toxicity. By cross-reference it can be determined if biologically active toxin is secreted. W. saturnus NCYC 22 is active against C. albicans only at the higher pH regardless of temperature. In contrast, the same killer yeast is active against C. glabrata only at pH 4.5.

This complex pattern of activity may be explained by the following: a) there is more than one toxin produced by each yeast, characterized by different optimal conditions for activity (toxin multiplicity); b) the target yeast is in a "receptive state" governed by pH, i.e., "conditional killing".

The screening technique used is very much dependent on growth and secretion of pharmacologically active proteins. With this in mind, the factors which affect activity can be listed as follows:

- (i) relative growth rate of yeasts and competition for nutrients (non-specific yeast-yeast interactions);
- (ii) temperature and pH;
- (iii) target yeast being in a "receptive state";
- (iv) quantity of toxin secreted;
- (v) number of distinct toxins secreted.

SECTION 3.1.2.3 USE OF KILLER YEAST SCREENING IN BIOTYPING C. albicans STRAINS

Table 3.1.6 illustrates the principles involved in the killer typing system of Polonelli et al. (1983). This data (derived from Table 3.1.1) allowed the differentiation of six strains of C. albicans into 4 biotypes (A-D), based on their phenotypic response to the killer yeasts under standard conditions (25°C/pH 4.5).

Killer strain selection is of prime importance in biotyping. Good "killers" of C. albicans like W. mrakii NCYC500 and D. vanriji NCYC 577 do not differentiate strains well. On the other hand, killer yeasts which are weakly active against this species differentiate strains much better. Strong "killers" may produce a typical picture which could aid in speciation of medically-important yeasts (Lehmann et al., 1987).

TABLE 3.1.6 BIOTYPING C. ALBICANS STRAINS

<u>Candida albicans</u>	KILLER YEAST STRAIN					BIOTYPE
	UCSC 0	NCYC 577	NCYC 500	NCYC 333	NCYC 22	
NCYC 854	- ¹	+ ²	+	+	+	A
ATCC 10261	-	+	+	-	-	B
CA 2	-	+	+	+	-	C
A 72	+	+	+	-	+	D
HOG 301	-	+	+	+	+	A
UCSC 10	-	+	+	+	+	A

1. RESISTANT

2. SENSITIVE

SECTION 3.1.3 SUMMARY OF SCREENING ASSAYS

Twenty strains of killer yeasts were screened for antimicrobial activity against 8 strains of C. albicans and 2 strains of C. glabrata using an improved medium for detection of killer activity. This medium - based on Sabouraud Liquid Medium (OXOID) - had excellent colour characteristics at pH 4.5 and was adequate at pH 7.0. Screening at pH 4.5 and pH 7.0 - both at 37°C - was considered physiologically relevant. Killer activity against C. albicans was demonstrated at pH 7.0 (37°C). However, activity was most abundant at pH 4.5 (25°C).

From comparative effects of different killer strains it was apparent that specific killer yeasts secrete distinct toxins. Conditional killing is proposed, such that the target yeast must be in a "receptive state". In addition, from the complex pattern of activity it is proposed that one killer yeast may secrete several distinct toxins.

Ten killer yeasts were found to be active against C. albicans (see Table 3.1.7) and 11 strains were active against C. glabrata (Table 3.1.8). W. mrakii NCYC 500 was found to have good antimicrobial activity against C. albicans, and had the broadest range of activities. W. mrakii LKB 169 was found to have moderate activity against C. albicans. In addition, Debaryomyces vanriji NCYC 577, Pichia anomala NRRL Y-2153 and Pichia anomala NRRL Y-2153-4 exhibited moderate anti-Candida albicans activity.

In view of these findings, Williopsis mrakii strains NCYC 500 and LKB 169 were selected for further investigation.

TABLE 3.1.7 KILLER YEASTS FOUND TO BE ACTIVE AGAINST
CANDIDA ALBICANS

<u>C. glabrata</u> UCSC 0	*
<u>Debaryomyces vanriji</u> NCYC 577	**
<u>P. anomala</u> NRRL Y-366	*
<u>P. anomala</u> NRRL Y-2153	**
<u>P. anomala</u> NRRL Y-2153-4	**
<u>P. membranaefaciens</u> NCYC 333	*
<u>W. mrakii</u> NCYC 500	***
<u>W. mrakii</u> LKB 169	**
<u>W. saturnus</u> NCYC 22	*
<u>W. saturnus</u> IFO 0117	*
Good Activity	***
Moderate Activity	**
Weak Activity	*

TABLE 3.1.8 KILLER YEASTS FOUND TO BE ACTIVE AGAINST
CANDIDA GLABRATA

- D. vanriji NCYC 577
- K. lactis NRRL Y-1140
- P. anomala NRRL Y-2153
- P. anomala NRRL Y-2153-4
- P. membranaefaciens NCYC 333
- S. cerevisiae c.d.c. 4
- S. cerevisiae 347
- W. mrakii NCYC 500
- W. mrakii LKB 169
- W. saturnus IFO 0117
- W. saturnus NCYC 22

SECTION 3.2 INSTABILITY OF KILLER YEAST STRAINS NCYC 500 AND LKB 169

Much of the fermentation work was carried out with W. mrakii strains NCYC 500 and LKB 169; however, optimization studies of killer toxin production (Section 3.4) were hampered by unstable toxin activity in liquid broth.

During initial studies with W. mrakii NCYC 500, a high titre of anti-C. albicans activity was readily produced in minimal medium and this was stable in fermentation broth for 72 hours. Twelve months later, toxin production from this strain was found to be negligible. Subsequent studies showed that the non-ionic detergent Brij 58 (Section 3.4.2.2) produced a transient stimulation of toxin secretion from the strain; however by the end of the studies, even this treatment was ineffective.

Following receipt of W. mrakii LKB 169, it became apparent that this yeast secreted multiple toxins (defined later as toxins KA, KG and KS; Section 3.6.5). Initial studies showed that LKB 169 was inhibitory toward C. albicans, C. glabrata and S. cerevisiae. After just one month as a laboratory maintained culture, the antimicrobial activity of LKB 169 against C. albicans and S. cerevisiae became undetectable.

Secretion of killer toxin has been shown to be growth dependent (Ohta et al., 1984; Palfree & Bussey, 1979; Sugisaki et al., 1984) and there have been no reports of toxin secretion in stationary phase. Thus it was of interest to find that the attenuated LKB 169 produced anti-C. glabrata activity during the stationary phase of growth. This strain, here called LKB 169A, was indistinguishable morphologically from the original strain LKB 169. The killer activity of this secretion mutant was compared with the original

stock culture by the streak plate assay (Table 3.2.1). In view of the strain instability found, it was fortunate that a stock of cell-free culture broth containing toxins from the original strains had been stored and this was used for experiments months later.

Table 3.2.1 Killer phenotype of a secretion mutant derived from W. mrakii LKB 169

INDICATOR STRAIN	LKB 169	LKB 169A
<u>C. albicans</u> A 72	+	-
<u>C. glabrata</u> NCYC 388	+	+
<u>S. cerevisiae</u> NCYC 1006	+	-

(+, active; -, inactive using streak plate assay, Section 3.1.1)

SECTION 3.3 DEVELOPMENT OF A QUANTITATIVE BIOASSAY

3.3.1 INTRODUCTION

Use of the agar diffusion assay for analysis of antimicrobial agents has been the subject of two important reviews (Kavanagh, 1972; Hewitt, 1977). This type of bioassay consists of nutrient agar inoculated with a uniform suspension of a test organism. The basis of the analytical technique is radial diffusion of the antimicrobial agent from a central reservoir through the agar gel. Thus there is progressive dilution of the agent which inhibits the growth of the indicator strain. After a lag period, there is sufficient biomass to absorb all the antibiotic, thus preventing further diffusion outwards. At this time the size of the inhibition zone is limited. Therefore, the agar diffusion assay is both a microbiological and a physico-chemical technique. In general the square of the width of the inhibition zone is approximately linearly related to the logarithm of the applied dose of a freely diffusible antibiotic (Mitchison & Spicer, 1949).

The most commonly employed method to quantify killer toxin is the "well test" agar diffusion bioassay used by Woods & Bevan (1968). The aim of this analytical technique is to quantify biologically active protein. In addition to cylindrical wells cut out of the agar, two other toxin reservoirs have been used in the agar diffusion bioassay: stainless steel cylinders (Ouchi et al., 1979) and absorbent discs (Kandel & Koltin, 1978; Sugisaki et al., 1984). In addition, there are many liquid broth bioassay variations. For example Bussey (1974) used one based on viable count determination and Kandel & Koltin (1978) used a critical dilution method.

Recently Spacek & Vondrejs (1986) reported the use of the fluorescent dye, rhodamine B to quantify the lethal action of S. cerevisiae toxin.

FACTORS AFFECTING THE AGAR DIFFUSION BIOSAY

Numerous factors influence the zone size and reproducibility of this analytical technique. Factors common to all agar diffusion assays are summarized in Table 3.3.1.

TABLE 3.3.1 FACTORS AFFECTING ZONE SIZE IN THE AGAR DIFFUSION ASSAY

(After Lees & Tootill, 1955)

1. CHOICE OF TEST ORGANISM: its inherent sensitivity; can also influence the sharpness of the zone boundary.
2. CONDITION OF TEST ORGANISM: phase of growth; viability and preparation of inoculum.
3. DENSITY OF SEEDING: the zone width is related inversely to the size of inoculum.
4. FORMULATION & CONDITION OF THE MEDIUM: a rich medium results in more rapid growth with consequent smaller zone sizes; water content of the medium; drying out of the agar gel at the edges of large plates may lead to inflated zone diameters; pH; autoclaving regime; pouring technique and elimination of bubbles; integrity of the wells.
5. THICKNESS OF THE AGAR MEDIUM: as thickness increase zone width decreases (in practice, this is the volume of agar per plate).
6. POTENCY OF TOXIN IN TEST SOLUTION
7. VOLUME OF THE TEST SOLUTION APPLIED TO THE PLATE: This should be large enough to act as a reservoir of constant concentration or should be a standard volume.
8. AREA OF SEEDED AGAR TO WHICH THE TEST SOLUTION IS APPLIED: a larger cylindrical well cut out of the agar naturally leads to a zone of greater diameter.
9. TIME OF APPLICATION OF THE TEST SOLUTIONS: solutions applied to the plate appreciably later than the first have less time for diffusion before the critical population is achieved, thus producing relatively smaller zones.
10. TEMPERATURE OF INCUBATION: more rapid growth results in smaller zone sizes so that uniformity of incubation temperature is of great importance.

It is appropriate to highlight a number of points in relation to killer toxin assays:

- (i) In general, killer toxins are thought to show a high degree of specificity with a narrow spectrum of action. Moreover, for a killer yeast to show activity, not only must the assay conditions be appropriate, but the choice of indicator strain is critical (Young & Yagiu, 1978; Middelbeek et al., 1980a).

- (ii) It is assumed that killer toxins, like many antibiotics, affect only actively dividing cells (Bussey, 1972; 1974; Bussey & Skipper, 1975). Therefore, it is important to avoid "phenotypic tolerance" by ensuring sensitive strains are in the log phase of active growth.

- (iii) The agar medium as used by many authors and herein contains methylene blue. This feature was introduced by Woods & Bevan (1968) and has been retained without question by subsequent workers.

- (iv) There is no standard reference material available. In common with all fundamental and elemental research with products of natural origin, the usual protocol is to set aside a quantity of purified material to be the standard reference.

This type of internal standard was used in an agar diffusion assay for killer toxin activity (Young & Yagiu, 1978).

ARBITRARY UNITS OF BIOLOGICAL ACTIVITY

When purification of the product in question remains elusive, the typical strategy has been to assign an arbitrary unit of biological activity. In killer toxin research, the approach adapted depends on whether the bioassay is

in liquid broth or agar. Bussey (1974) defined the "killing unit" (m), calculated from the survival ratio: $S/S_0 = e^{-m}$. This method is based on the viable plate count. Middlebeek et al. (1979) used a turbidimetric method in which 100 ul toxin - from a serial 2-fold dilution - was added to sensitive cells. The arbitrary unit (per 100 ul) was defined as the reciprocal of the dilution yielding 50% increase in turbidity compared to the control (100%). In a further study, using an agar diffusion assay, de la Pena et al. (1980) defined a unit of killer toxin as the amount producing a 13 mm clear zone from a 5 mm well.

In the present research, arbitrary units have not been assigned to toxin activity, since the existence of multiple toxins was a working hypothesis. Thus it was expedient to develop differential assays for this toxin mixture.

SECTION 3.3.2. DEVELOPMENT OF AGAR DIFFUSION BIOASSAYS

SECTION 3.3.2.1 BIOASSAYS TO QUANTIFY TOXIN(S) FROM W. MRAKII

The medium developed for the screening assay (see Section 2.3.1) was adopted for the bioassay. The time (48h) and temperature (25°C) of incubation were similar, as was the seeding density (5×10^4 cells/ml). Considerations such as indicator strains, pH and choice of assay plate are discussed in the next sections. The agar diffusion bioassay has been described (Section 2.5).

TABLE 3.3.2 CANDIDATES FOR INDICATOR STRAIN - ACTIVITY OF CELL-FREE
BROTH FROM WILLIOPSIS MRAKII LKB 169

INDICATOR STRAIN	pH OPTIMUM	MAXIMUM INHIBITION ZONE IN BIOASSAY (mm)
<u>C. albicans</u> A 72	4.6 \pm 0.2	17
<u>C. albicans</u> UCSC 10	4.7 \pm 0.1	16.5
<u>C. albicans</u> ATCC 10261	4.8	16.5
<u>C. albicans</u> #80 A	4.8	16
<u>C. albicans</u> CA 2	4.8	15.2
<u>C. albicans</u> # 12 A	4.6 \pm 0.2	14.25
<u>C. glabrata</u> NCYC 388	4.5 \pm 0.1	21
<u>S. cerevisiae</u> NCYC 1006	4.6 \pm 0.2	23

SECTION 3.3.2.2 SELECTION OF INDICATOR STRAIN FOR ANTI-CANDIDA ALBICANS ACTIVITY

The prime consideration in the selection of an indicator strain is its inherent sensitivity. It should be predominantly blastospore in morphology so that uniform dispersion of inoculum is possible. In view of the high specificity of killer toxin, an inappropriate choice could lead to a lack of detection of certain toxins.

Eight strains of C. albicans were considered as test organisms against crude toxin preparation from W. mrakii LKB169, and the results are shown in Table 3.3.2. Strain NCYC 854 and strain HOG 301 were unacceptable because of their predominantly mycelial morphology.

Preliminary studies in this research employed C. albicans ATCC 10261 as the indicator strain to quantify anti-Candida albicans activity in cell-free broth of killer yeasts. However, it became apparent that C. albicans strain A72 was a more sensitive organism (Table 3.3.2). In addition, it was subsequently shown that A72 had the lowest intrinsic proteolytic activity among C. albicans strains tested (Table 3.5.2). Although C. glabrata NCYC 388 and S. cerevisiae were far more sensitive to the cell-free broth of W. mrakii, it could not be assumed there was only one toxin type present.

SECTION 3.3.2.3 EFFECTS OF pH

The choice of optimal pH for activity in the bioassay is a function of both the toxin and the test organism. Unfortunately, accurate measurement of toxin activity was not possible below pH 4.4 since the agar loses its gelling properties (Young & Philliskirk, 1977). Apparent increases in activity below pH 4.4 were ignored and ascribed to facilitated diffusion of toxin (see Figure 3.6.1). Cell-free broth from either W. mrakii NCYC 500 or LKB 169 (with

production at pH 4.5) showed little activity above pH 5.0 (Figure 3.6.1). With these two points in mind, limits were set for the agar diffusion bioassay, such that it could have a pH between 4.5 and 5.0.

It seemed prudent to buffer the bioassay medium at the pH optimum for toxin activity. The optimal pH for toxin(s) from Williopsis strains occurred at pH 4.5-4.8; i.e., within the adopted pH window (Table 3.3.2).

The low pH limitation of this bioassay could be avoided in future if the agar is autoclaved separately from the medium and these are then mixed after cooling to 55°C (P.F. Lehmann, personal communication, 1986). In addition, by this method, the agar concentration can be substantially reduced.

SECTION 3.3.2.4 LARGE PLATE ASSAY

Large square assay dishes are better equipped to handle large numbers of samples than are standard plastic petri dishes. The latter have a tendency to warp (Shadomy & Espinel-Ingroff, 1984). This is important for fermentation studies, especially when data are required from a large number of samples, and processing column chromatography samples.

One disadvantage with the large plate assay was splitting of wells due to irregular drying, particularly at the corners of the plate. In the routine assay of ketoconazole with S. cerevisiae, plates can be sealed to eliminate this problem (J. R. Perfect, personal communication, 1986). However, this is not appropriate for the aerobic C. albicans. This difficulty was partially resolved by humidifying the incubator and increasing the volume of the agar. This latter modification to the bioassay reduced its sensitivity (see point 5, Table 3.3.1). A compromise was reached in which the volume of sample was increased to account for the increased depth of agar.

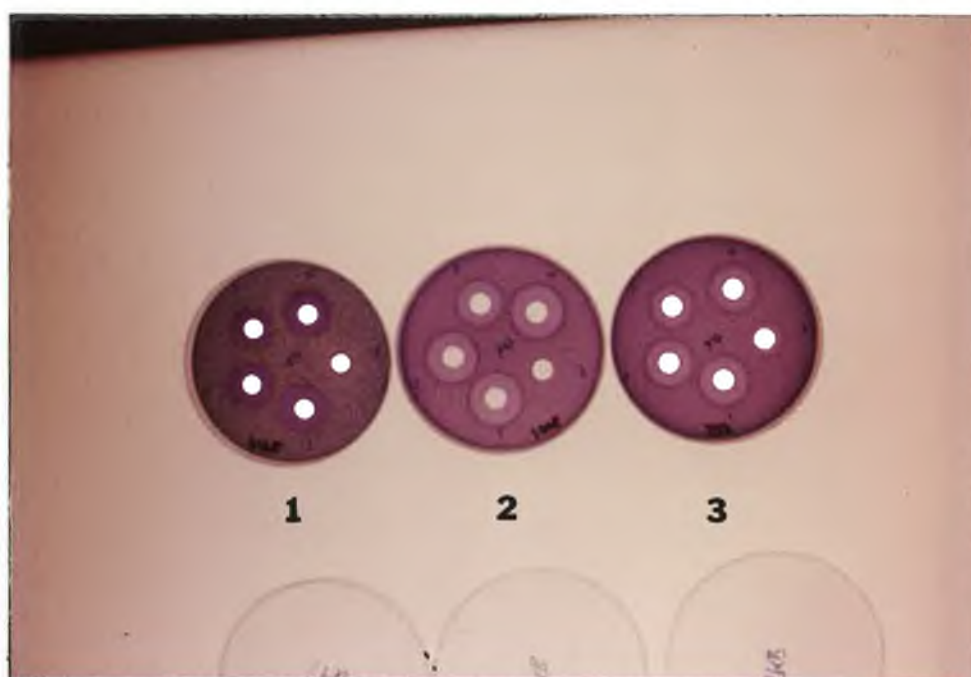


PLATE 4 TRIPLET BIOASSAY TO DIFFERENTIATE TOXINS IN A MIXTURE

Left to right (indicator strains in brackets):

1, KA toxin bioassay (C. albicans strain A72 ; 10% glycerol)

2, KS toxin bioassay (S. cerevisiae NCYC1006)

3, KG toxin bioassay (C. glabrata NCYC388).

SECTION 3.3.2.5 SELECTIVE MICROBIOLOGY

In view of the large number of samples that could be tested on mast assay plates (e.g., 36, 64 or 100 wells per plate), fermentation samples were routinely sedimented by centrifugation to avoid filter sterilization. However, this did not avoid carry over of yeasts to the assay plate. In fact, W. mrakii often formed colonies on the walls of the wells, only to secrete toxin in situ, distorting the zone of inhibition. To circumvent this problem, the medium was made selective for growth of the C. albicans indicator by the inclusion of 0.001% (w/v) cycloheximide (this concentration determined appropriate from preliminary inhibition studies). Cycloheximide is routinely used in medical mycology (Prince, 1984) and since it is heat stable it can be autoclaved with the medium without loss of activity. A similar approach using crystal violet has been adopted by Young & Philliskirk (1977) to quantify the viability of a killer yeast in the presence of a sensitive brewing strain.

Use of cycloheximide was discontinued with the introduction of syringe filters in the sampling technique to provide cell-free broth from the fermentations. This was considered appropriate when it became necessary to concurrently bioassay toxin solutions against three different test organisms.

SECTION 3.3.2.6 DIFFERENTIAL BIOASSAYS

An integral part of this thesis involves a working hypothesis of the existence of multiple toxins produced by one yeast. This supposition is based on numerous observations, summarized in Section 3.6.1. One such observation, was loss of antimicrobial activity against C. albicans, with retention of activity against C. glabrata and S. cerevisiae. This and other pieces of evidence substantiated the view that different toxins from one yeast could show target-yeast specificity, and hence, a mixture of toxins should not be analyzed with one indicator strain.

In addition to C. albicans strain A72, C. glabrata NCYC 388 and S. cerevisiae NCYC 1006 were selected as comparative strains because they were strongly sensitive to cell-free broth of W. mrakii (see Table 3.3.2). A triplet bioassay with these dissimilar test organisms is depicted in Plate 4.

SECTION 3.3.2.7 OPTIMIZATION OF BIOASSAY

(a) GLYCEROL - to retard growth of C. albicans

15% glycerol has been found to improve the sharpness of zone boundaries in an agar diffusion assay seeded with C. albicans (Lehmann et al., 1987). In the present study, the performance of C. albicans, A 72 as an indicator strain was greatly improved by inclusion of 10% (v/v) glycerol (see Figure 3.3.1). It is thought that the effect is due to growth retardation, possibly secondary to limitation of oxygen availability. Glycerol may also facilitate toxin diffusion or binding to C. albicans. Glycerol can both potentiate (Sugisaki et al., 1984) and interfere with toxin activity from K. lactis (P. F. Lehmann, pers. commun., 1987). Ouchi et al. (1978) included 10% glycerol in an agar diffusion bioassay to stabilize a S. cerevisiae toxin.

(b) HEAT SHOCK - to stimulate growth of S. cerevisiae

S. cerevisiae NCYC 1006 grew slowly in the modified Sabouraud medium which meant a three-day incubation was required before zone diameters could be measured. The performance of S. cerevisiae as an indicator strain in the new medium, was greatly improved by the following heat shock treatment: yeast in log phase of growth was shifted from 25°C to 37°C for two hours, just prior to seeding. The basis of this innovation was a report by McAlister & Finkelstein (1980) which showed that heat shock treatment confer thermal

resistance in yeast. This is an important consideration in view of the fact that seeding takes place between 45-55°C.

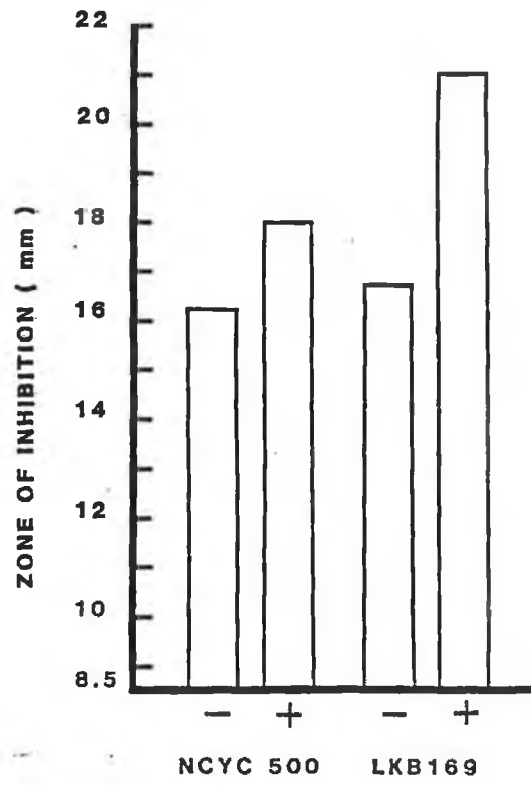


Fig 3.3.1 STIMULATION OF ZONE SIZE BY 10% GLYCEROL IN THE KA BIOASSAY

SECTION 3.3.3 INVESTIGATION INTO A LIQUID ASSAY SYSTEM

Development of a broth assay system for killer toxin action is desirable to facilitate both viability studies and receptor competition studies (Hutchins & Bussey, 1983). Attempts in both YEPD and Sabouraud Liquid broth (pH 4.5) were unsuccessful. In particular, cell-free broth from W. mrakii NCYC 500 did not inhibit growth of C. albicans ATCC 10261 (early log phase), although it was very effective in agar medium. Excess toxin was used - as determined by the agar diffusion bioassay. Further study showed the anti-C. albicans activity to be stable at 25°C in Sabouraud Liquid Medium (pH 4.5).

Different morphological forms of C. albicans in the broth and in the agar may explain some of the differences seen for toxin sensitivity in the two systems. In liquid broth, C. albicans ATCC 10261 grew as a budding yeast (blastospore). In agar, it was a mixture of blastospores and pseudomycelium with occasional chlamydospores.

In retrospect, the main difference between the two systems was the presence of methylene blue in agar and its absence in broth. Bilinski et al. (1985) found that the presence of methylene blue in their culture medium was a prerequisite for antibacterial activity by two yeast species. They proposed that methylene blue may be converted into a pharmacologically-active form by the yeast. It is possible that methylene blue is also pharmacologically active against C. albicans.

Kandel & Stern (1979) failed to find killer yeasts active against C. albicans, in their screening program without methylene blue. In contrast, Middelbeek et al. (1980a) reported a high incidence of sensitivity of C. albicans in the presence of methylene blue. It is possible that this is

methylene blue-dependent "killing" and this apparently innocuous blue dye is pharmacologically active.

SECTION 3.3.4 SUMMARY OF SECTION 3.3

A triplet bioassay system was proposed which utilizes three dissimilar test organisms to differentiate toxins in cell-free broth from Williopsis mrakii (see Section 2.5.2). C. albicans strain A72 was selected to serve as indicator, to quantify anti-Candida albicans activity in a bioassay medium containing 10% (v/v) glycerol to retard growth of this test organism. Antimicrobial activities against C. glabrata and S. cerevisiae were quantified with strains NCYC 388 and NCYC 1006 respectively. The performance of S. cerevisiae NCYC 1006 as an indicator strain was greatly improved by a heat shock treatment.

Cycloheximide (0.001%; w/v) inhibited growth of W. mrakii, making the bioassay medium selective for growth of C. albicans; however, it excluded use of C. glabrata NCYC 388 and S. cerevisiae NCYC 1006 as test organisms. When the aim is to quantify antimicrobial activity against C. albicans, a methodology which includes cycloheximide, avoids the need to filter-sterilize fermentation broth, since it circumvents the problem of yeast carry-over.

In contrast to assay in agar, assay for toxins in broth disclosed no detectable anti-C. albicans activity from W. mrakii.

SECTION 3.4 STUDIES AIMED AT OPTIMIZING THE PRODUCTION OF YEAST KILLER TOXIN

SECTION 3.4.1 INTRODUCTION

PRODUCTION OF KILLER TOXIN BY FERMENTATION

Optimization of killer toxin production from W. mrakii, was a major objective of this study (see Section 1.5). Killer toxin protein is known to be secreted during the exponential phase of growth (Young & Philliskirk, 1977; Ouchi et al., 1978; Palfree & Bussey, 1979). It was confirmed that toxin secretion from W. mrakii NCYC 500 was also growth-linked (see Figure 3.4.7). Two principal tactics to improve production of a microbial metabolite have been outlined by Grafe (1982):

- (i) increase biomass concentration, and
- (ii) increase cellular productivity by manipulating control mechanisms of biosynthetic pathways.

For instance the growth-associated secretion of proteins from microbes may be enhanced by improving ammonia assimilation. Three types of culture media were examined in this study for the production of killer toxin: (i) rich and undefined, (ii) chemically defined, and (iii) synthetic medium supplemented with ultra-filtered yeast-extract and peptone (UYEP).

RICH CULTURE MEDIUM

Complete medium like YEPD (Yeast Extract, Peptone, Dextrose) is reliable for toxin production. First reported by Woods & Bevan (1968), it is well documented that yeast extract stimulates production by 3-4 fold (Young & Philliskirk, 1977; Middelbeek, et al., 1979; Palfree & Bussey, 1979). Media which contains peptones can cause problems in purification stages because of the ability of toxin to bind macromolecules (Middelbeek et al., 1979; Palfree & Bussey, 1979). Many workers have added excess glucose (final concentration

50g/L) to the culture medium as a strategy to promote biomass (Ohta et al., 1984; Pfeiffer & Radler, 1972).

MINIMAL DEFINED MEDIUM

W. mrakii has simple nutritional requirements; growing in the absence of added vitamins (Barnett et al., 1983). Production of toxin in minimal medium would yield a very high initial specific activity. However, the major problems encountered with chemically-defined media are the low toxin yield and toxin instability (Woods & Bevan, 1968). Attempts to overcome these difficulties have included the use of stimulants of toxin production (Table 3.4.1 and stabilizers in the fermentation and downstream processing (Table 3.4.2)). Ouchi et al. (1978) established the use of glycerol as a stabilizer of killer toxin, though Middelbeek et al. (1979) have reported the non-ionic detergents to be superior.

MINIMAL MEDIUM SUPPLEMENTED WITH UYEP

Bussey & Skipper (1975) described the use of yeast extract and peptone which was pre-filtered through an ultra-filtration membrane with a 30,000 molecular weight cut-off. The product could be recovered in one step by concentrating the cell-free broth on the same membrane. Consequently the retentate would contain no extraneous macromolecules with an apparent molecular weight greater than 30,000 and this strategy produces a product with a high initial specific activity, even though a rich medium was used. The use of this pretreated growth medium is a good attempt to integrate fermentation with downstream processing, since it reduces the number of steps required and increase the throughput on the ultra-filtration membranes.

TABLE 3.4.1 ITEMS REPORTED TO STIMULATE TOXIN PRODUCTION

STIMULANT	REFERENCE
AMMONIUM SULPHATE	Sugisaki <i>et al.</i> , 1984
BRIJ 58	Middelbeek, Hermans & Stumm, 1979; this study
GELATIN	Middelbeek, Hermans & Stumm, 1979; this study
SODIUM DODECYL SULPHATE	Middelbeek, Hermans & Stumm, 1979
TRITON X100	Middelbeek, Hermans & Stumm, 1979; this study
TWEEN 80	Middelbeek, Hermans & Stumm, 1979; this study
YEAST EXTRACT	Woods & Bevan, 1968; Young & Philliskirk, 1977; Middelbeek, Hermans & Stumm, 1979
MAGNESIUM SULPHATE	This study

TABLE 3.4.2 STABILIZATION OF KILLER TOXIN

STABILIZER	REFERENCE
AMMONIUM SULPHATE	Ouchi <i>et al.</i> , 1978; Palfree & Bussey, 1979
BRIJ 58	Middelbeek, Hermans & Stumm, 1979
BOVINE SERUM ALBUMIN	Young & Philliskirk, 1977
ERYTHRITOL	Ouchi <i>et al.</i> , 1978
GELATIN	Woods & Bevan, 1968; Young & Philliskirk, 1977
GLYCEROL	Ouchi <i>et al.</i> , 1978
INOSITOL	Ouchi <i>et al.</i> , 1978
MANNITOL	Ouchi <i>et al.</i> , 1978
POLYETHYLENE GLYCOL 7000	Kotani <i>et al.</i> , 1977
POLYVINYL ALCOHOL 22000	Kotani <i>et al.</i> , 1977
PROPYLENE GLYCOL	Ouchi <i>et al.</i> , 1978
SUCROSE	Ouchi <i>et al.</i> , 1978
YEAST EXTRACT/PEPTONE	Middelbeek, <i>et al.</i> , 1980

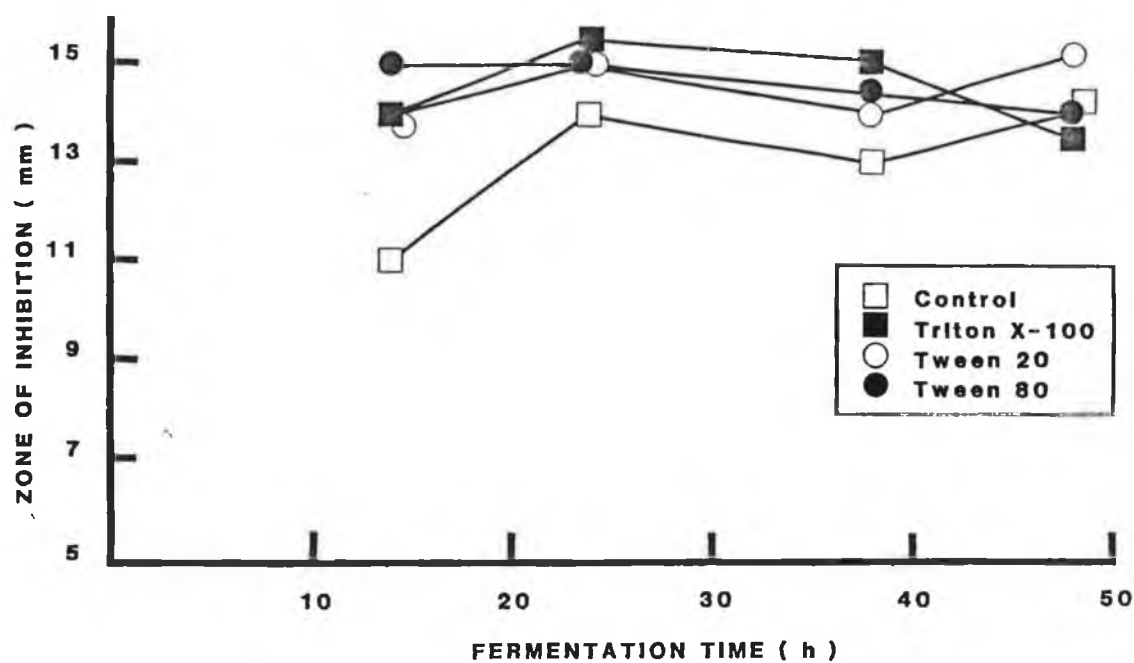


Fig 3.4.1 STIMULATION OF ANTI-*C. albicans* TOXIN PRODUCTION FROM *W. mrakii* NCYC 500 IN MINIMAL MEDIUM BY NON-IONIC SURFACTANTS(0.05%,w/v)

SECTION 3.4.2 THE ROLE OF SURFACTANTS IN FERMENTATION

SECTION 3.4.2.1 THE EFFECT OF BRIJ 58 ON TOXIN PRODUCTION

Middelbeek et al. (1979) determined the optimum concentration of the non-ionic detergent, Brij 58 for killer toxin production in minimal medium to be 0.001% (w/v). It was suggested that the enhancing effect on toxin titre was due to stabilization of the killer toxin produced during growth (Middelbeek et al., 1979). However, results from the present study do not support this explanation.

For example, Figure 3.4.2 shows the effect of Brij 58 on the growth and toxin secretion from an attenuated W. mrakii strain. Brij 58 stimulated the growth of W. mrakii NCYC 500. The detergent did not alter the maximal cell density in minimal medium but was shown to have a stimulating effect on biomass production; a cellular dry weight of 8.19 mg/ml was achieved at 48 hours as opposed to a control value of 2.69 mg/ml.

In addition Brij 58 produced a net secretion of protein compared to the culture without this surfactant. The difference in toxin production was a striking feature: only the Brij 58 supplemented culture produced detectable toxin, but this was transitory in nature. Brij 58 was a powerful stimulant of toxin secretion at low concentration and its role in potentiating the toxin titre from an attenuated strain is clearly demonstrated in Figure 3.4.2. No evidence of toxin stabilization by Brij 58 was seen.

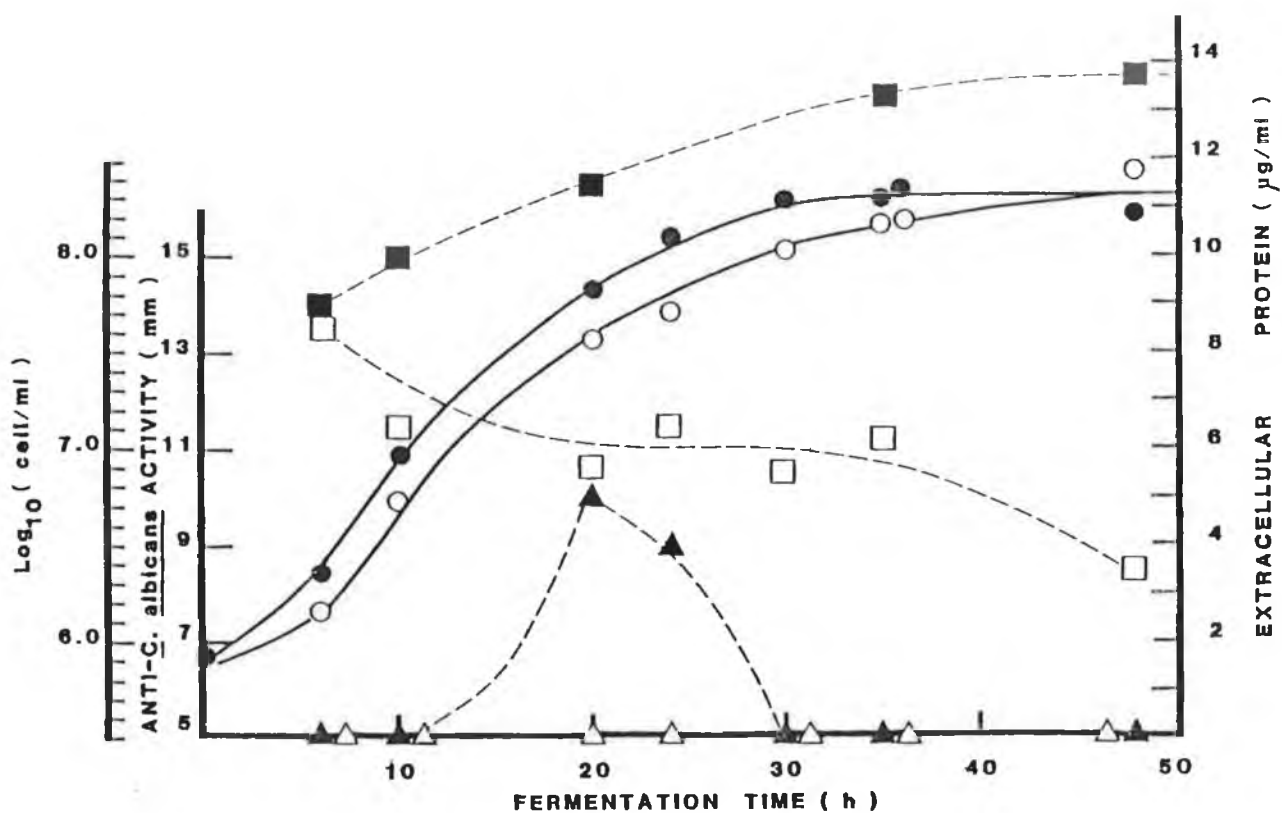


Fig 3.4.2 Effect of Brij 58 on toxin production by *W. mrakii*

W. mrakii NCYC 500 was grown in minimal medium with & without Brij 58 (0.001%, w/v) -- open & closed symbols respectively.

GROWTH, ○ ● ; EXTRACELLULAR PROTEIN, □ ■ ; ANTI-*C. albicans* ACTIVITY, △ ▲ .

The molecular basis of the stimulation of protein secretion by non-ionic detergents is not only due to their surfactant nature but may also result from an increase in the unsaturated fatty acid content of the membrane lipids (Jacques et al., 1985). Thus in this W. mrakii fermentation, the fatty acid moiety of Brij 58 may have been used as a lipid nutrient and incorporated into the lipids of the yeast membrane. This may explain the higher dry-weight per cell and also the faster specific growth rate, since a more fluid plasma membrane would promote nutrient absorption and similarly promote protein secretion. The detergent effect of Brij 58 which dissociates bound toxin is an important factor in enhancing toxin titre.

A significant difference was found between the effect of Brij 58 which had been filter-sterilized compared to that which was autoclaved with the medium. The autoclaved Brij 58 (0.001%, w/v) suppressed the maximal attained cell density in a culture of W. mrakii (data not shown). This was not a problem when filter-sterilized Brij 58 was supplemented to the autoclaved culture broth (see Figure 3.4.6).

Referring to the working hypothesis of toxin multiplicity, an important facet of analytical microbiology becomes evident; that of "experimental blindness" when an inappropriate indicator strain is used in the bioassay. If different toxins show a high degree of antimicrobial specificity, then one indicator strain may not be enough to detect all activity. For example, in Figure 3.4.2 with a net secretion of protein in the presence of Brij 58, activity against C. albicans was transient. It could not be assumed that this was also true for antimicrobial activity against other yeasts.

For this reason, the methodology was revised so that in future analyses of toxin, two or three test organisms would be used (Section 3.3.2.7).

SECTION 3.4.2.2 THE EFFECT OF GLYCEROL ON TOXIN PRODUCTION

Since Brij 58 was not found to stabilize the toxins from W. mrakii, the stabilizing action of glycerol (Wiseman, 1973; Ouchi et al., 1978) was investigated in fermentation. The objective was to determine the optimal concentration of glycerol which could be used in the fermentation but which would not suppress growth. This was considered important since toxin secretion is growth-associated (Palfree & Bussey, 1979). Figure 3.4.3 shows the effect of increasing concentrations of glycerol on the growth of W. mrakii in UYEPD medium (0.001% BRIJ 58), and Table 3.4.3 shows the corresponding toxin titres after 24, 48 and 72 hours fermentation. At glycerol concentrations above 5% (v/v), growth and toxin production were adversely affected. This growth suppression is possibly due to reduction in mass transfer of oxygen (Grafe, 1982).

The addition of glycerol at a concentration of 5% (v/v) in the presence of 0.001% Brij 58, did not adversely affect growth at 25°C (Figure 3.4.3) or toxin production (Table 3.4.3).

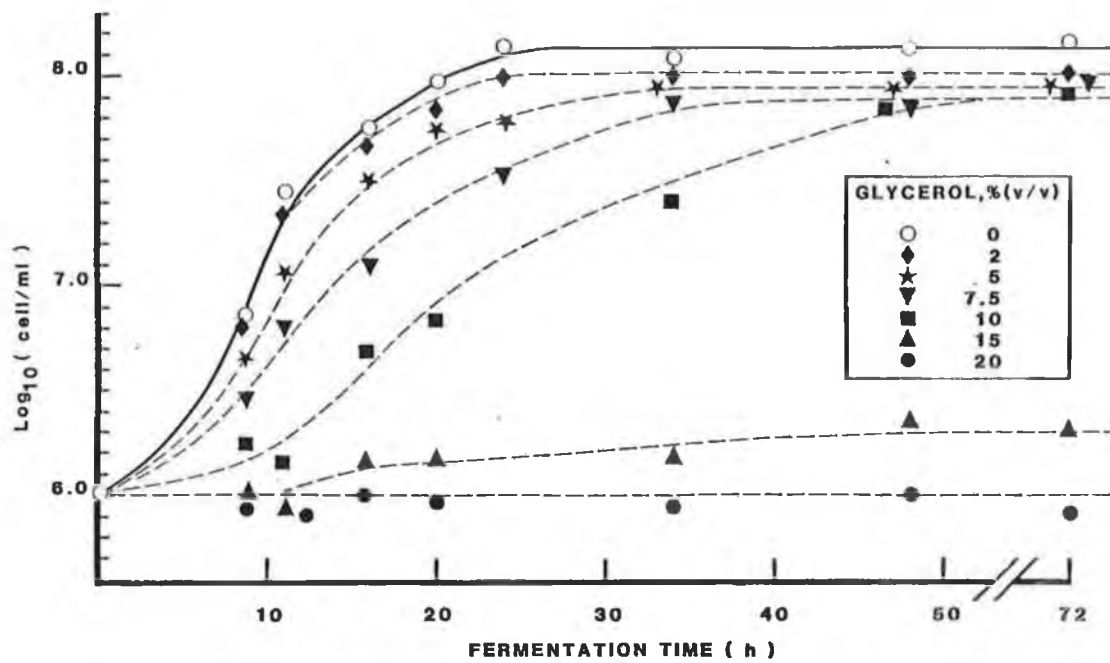


Fig 3.4.3 Effect of glycerol on growth of *W. mrakii* LKB169 in UYEPD medium containing Brij 58 (0.001%,w/v)

TABLE 3.4.3 EFFECT OF GLYCEROL ON TOXIN SECRETION FROM W. MRAKII LKB 169

GLYCEROL % (v/v)	RELATIVE Anti- <u>C. glabrata</u> ACTIVITY (mm)		
	24 h	48h	72 h
0	(10)*	12	13.5
2	(11)	11.5	13
5	(10)	12	13.5
7.5	---	(10)	11
10	---	13.5	---
15	---	---	---
20	---	---	---

UYEPD, pH 4.0 (2% GLUCOSE), 0.001% (w/v) Brij 58; 0.02% Mg SO₄

* PARENTHESIS INDICATE ILL-DEFINED ZONES OF INHIBITION IN THE AGAR DIFFUSION ASSAY

TABLE 3.4.4 THE EFFECT OF CULTURE MEDIUM ON GROWTH AND TOXIN PRODUCTION BY W. MRAKII NCYC 500

CULTURE MEDIUM	TWEEN 80 % (w/v)	GROWTH AT 24H (cells/ml)	ANTI- <u>C. ALBICANS</u> * ACTIVITY (mm)	ANTI- <u>S. CEREVISIAE</u> ** ACTIVITY (mm)
UYEPD	0	1.010x10 ⁸	17	23
	0.05	1.227x10 ⁸	18	23
	0.1	1.320x10 ⁸	19	23
MINIMAL MEDIUM	0	1.600x10 ⁷	--	--
	0.005	2.410x10 ⁷	8	16
	0.01	2.760x10 ⁷	9	16
SABOURAUD LIQUID MEDIUM	0	7.500x10 ⁶	--	10
	0.05	8.900x10 ⁶	--	11.5
	0.1	9.500x10 ⁶	--	12.5

Each culture medium (pH 4.5, 2% glucose) was inoculated with 8.9×10^4 cells/ml and incubated at 25°C for 24h.

Bioassay test strains (at 24h): *C. albicans ATCC 10261; ** S. cerevisiae NCYC 1006.

SECTION 3.4.3 NUTRITIONAL REQUIREMENTS FOR TOXIN PRODUCTION

Table 3.4.4 shows how the quality of growth medium affects toxin production and the ability of a non-ionic detergent to stimulate toxin production in a poor medium. The results of this aerobic shake-flask experiment are shown for a single sample point 24 hours after inoculation. There was no significant difference in lag phase between the cultures and although only the UYEPD culture had reached early stationary phase, the relative values remained the same at 48 hours. A direct cell count was taken as an index of biomass accumulation.

Williopsis mrakii grown in the poorest growth medium (SLM) produced the least toxin; in the richest medium (UYEPD), it produced most toxin. Since toxin production is growth-linked, there was a good correlation between biomass accumulation and toxin titre. With SLM, in the absence of TWEEN 80, W. mrakii NCYC 500 produced antimicrobial activity against S. cerevisiae at a very low cell density. This contrasted with the lack of toxin when it was grown in minimal medium. Undefined media like Sabouraud and UYEPD may contain stimulants of toxin secretion.

Addition of TWEEN 80 led to a dose-dependent increase in the growth of W. mrakii with a concomitant rise in toxin titre. This effect was least marked in UYEPD, possibly because yeast extract is a powerful stimulant of production (Middelbeek et al., 1979); however, the effect of TWEEN 80 is still evident in its potentiation of anti-C. albicans activity.

In general, S. cerevisiae was more sensitive than C. albicans to cell-free culture broth of W. mrakii (Figure 3.7.1). However, changes in the relative

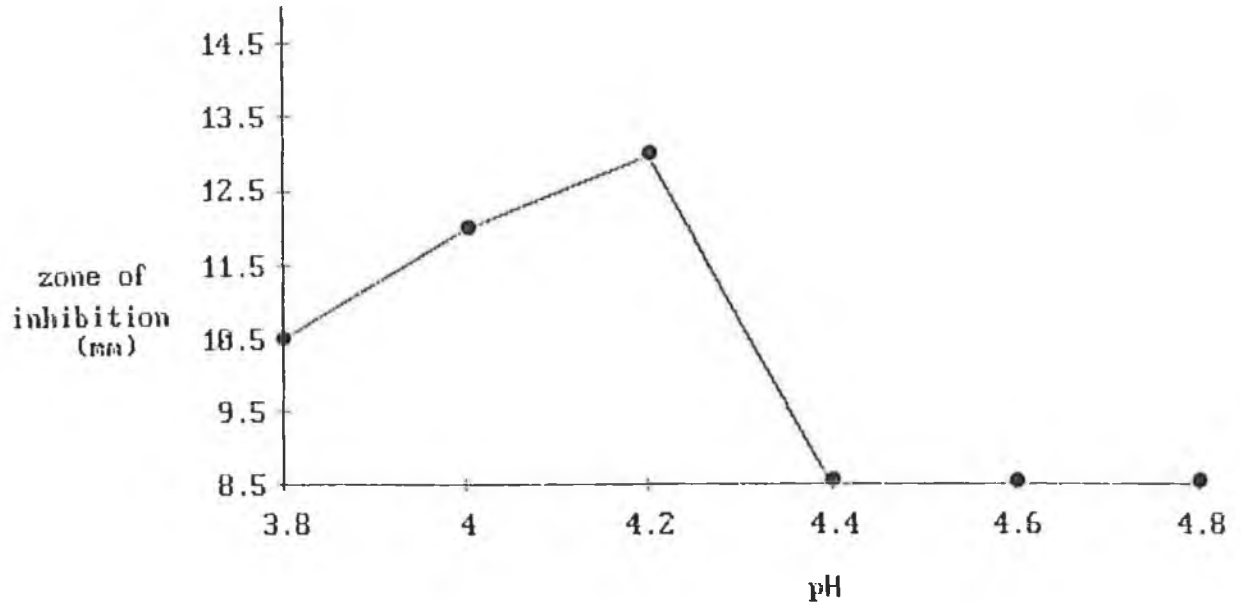


Figure 3.4.4 EFFECT OF pH ON PRODUCTION OF TOXIN FROM *W.mrakii* LKB169A*

Activity against *C. glabrata* NCYC 388 was bioassayed after 36 hours growth in UYEPD medium (25 C).

*Attenuated strain ; identified as a secretion mutant.

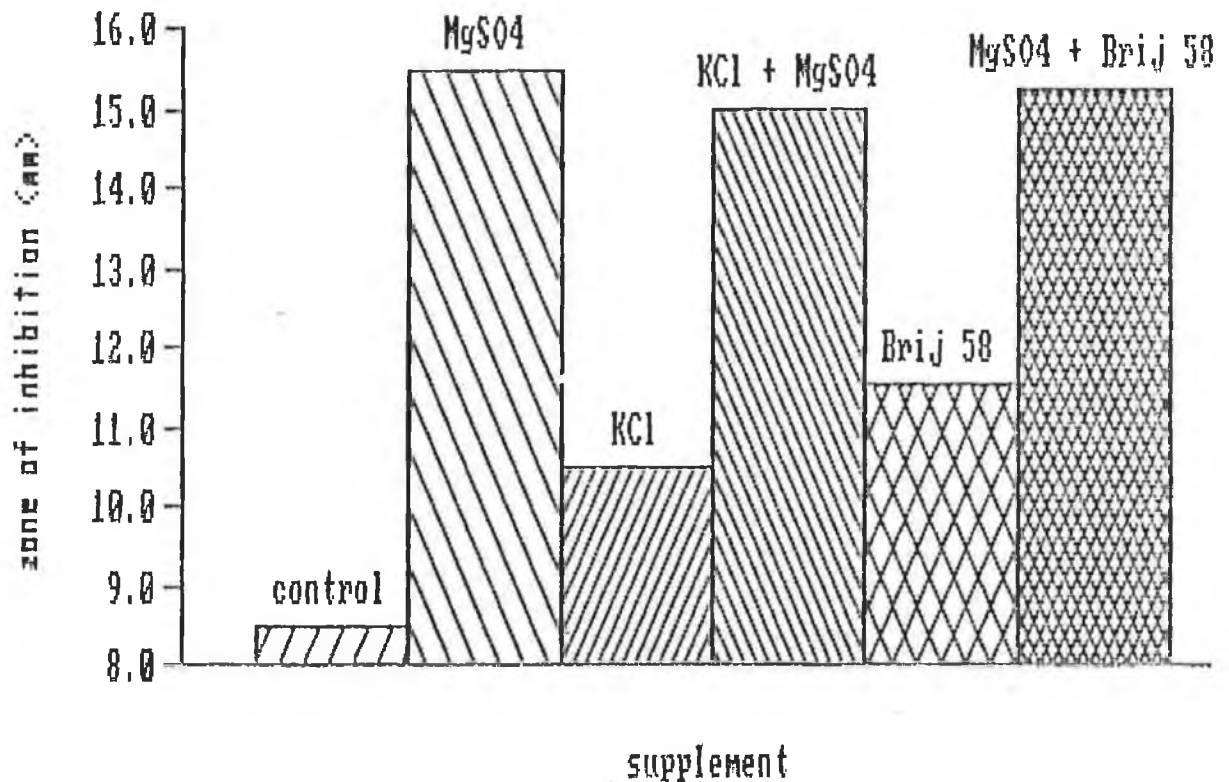


Figure 3.4.5 Stimulation of toxin production from *W.mrakii* LKB 169A in UYEPD medium (pH 4.5)

Nutrients were supplemented at 0.02%(w/v);

Brij 58 was supplemented at 0.001%(w/v).

sensitivity of these two yeasts to toxin produced in the UYEPD culture is noteworthy: there was a quantitative increase in activity against C. albicans with increasing dose of TWEEN 80, while the anti-S. cerevisiae activity remained constant. This variance in activity may reflect the existence of two separate toxins in culture broth of W. mrakii NCYC 500.

Optimization studies of toxin production were not able to be completed due to strain instability (Section 3.2), for the ability of W. mrakii LKB 169 to produce toxin declined within a month of study. The following experiment established that the yeast strain in use had altered properties.

The initial work with W. mrakii LKB 169 showed that abundant toxin was produced in UYEPD medium (pH 4.5) after 24 hours (25°C). Cell-free culture broth was active against C. albicans, C. glabrata and S. cerevisiae. Three months later, using the same conditions W. mrakii produced no toxin activity against C. albicans. Figure 3.4.4 shows a pH-production profile with the strain in use. Antimicrobial activity against C. glabrata was only produced below pH 4.4 and no activity was detected against C. albicans or S. cerevisiae. The nature of this strain, designated LKB 169A was elucidated (see Section 3.4.4).

An attempt was made to stimulate toxin production at pH 4.5 in this attenuated strain (arrow in Figure 3.4.4). Salts of the major intracellular cations, K^+ and Mg^{++} had a positive effect. $MgSO_4$ potentiated toxin production to a greater extent than the non-ionic detergent Brij 58 (see Figure 3.4.5). At 24 hours, the stimulant effect of Brij58 was seen only in the absence of additional magnesium sulphate. Stimulation of toxin secretion by magnesium

sulphate may be secondary to improved growth rate and biomass accumulation. Likewise, although potassium chloride produced a moderate stimulation in toxin titre, a combination of the potassium and magnesium salts did not surpass the effect of MgSO_4 alone.

Two groups of workers have used 0.05% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in their toxin production medium (Palfree & Bussey, 1979; Ohta et al., 1984). The protease induction medium of MacDonald & Odds (1980) used in this study, also contained 0.05% magnesium sulphate (see Section 3.6). The toxin production medium of Palfree & Bussey (1979) was a combination of a minimal salts medium with UYEP. The magnesium dependence of W. mrakii may not be seen in normal YEPD medium (containing 1% yeast extract and 2% peptone) but it is an important factor in UYEPD alone (0.5% yeast extract and 0.5% peptone); in later studies 0.02% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was routinely added as a supplement.

Inclusion of KCl in the toxin production medium may not be critical. It may, however, be beneficial to substitute the sodium buffer salt (Na_2HPO_4) with the potassium equivalent. It is probable that the toxin production medium could be further improved by inclusion of 2% ammonium sulphate to stimulate biomass. Improvement of ammonium assimilation can stimulate protein synthesis by promoting glutamic acid availability as a basic intermediate of the amino acid precursors (Grafe, 1982). Ammonium sulphate is possibly the best nitrogen source for killer toxin production; not only has it been claimed to induce a 5-fold increase in yield (Sugisaki et al., 1984), but high concentrations were found to be non-destructive (Palfree & Bussey, 1979) and can even stabilize toxin (Ouchi et al., 1978).

SECTION 3.4.4. PRODUCTION OF TOXIN FROM W. MRAKII LKB 169ASECTION 3.4.4.1 LKB 169A - A Secretion Mutant Derived From W. mrakii LKB169

Toxin secretion from W. mrakii LKB 169A was examined by differential bioassays (see Section 3.3.2.7) in UYEPD medium supplemented with 0.02% (w/v) $MgSO_4 \cdot 7H_2O$. From the kinetics of toxin production (Figure 3.4.6), it was established that strain LKB 169A was a secretion mutant. Three differences were found between strain LKB 169A and the parent strain, W. mrakii LKB 169:

- (i) antimicrobial activity against C. albicans and S. cerevisiae could not be detected;
- (ii) LKB 169A had antimicrobial activity against C. glabrata but the toxin titre was depressed;
- (iii) the anti-C. glabrata toxin from LKB 169A appeared in late log phase and the titre increased gradually during stationary phase.

Secretion of killer toxin is thought to be growth associated and confined to the exponential phase of growth (Young & Philliskirk, 1977; Ouchi et al., 1978; Palfree & Bussey, 1979). There have been no reports of yeast killer toxin produced in stationary phase. Strain LKB 169A may have a defective secretion mechanism. This would explain the reduced toxin titre produced by this attenuated strain. The toxin which gradually appears during stationary phase may be released by a non-specific mechanism. This efflux was facilitated by the detergent action of Brij 58 or glycerol (Figure 3.4.6).

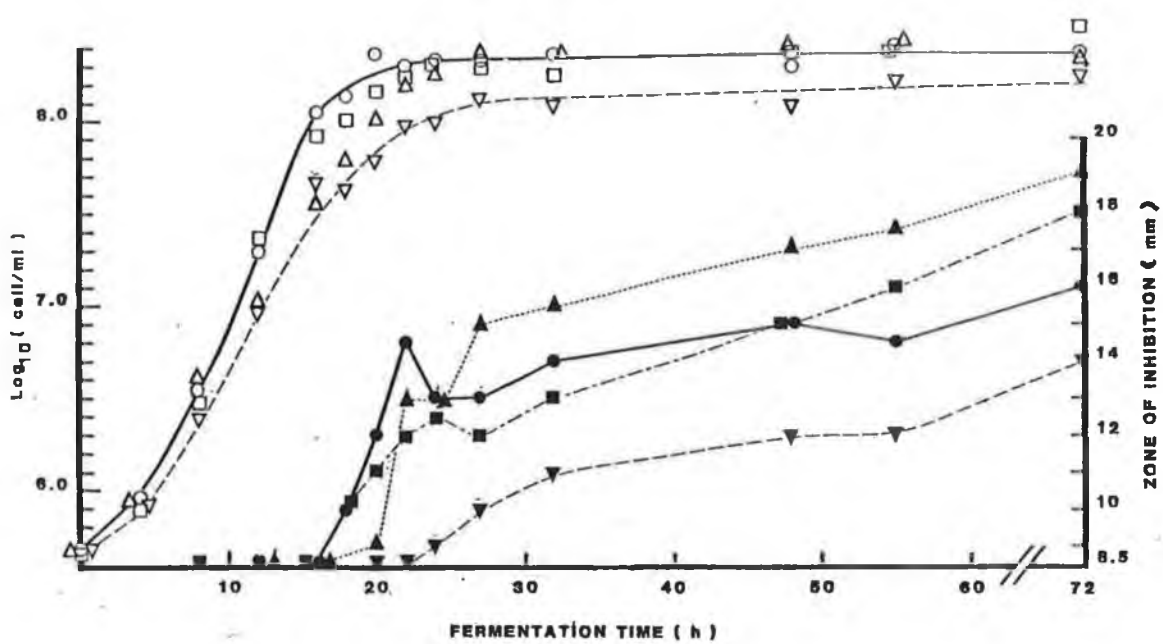


Fig 3.4.6 Production of KG Toxin from *W. mراكلي* LKB169A
In UYEPD broth (pH 4.3) with 0.02%(w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

	Growth	KG Toxin
Control	○	●
Brij 58 ,0.001%(w/v)	□	■
Glycerol ,5%(v/v)	△	▲
Brij 58 + Glycerol	▽	◆

SECTION 3.4.4.2 TOXIN MULTIPLICITY IN W. MRAKII LKB 169

Qualitative differences in activity between the parent strain of W. mrakii and the secretion mutant support a hypothesis of toxin multiplicity. The kinetics of toxin secretion was not recorded for the wild type strain LKB 169 but it was established that at least three distinct toxins are secreted (Section 3.6.5). The anti-C. glabrata toxin, designated KG toxin (Section 3.6.5), may be a major protein component secreted by W. mrakii LKB 169. Toxins active against C. albicans or S. cerevisiae (KA and KS toxins respectively) may possibly be minor extracellular proteins. It is possible that in the secretion mutant LKB 169A, these latter toxins accumulate intracellularly, and a diminished release of such minor proteins would make detection extremely difficult. This hypothesis could be tested by analysis of intracellular toxin in cell-free extracts (Pfeiffer & Radler, 1982).

SECTION 3.4.4.3 THE EFFECT OF A STIMULANT (BRIJ 58) WITH A STABILIZER (GLYCEROL) ON PRODUCTION OF TOXIN FROM W. MRAKII LKB 169A

The effect of both Brij 58 and glycerol on toxin production from W. mrakii LKB 169A in UYEPD (0.02% $MgSO_4$) medium was examined. Figure 3.4.6 depicts the kinetics of KG toxin secretion by W. mrakii LKB 169A. The toxin titre of the control culture rose rapidly during late exponential phase (18-22h) and following a slight drop in titre at true stationary phase (24h), the KG toxin activity gradually rose over the next 48 hours. This would be considered an abnormal secretion pattern for killer toxins.

Brij 58 had no effect on growth of this yeast, and a stimulant effect on toxin production was only evident in late stationary phase (48-72h, Figure

3.4.6). This non-ionic detergent was discussed earlier. Glycerol (5%, v/v) potentiated toxin production in stationary phase also and was discussed earlier.

It was hoped that a combination of a stimulant and a stabilizer of toxin might have a synergistic effect on toxin production. However, the combination of Brij 58 (0.001%) and glycerol (5%) appeared to have a toxic effect (Figure 3.4.6). A small reduction in growth was accompanied by a large depression in toxin titre. Therefore, such a combination cannot be recommended unless the working concentrations are appropriately reduced.

Figure 3.4.6 shows that glycerol was the additive of choice for the toxin production medium. This effect must be predominantly due to stabilization of toxin, since glycerol is a very weak surfactant. In the absence of Brij 58, the optimum concentration of glycerol for toxin stabilization in fermentation is undoubtedly much higher than 5%.

SECTION 3.4.5 MULTIPLICITY OF TOXINS FROM W. MRAKII NCYC 500

A second strain of W. mrakii was investigated. Production of killer toxin from W. mrakii NCYC 500 was assayed using three test organisms (Figure 3.4.7). By comparing the magnitude of toxin activities at 24 hours from Figure 3.4.7 with those found in earlier work (Figure 3.6.1), it can be seen that this W. mrakii was also an attenuated strain. In particular, its ability to produce KA¹ activity was much reduced (strain instability is discussed in Section 3.2).

Toxin production from W. mrakii NCYC 500 was growth-linked; no activity was detected before 18 hours of fermentation and net accumulation of

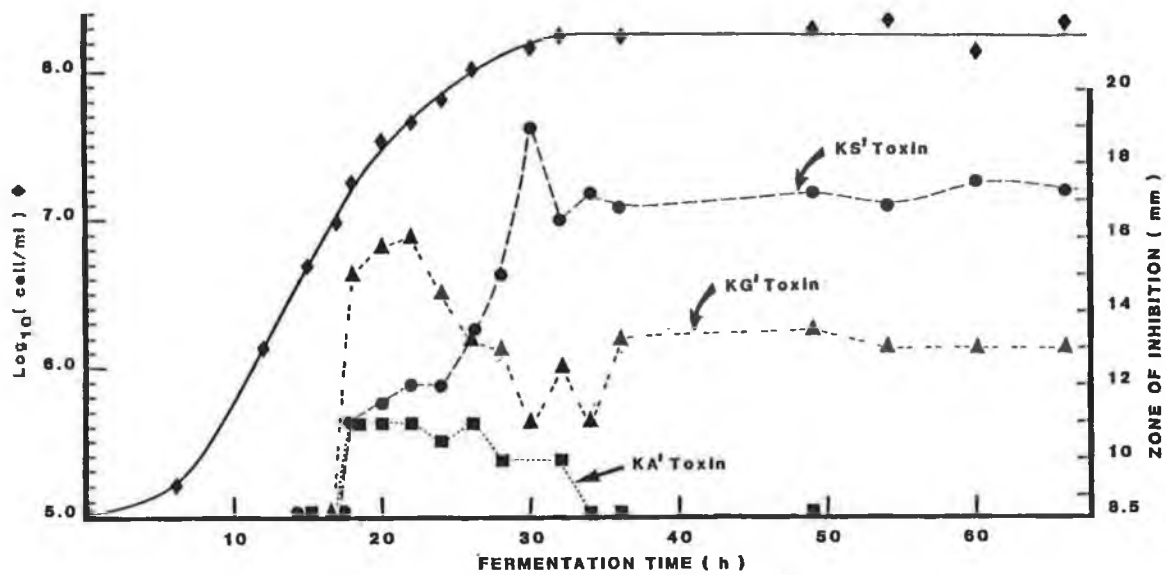


Fig 3.4.7 Multiple toxin production by *W. mrakii* NCYC 500

toxin ceased with onset of stationary phase (Figure 3.4.7, 32h). The dynamic changes in activity profile may reflect production of multiple toxins or toxin interconversion. Notably the order of potency at 24 hours ($\underline{KG}^1 > \underline{KS}^1 > \underline{KA}^1$) was reversed at 48 hours with the loss of anti-C. albicans activity ($\underline{KS}^1 > \underline{KG}^1$).

From 22 hours onwards (Figure 3.4.7), the \underline{KG}^1 and \underline{KS}^1 titres appear to be mirror images of each other. In particular, the rate of decline of the \underline{KG}^1 toxin activity was matched by the rate of increase of \underline{KS}^1 activity (22-30h). Subsequently these activities remained stable in stationary phase. This is suggestive of toxin interconversion, possibly mediated by a cell surface protease.

Figure 3.4.7 shows the evidence for two or three distinct killer toxins secreted by W. mrakii NCYC 500. A two-toxin hypothesis could explain the secretion pattern solely if one toxin was active against both C. albicans and C. glabrata, and the second toxin had anti-S. cerevisiae activity. This cannot adequately explain the early loss of anti-C. albicans activity. Therefore a three-toxin hypothesis (\underline{KA}^1 , \underline{KG}^1 and \underline{KS}^1) is proposed. The \underline{KA}^1 toxin is active against both C. albicans and C. glabrata; \underline{KG}^1 toxin is active against C. glabrata, and the \underline{KS}^1 toxin is active against S. cerevisiae. The fall in activity against C. glabrata (Figure 3.4.7, 22-34h) would be due to loss of \underline{KA}^1 toxin which was unstable. Thus, there is evidence for the accumulative action of two distinct toxins (\underline{KA}^1 and \underline{KG}^1) against one yeast (C. glabrata NCYC 388).

In conclusion, a three-toxin hypothesis is favored. The three toxins (KA¹, KG¹, and KS¹) can act independently or in an additive fashion. Also, in analogy to the bacteriocin, nisin (Rayman & Hurst, 1984), the killer toxins from W. mrakii could be a group of closely related polypeptide toxins whose relative levels may be altered by manipulating culture conditions.

SECTION 3.4.6 DOWNSTREAM PROCESSING

This section deals with two unit processes - filtration and ultrafiltration - which are crucial to the downstream processing of killer toxin.

SECTION 3.4.6.1 FILTRATION

A fine filtration step (0.45 μm) was necessary when processing culture broth from W. mrakii, since centrifugation failed to remove all the viable cells. The pellicle which is a normal part of this culture was particularly difficult to sediment. Centrifugation was an important first step to primary separation in order to avoid rapid clogging of the filters. For analysis of anti-C. albicans activity, centrifugal separation was adequate when the selective medium containing cycloheximide was used (Section 3.3.2.5), however the analysis fo multiple toxins required the use of cycloheximide sensitive test organisms, C. glabrata and S. cerevisiae (Section 3.3.2.7).

Woods & Bevan (1968) reported the loss of killer activity on filter sterilization and suggested that this was due to surface inactivation. More recently, Shimizu et al. (1985) reported that nitrocellulose membrane filters absorb most of the killer activity. Membrane filters made of mixed esters of cellulose (0.45 μm) used for clarifying culture broth of W. mrakii were found to be adequate but there were indications of selective absorption of toxin types. Therefore, cellulose acetate syringe filters (0.2 μm , Flow Pore D26), known to be low in protein binding, were used for analytical work.

An attempt was made to process large volumes of culture broth using a filter-aid. Dicalite 4158 was found to absorb all toxin activity and reduce

Bradford protein by 30-40% from a culture of W. mrakii NCYC 500.

SECTION 3.4.6.2 ULTRAFILTRATION

Ultrafiltration as a method for concentrating the extracellular killer toxin protein, was first used by Bussey et al. (1973), and is now widely used. Toxin denaturation has been a problem, especially in minimal medium. Middelbeek et al. (1979) reported that concentration of culture supernatant greater than 10-fold caused a substantial loss of a Pichia kluyveri toxin. Middelbeek et al. (1980d) found that a high titre of a Cryptococcus laurentii toxin was only obtained when 0.1% Yeast Extract and Peptone were present during the concentration step.

Loss of toxin activity from W. mrakii occurred during ultrafiltration. Brij 58 (1%, w/v) did not stabilize the Williopsis toxins, but glycerol (0.01%, w/v) had a stabilizing effect on the anti-C. albicans activity. This contrasts with the higher concentration (5%, v/v) glycerol needed for a stabilizing effect during fermentation (Section 3.4.2.4). However, for ultrafiltration, high levels of glycerol may be more effective for toxin stabilization but these were not tested.

Dependence on pH for thermal stability was identified as an important factor for a killer toxin from W. mrakii LKB 169 and is discussed in Section 3.6.4.3. in particular, the KG toxin showed remarkable thermostability at pH 4.0, but was labile at pH 4.5. Therefore culture broth from W. mrakii LKB 169 was processed at pH 4.0

In contrast to the S. cerevisiae toxin (Palfree & Bussey, 1979), only part of the killer activity from W. mrakii LKB 169 was retained by a membrane of 30,000 molecular weight cut-off. A diafiltered YM 30 retentate of LKB 169 culture broth (pH 4.0, 1% (w/v) Brij 58) was active against C. glabrata and S. cerevisiae. Activity against C. albicans was lost early in the fermentation. Addition of one volume 8M urea buffer (pH 4.0) - a procedure used to dissociate S. cerevisiae killer toxin (Palfree & Bussey, 1979) - released to the permeate some of the anti-C. glabrata activity but release of anti-S. cerevisiae activity was undetectable. The YM 30 retentate still retained both activities. Hence 4M urea treatment may allow bound toxin to pass through a membrane of critical pore size. Ultrafiltration experiments gave an early indication that more than one toxin may be present in culture broth from W. mrakii.

SECTION 3.4.7 SUMMARY

The role of surfactants in fermentation was examined. The non-ionic detergents (Brij 58, Triton X100, TWEEN 20 & TWEEN 80) were found to potentiate toxin production in minimal medium. The enhanced production of killer toxin by Brij 58 has been attributed to stabilization (Middelbeek et al., 1979); however, 4.5% Triton X100 was not found to stabilize killer toxin (Ouchi et al., 1978). No evidence for toxin stabilization by the non-ionic surfactants was found in this study.

Brij 58 and TWEEN 80 were found to stimulate growth, biomass accumulation and protein secretion. These effects may be due to metabolism of the surfactant and incorporation of the unsaturated fatty acid moiety into the cell membrane of the yeast. The detergent effect of Brij 58 was evident in a secretion mutant of W. mrakii where this non-ionic detergent facilitated the efflux of toxin during the non-growth stationary phase (Figure 3.4.6).

Magnesium sulphate was found to be a limiting nutrient in UYEPD medium and this nutrient stimulated toxin production to a greater extent than Brij 58. This effect was thought to be secondary to improved growth and biomass accumulation. Glycerol potentiated the toxin yield in fermentation at a concentration (5%, v/v) which did not affect growth of W. mrakii. Evidence for secretion of three distinct toxins by W. mrakii NCYC 500 was found. It is proposed that the KG¹ toxin is an antimicrobial agent active against C. glabrata; the KS¹ toxin is active against S. cerevisiae, and the KA¹ toxin is active against both C. albicans and C. glabrata.

SECTION 3.5. STUDIES ON THE SECRETED ACID PROTEINASE FROM C. ALBICANSSECTION 3.5.1 INTRODUCTION

Many yeast species can secrete proteolytic enzymes, particularly those belonging to the genera Candida, Kluyveromyces and Hansenula (Nelson & Young, 1986). Evidence has accumulated which suggests that the secreted acid proteinase from C. albicans contributes greatly to the pathogenicity of this organisms and this is reviewed in Section 1.2. Although much of the in vivo experimental work has involved disseminated candidosis, it is expected that the extracellular acid proteinase is especially important as a virulence factor in vaginal candidosis where the low pH favours proteolytic activity.

In the present study, the nature of extracellular proteolytic activity in C. albicans was of particular interest since it is a mechanism whereby the yeast might destroy or reduce the action of the protein toxins.

TABLE 3.5.1 DETECTION OF PROTEOLYTIC ACTIVITY BY THE SKIM MILK POWDER AGAR TECHNIQUE (ph 4.0)

STRAIN	PROTEOLYTIC ACTIVITY
<u>C. albicans</u> ATCC 10261	+
<u>C. albicans</u> UCSC 10	+
<u>C. albicans</u> NCYC 854	+*
<u>C. albicans</u> A 72	+
<u>C. albicans</u> CA 2	+
<u>C. albicans</u> HOG 301	++
<u>C. glabrata</u> NCYC 388	-
<u>C. glabrata</u> UCSC 0	-
<u>W. mrakii</u> NCYC 500	-

+, positive; -, negative; *, slight

SECTION 3.5.2 DETECTION OF PROTEOLYTIC ACTIVITY IN C. ALBICANS

SECTION 3.5.2.1 SCREENING FOR PROTEOLYTIC CANDIDA

Rapid screening for extracellular proteolytic activity in Candida strains can be achieved with a solid protease screening medium such as the bovine serum albumen (BSA) agar used by Staib (1965). Other substrates which have been used in solid screening medium include casein and gelatin (Nelson & Young, 1986). Using BSA agar, Schonborn et al. (1985) reported detection of secretory proteases in 23% of C. albicans strains, 48% of C. tropicalis strains and 64% of C. parapsilosis strains. However, protease secretion is strongly dependent on culture conditions (Ruchel et al., 1982). In the present study skim milk powder was used as the substrate for a solid protease screening medium. Table 3.5.1 demonstrates that C. albicans secretes an extracellular proteinase which is active at pH 4. All 6 strains tested show proteolytic activity, though this was most marked with strain HOG 301. As also observed by MacDonald (1984), C. glabrata did not show proteolytic activity.

In addition, no proteolytic activity was detected with the killer yeast W. mrakii NCYC 500 after 7 days' incubation. Nelson and Young (1986) reported proteolytic activity from W. saturnus NCYC 22 only after 25 days' incubation at 29°C using casein as the substrate (pH 4.2). This may be due to death of yeasts with subsequent liberation of intracellular proteases. A long incubation (10-15 days) was determined by Nelson & Young (1986) to be necessary for most yeasts to exhibit clear zone of proteolysis; however, in the present study a nutritionally richer medium was used which resulted in detection of proteolysis within 7 days.

SECTION 3.5.3 INDUCTION OF ACID PROTEINASE & STRAIN VARIATION IN SECRETION BY

C. ALBICANS

The medium of MacDonald & Odds (1980) supplemented with Brij 58 was used to induce proteinase production by C. albicans. Strain variation in proteolytic activity was studied after 7 days, growth at 26°C. Results are presented in Table 3.5.2. All strains digested BSA (2 mg/ml) to a low level (Range: 7.4-16.4 ug/ml protein) as determined by the Bradford protein assay (Bradford, 1976).

From a measure of the absorption at 275 nm of the TCA soluble fractions, it can be seen that C. albicans NCYC 854 was the most proteolytic yeast and strain CA 2 showed the least activity. It is noteworthy that A 72, the most sensitive strain used to detect KA toxin activity, had a low specific activity of acid proteinase.

Pepstatin A (20 ug/ml) was found to inhibit completely the activity of all 8 acid proteinase preparations.

C. albicans NCYC 854 had the highest specific activity of acid proteinase. This contrasted with the results from the solid proteinase screening medium in which this strain showed the weakest proteolytic activity. To understand this discrepancy, the effect of Brij 58 on enzyme production was examined. It was evident from this that the answer lay in stimulation of biomass. Growth of strain A72 was unaffected by Brij 58; however, this non-ionic detergent produced a marked increase in the biomass of NCYC 854.

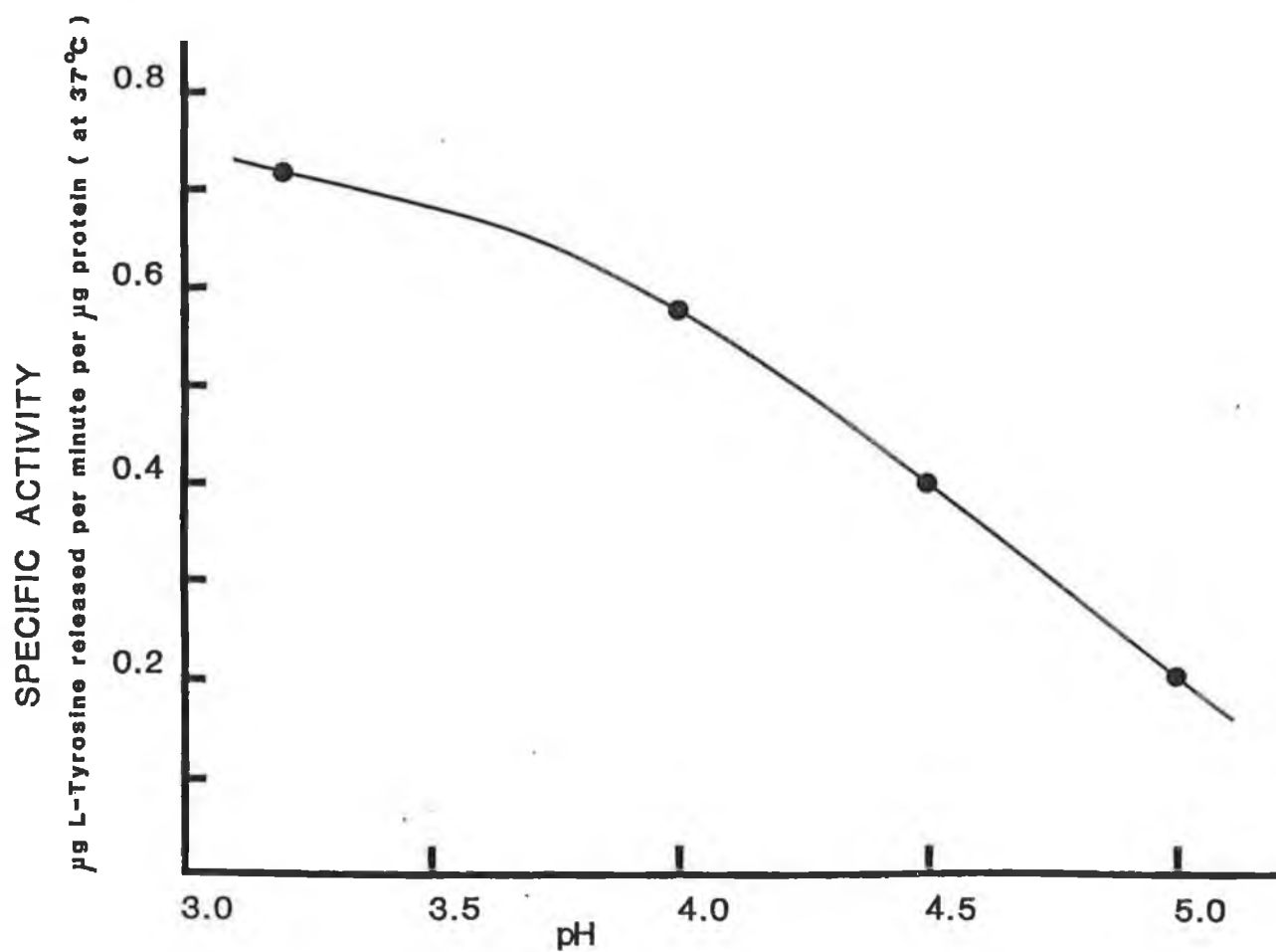


Fig 3.5.1 pH-ACTIVITY PROFILE OF THE ACID PROTEINASE FROM Candida albicans NCYC854

SECTION 3.5.4 pH-ACTIVITY PROFILE OF THE SECRETED ACID PROTEINASE FROM

C. ALBICANS NCYC 854

The pH optima for enzymatic activity of Candida acid proteases lie between pH 3.0 and 3.5 (Ruchel, 1986), although they can be as low as pH 2.2 (Ruchel et al., 1982), or in the case of a keratinase, as high as pH 4.0 (Hattori et al., 1984). Figure 3.5.1 shows the pH-activity profile for the NCYC 854 acid proteinase. The activity between pH 4.0 and pH 5.0 was of particular interest since these might therefore be active in the vaginal environment (Schnell, 1982; Sobel, 1985).

The activity of the acid proteinase from C. albicans NCYC 854 increases as the pH decreases over the pH range 3-5, see Figure 3.6.1. There was significant proteolytic hydrolysis of acid denatured haemoglobin at pH 5.0, which suggests this enzyme could be a virulence factor in Candida vaginitis. Not only does the low pH of the vagina favour proteinase activity from C. albicans but it also encompasses the pH optima for activity of the W. mrakii killer toxins (see Section 3.6).

Since vaginal candidosis was seen as a practical application for yeast killer toxins, it was important to examine the interaction between the protein toxins and extracellular proteases from Candida and this is discussed next.

SECTION 3.5.5 EFFECT OF THE SECRETED ACID PROTEINASE FROM NCYC 854 ON

KILLER TOXINS FROM W. MRAKII

This line of study arose from a failure to demonstrate toxin activity against C. albicans ATCC 10261 in liquid medium, although it was sensitive to culture broth of NCYC 500 in agar (see Section 3.3.3).

Plate 5 shows that the ATCC 10261 acid proteinase had no effect on the KA^1 toxin activity (see Section 3.6.6) following incubation for one hour at 25°C (pH 4.5). Therefore, secretion of acid proteinase seems not to be the reason for resistance of C. albicans ATCC 10261 to toxin in liquid broth. Hence, a pharmacological role for methylene blue is implicated and this was discussed further in Section 3.3.3.

Killer toxins from LKB 169 or NCYC 500 were co-incubated with NCYC 854 acid proteinase at 37°C (pH 4.5). Three distinct protein toxins in culture broth of W. mrakii LKB 169 are described in Section 3.6.5., and the natural decay of these toxins at 37°C (pH 4.5) is discussed in Section 3.6.4. In addition, multiple toxins from W. mrakii NCYC 500 are described in Section 3.6.6. Finally, the experimental protocol is discussed in Section 2.7.2.

The acid proteinase from NCYC 854 was found to have no effect on the activity of the putative toxins described from W. mrakii, namely the KA, KG and KS toxins from LKB 169 and the KA¹, KG¹ and KS¹ toxins from NCYC 500. For example, Figure 3.6.5 shows it to have no effect on the thermal decay of the KA toxin. Likewise, the KG toxin was not influenced by this enzyme over the two hour experiment in which the theoretical specific activity of the proteinase was 0.036 ug L-tyrosine equivalent/min/ug protein.

Since the NCYC 854 acid proteinase alone had no effect on toxins from W. mrakii, the accelerated decay of toxin activity with a combination of protease and pepstatin A was likely to be due to the presence of 1% DMSO used as a solvent for the enzyme inhibitor.

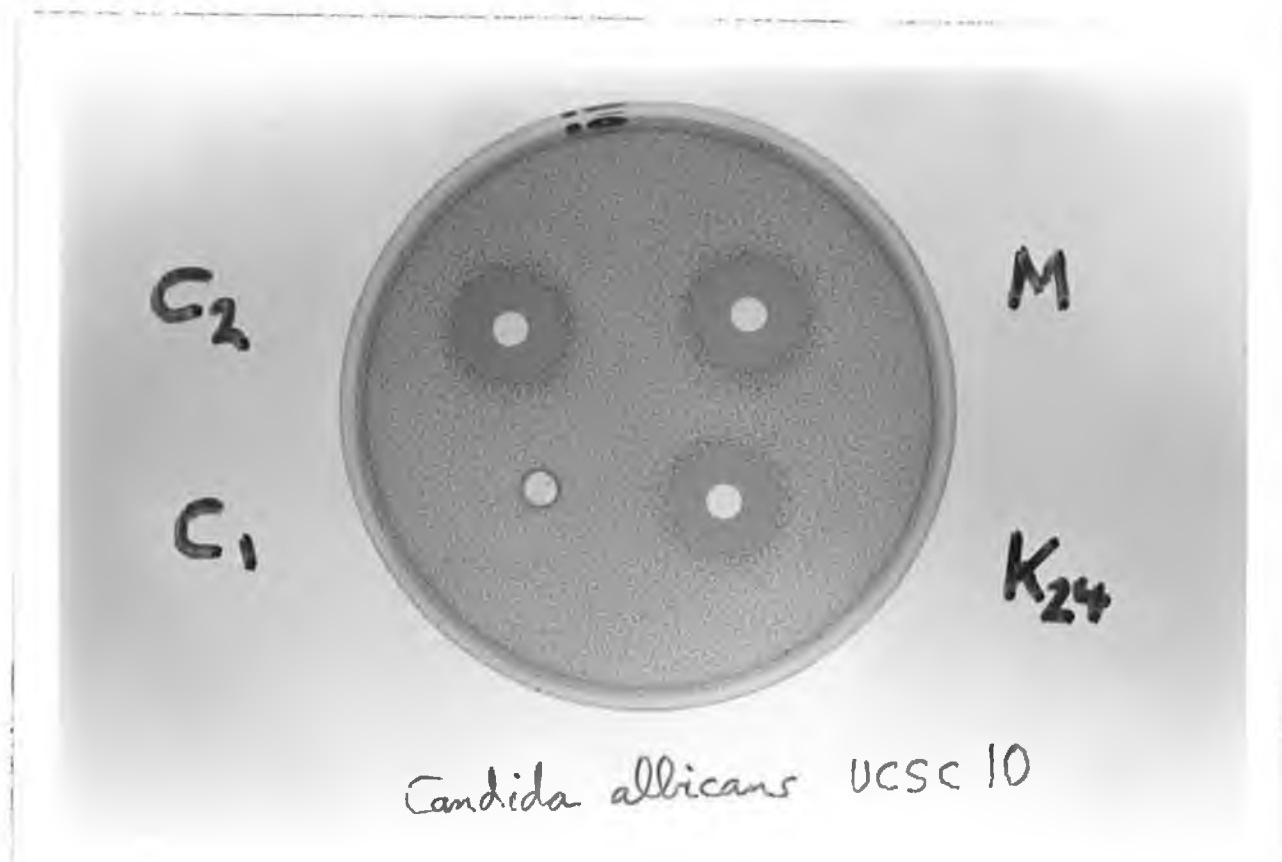


PLATE 5 *Candida* proteinase does not affect the Williopsis toxin at 25°C

- C_1 Cell-free broth from a 7-day culture of *C. albicans* ATCC 10261 (Protease-induction medium).
- K_{24} Cell-free broth from 24-hour culture of *W. mrakii* NCYC 500 (refrigerated)
- C_2 Incubation control; K_{24} incubated at 25°C for 60 min.
- M 1:1 mixture of C_1 and K_{24} , incubated at 25°C for 60 minutes (pH 4.5) prior to assay.

1% DMSO was responsible for the accelerated decay of the KA, KG and KS toxins as shown in Figures 3.6.5, 3.6.6 and 3.6.7, respectively. The corresponding toxins from NCYC 500 were more stable to this solvent (data not shown). Methanol, which was used by Ruchel (1986), would have been a better choice of solvent for pepstatin A.

A common feature of Candida proteases is their ability to cleave human serum proteins (Ruchel, 1986). The selective cleaving of monomeric immunoglobulins is pepsin-like, producing Fab₂ fragments and a short Fc fragment (Ruchel 1986). Pepsin, a mammalian acid proteinase, was used by (Young & Yagiu, 1978) to characterize killer toxins. Of interest in this study was the toxin from W. mrakii NCYC 500 which was bioassayed with S. cerevisiae NCYC 1006: this "KS toxin activity" (see Section 3.6.5) has been reported to be resistant to the action of pepsin (Young & Yagiu, 1978).

A recent study of protease secretion by yeasts included a number of killer yeasts (Nelson & Young, 1986). Four killer yeasts used in the present work (NCYC 16, NCYC 22, NCYC 577 and NRRL Y-2153) were found to secrete a protease by Nelson & Young (1986). In particular, Pichia subpelliculosa NCYC 16 was strongly proteolytic for the haemoglobin substrate. No correlation was found between this protease activity and the ability to hydrolyse the polypeptide precursors of chill-haze complexes in beer (Nelson & Young, 1986). Extracellular proteases from yeast probably show strong substrate specificity.

The report by Nelson & Young (1986) provokes two important questions. Firstly, can secretion of a proteolytic enzyme be the basis of the killer phenomenon in certain yeasts? This hypothesis is supported by the finding that

papain (50 ug) produced a 35 mm zone of inhibition in a bioassay using C. glabrata NCYC 388. Secondly, it is not known if the extracellular protease from a killer yeast, such as NCYC 16 can destroy its own toxin, or those of other killer species. A "super killer" strain of S. cerevisiae (ski 5 mutant) has been reported to produce more killer toxin because of the absence of a cell surface protease (Wickner, 1985). In addition, a cell surface protease has been implicated in the ability to degrade BSA by two Candida species which lack extracellular proteinase (MacDonald, 1984).

SECTION 3.5.6 SUMMARY OF SECTION 3.5

C. albicans was shown to produce an extracellular acid proteinase. Production of proteinase was dependent on cultural conditions. Addition of Brij 58 potentiated protease production from NCYC 854 secondary to stimulation of biomass. Marked strain variation in proteolytic potential was found. C. albicans A72, the indicator strain of choice for KA toxin activity, had a low proteolytic potential.

Proteolytic activity of the NCYC 854 proteinase was examined over the vaginal pH range. Significant activity was found at pH 5.0 and the specific activity of the enzyme increased with decreasing pH. Therefore this acid proteinase may play an important role in the pathogenesis of vaginal candidosis.

Pepstatin A, a specific inhibitor of carboxyl proteinases, effectively inhibited the Candida proteinases at 20 ug/ml. The pharmacology and toxicology of pepstatin A is further considered in Section 4.

In conclusion, the secreted acid proteinases from C. albicans probably do not pose a limitation to therapeutic use of killer toxins. The NCYC 854 acid proteinase did not show substrate specificity for toxins from W. mrakii. However, variation in substrate specificity among extracellular Candida proteases has been reported (Ruchel et al., 1982), and the possibility that a cell-surface protease on the target yeast could cleave a killer toxin must be kept in mind.

SECTION 3.6 CHARACTERIZATION OF YEAST KILLER TOXINS FROM W. MRAKII

SECTION 3.6.1. INTRODUCTION

In order to substantiate the hypothesis that there were multiple toxins produced by W. mrakii, differential bioassays were developed using three different target yeasts. The selective microbiology of the "triplet bioassay" (as outlined in Section 3.3.2.6) will be discussed in this section.

Three different toxins?

It is proposed that three distinct protein toxins are secreted by W. mrakii LKB 169. The distinguishing features of these putative toxins are summarized in Table 3.6.2. Findings suggestive of multiple toxins produced by W. mrakii are presented below:

- (i) There is a complex pattern of killer phenomenon expression at different temperatures and pH (see Section 3.1.2.2).
- (ii) There are qualitative and quantitative differences in antimicrobial activities in culture supernatants from different growth media (Table 3.4.4).
- (iii) There are different activities of toxins produced at different times during growth in broth (Figure 3.4.7).
- (iv) During incubation of W. mrakii in broth, antimicrobial activity was characterized by a loss of activity against C. albicans with a concurrent reversal in the ratio of the activity against C. glabrata to the activity against S. cerevisiae (Section 3.4.5).

SECTION 3.6.2 pH-ACTIVITY PROFILE

The effect of pH on toxin activity against three yeasts was examined by the agar diffusion assay. Figure 3.6.1 compares the activity of cell-free broth (pH 4.5) from strains LKB 169 and NCYC 500 against C. albicans, C. glabrata and S. cerevisiae. A comparison of activity between pH 4.4 and pH 5.0 shows that the ratio of activities remains the same and there is no evidence to suggest the existence of multiple toxins based on pH profiles. That is, only marginal differences were seen in the pH optima indicating that cell-free culture broths of LKB 169 and NCYC 500 were equipotent. The highest activity was observed against S. cerevisiae (23 mm, pH 4.5), followed by C. glabrata (21 mm, pH 4.6), and C. albicans was the least sensitive (17.75 mm, pH 4.8).

The inherent sensitivity of the indicator stain is an important factor governing the pH optima for toxin activity. The supposition that the target yeast must be in a "receptive state" was put forward in Section 3.1.2.2. When cell-free broth from either LKB 169 or NCYC 500 - grown at pH 4.5 - was tested against 6 strains of C. albicans (data not shown), the toxin activity sharply declined above pH 5.0. This was surprising since NCYC 500 was shown to have activity against C. albicans ATCC 10261 at pH 7.0 (Table 3.1.3).

Perhaps a different toxin to that found at pH 4.5 is produced at pH 7.0. Such a phenomenon occurs in fermentation of Streptococcus lactis to produce the bacteriocin nisin, which is inhibitory to Streptococcus cremoris; when grown at 30°C but not at 20°C, the nisin produced is also active against Streptococcus agalactiae. In fact, a single preparation of nisin consists of a mixture of closely related polypeptides and the ratio of these can be altered by a variety of conditions (Rayman & Hurst, 1984).

It should be reemphasized that the pH range 4-5 is of interest since it reflects vaginal pH (Schnell, 1982). Figure 3.6.1 shows that an antimicrobial agent from W. mrakii is active in vitro against the two most common aetiological agents of vaginal candidosis: C. albicans and C. glabrata (Odds, 1979).

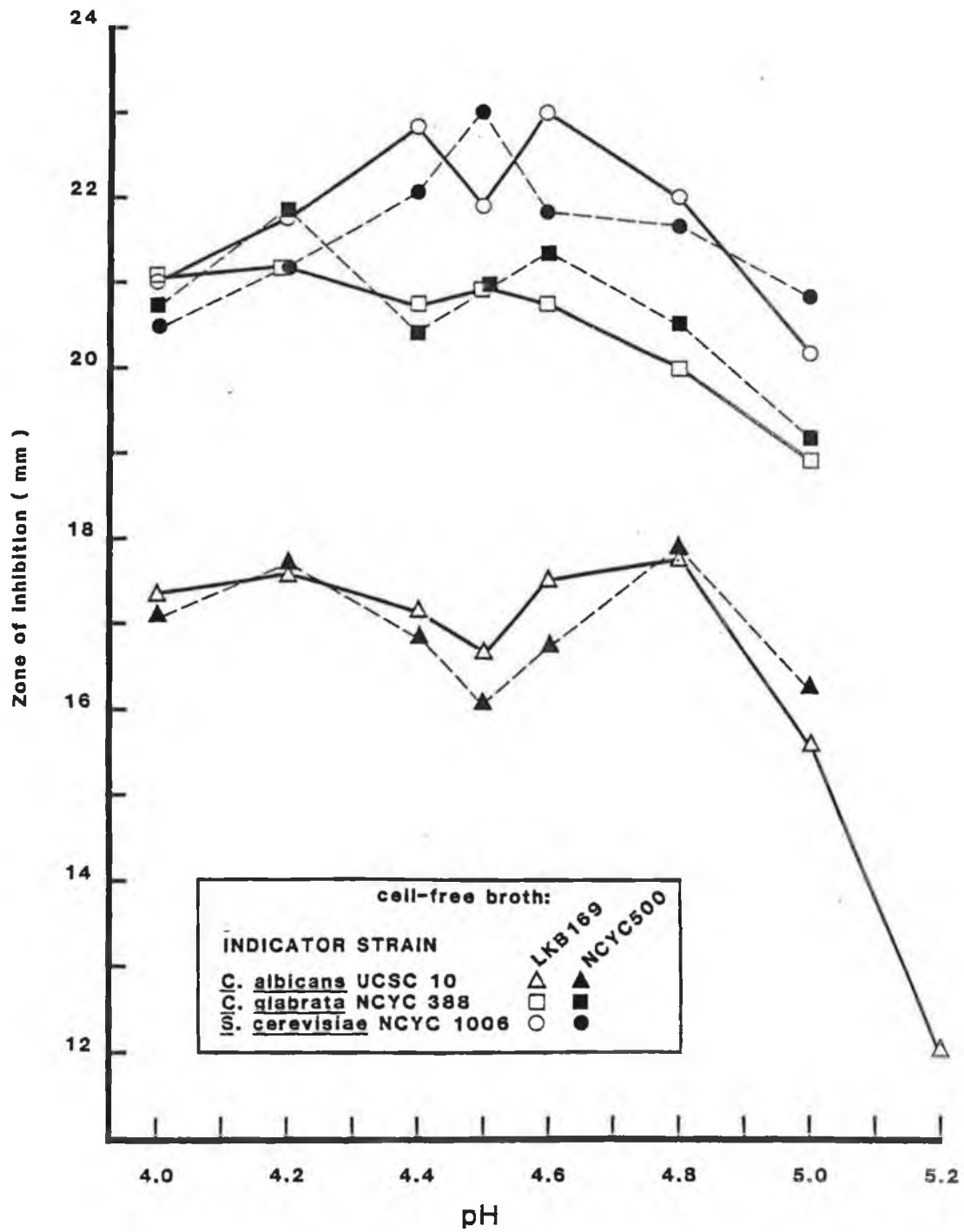


Fig 3.6.1 pH-activity profile of cell-free broth from *W. mrakii*

SECTION 3.6.3 THE EFFECT OF CALCIUM

Many killer toxins have been shown to be membrane active, producing proton, K^+ and ATP leakage in sensitive cells (Bussey & Skipper, 1975; de la Pena et al., 1981). This is supported by the electro-physiological work of Kagan (1983) who suggested killer toxin from Pichia kluyveri produced a pore in the cell membrane.

Kotani et al. (1977) showed that 100 mM Ca^{++} in agar medium inhibited the action of a sake yeast killer toxin. In particular, 100 mM Ca^{++} prevented leakage of ATP and this leakage resumed upon removal of extracellular Ca^{++} . This membrane stabilizing activity of extracellular calcium is well documented (Pasternak, 1985). The mode of action is possibly due to reversal or balance of the electro-chemical gradient, such that the major intracellular cations do not spontaneously flow out.

To investigate the effect of extracellular Ca^{++} on the action of toxins from W. mrakii, $CaCl_2$ was included in the agar diffusion bioassay. Figure 3.6.2 compares the effect of increasing calcium ion concentration on the action of the "KII fraction" with that of whole culture broth of LKB 169.

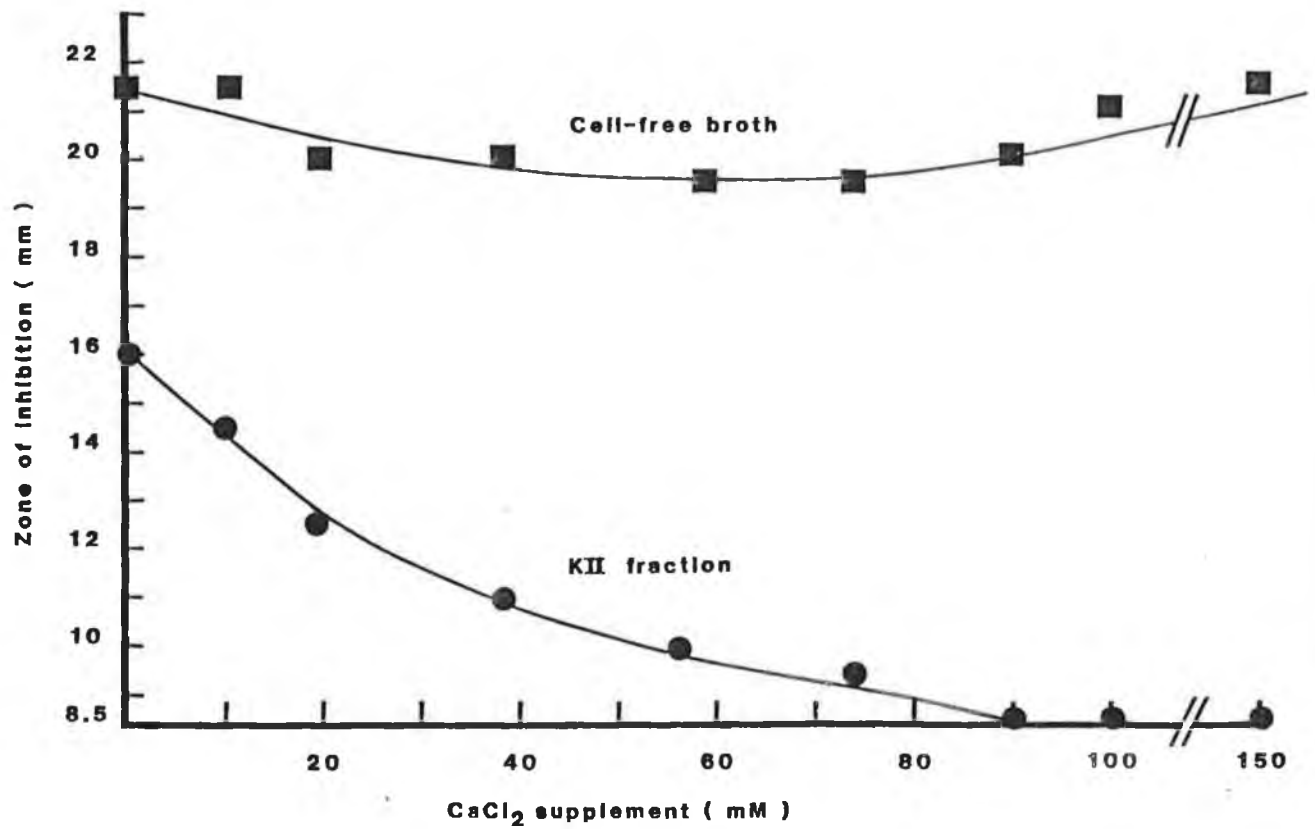


Fig 3.6.2 The effect of extracellular Ca^{++} on the activity of toxins from W. mrakii LKB169 with activity against C. glabrata NCYC 388

Figure 3.6.2 shows that the activity of the KII fraction which is assumed to contain the 8,900 molecular weight toxin characterized by Ashida et al. (1983), was depressed by calcium in a dose dependent fashion. In contrast, cell-free culture broth of LKB 169 was insensitive to calcium, suggesting that there may be another strongly active toxin which masks the effect of the KII fraction contribution. Alternatively, there may be synergism of toxin activities such as has been reported for the multiple defensins from mammalian leucocytes (Lehrer et al., 1986).

It has been established that whole broth of LKB 169 contains at least three toxins (outlined in Section 3.6.5). In this experiment the KII fraction was only active against C. glabrata, whilst whole broth was active against C. glabrata, C. albicans and S. cerevisiae. If the constituent toxins acted independently of each other; then inclusion of 100 mM CaCl₂ in the bioassay medium may selectively affect the KG toxin (defined in Section 3.6.6) and thus form the basis of a differential assay. The possibility exists that a constituent toxin of the LKB 169 broth is similar to the K. lactis toxin which inhibits adenylate cyclase and results in G₁ cell cycle arrest of sensitive cells (Sugisaki et al., 1983).

SECTION 3.6.4 STABILITY OF W. MRAKII TOXINSSECTION 3.6.4.1 STORAGE STABILITY OF KILLER TOXINSa. STORAGE AT -20°C

Cell-free broth of the killer yeast NCYC 500 which was stored frozen (-20°C) showed no loss of activity after 12 months (Table 3.6.1). The effect of successive freezer-thaw on toxin stability was not investigated.

TABLE 3.6.1 STABILITY OF NCYC 500 TOXINS ON STORAGE AT -20°C

INDICATOR STRAIN	CONTROL ACTIVITY (ZONE SIZE, mm)	ACTIVITY AFTER 12 MONTHS (mm)
<u>C. albicans</u> A 72	17	17.5
<u>C. glabrata</u> NCYC 388	21	20.5
<u>S. cerevisiae</u> NCYC 1006	23	22

b. STORAGE AT 4°C

Cell-free broth of the killer yeast LKB 169 showed no loss of constituent activities over a five-month period (Figure 3.6.3). the important implication of this is that samples taken from a fermentation can be conveniently refrigerated pending bioassay; freezing samples being unnecessary. Refrigerated broth can also be used for experiments months later.

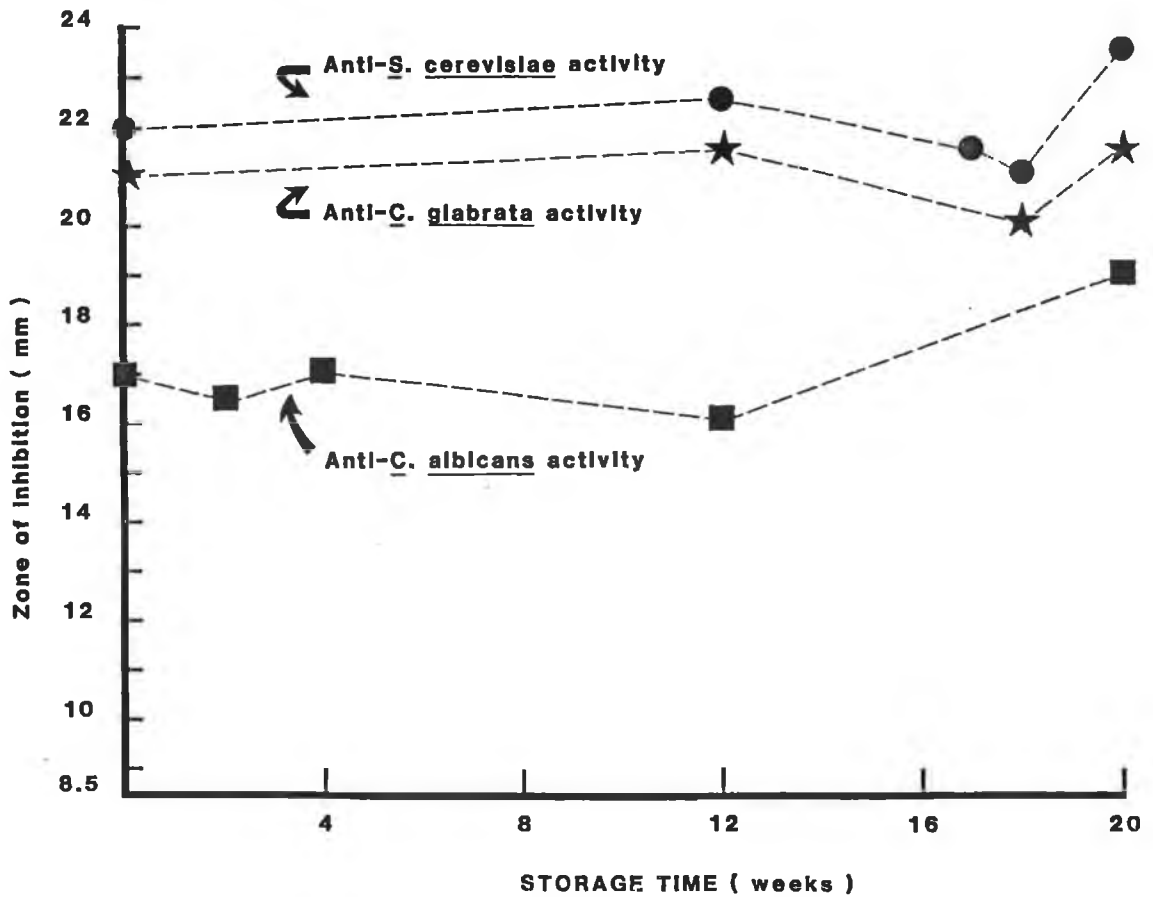


Fig 3.6.3 Storage stability (4°C) of toxins from LKB169 culture broth (UYEPD, pH 4.5)

SECTION 3.6.4.2 MECHANICAL STABILITY

Membrane filtration can subject biologically active proteins to high shear forces. Figure 3.6.4 shows the effect of filtration on toxin stability. Cell-free culture broth from W. mrakii LKB 169 was successively filtered through fresh disposable syringe filters (Flow Pore D, cellulose acetate, 0.2 um pore diameter). At each step, the filtrate was bioassayed for constituent toxins (see Figure 3.6.4).

The anti-C. albicans activity was unaffected by repeated filtration. The anti-C. glabrata activity was actually increased by this treatment. This may be due to separation of toxin dimers which may have formed during storage (4°C), or dissociation from macromolecules. The anti-S. cerevisiae activity was stable for three passes through a 0.2 um membrane but then a noticeable fall-off in activity was found. Absorption to the membranes was not a problem, but the anti-S. cerevisiae toxin appears to be more labile than the other putative toxins. This difference in physico-chemical behaviour, with the resultant change in ratio of activities, supports a hypothesis for toxin multiplicity.

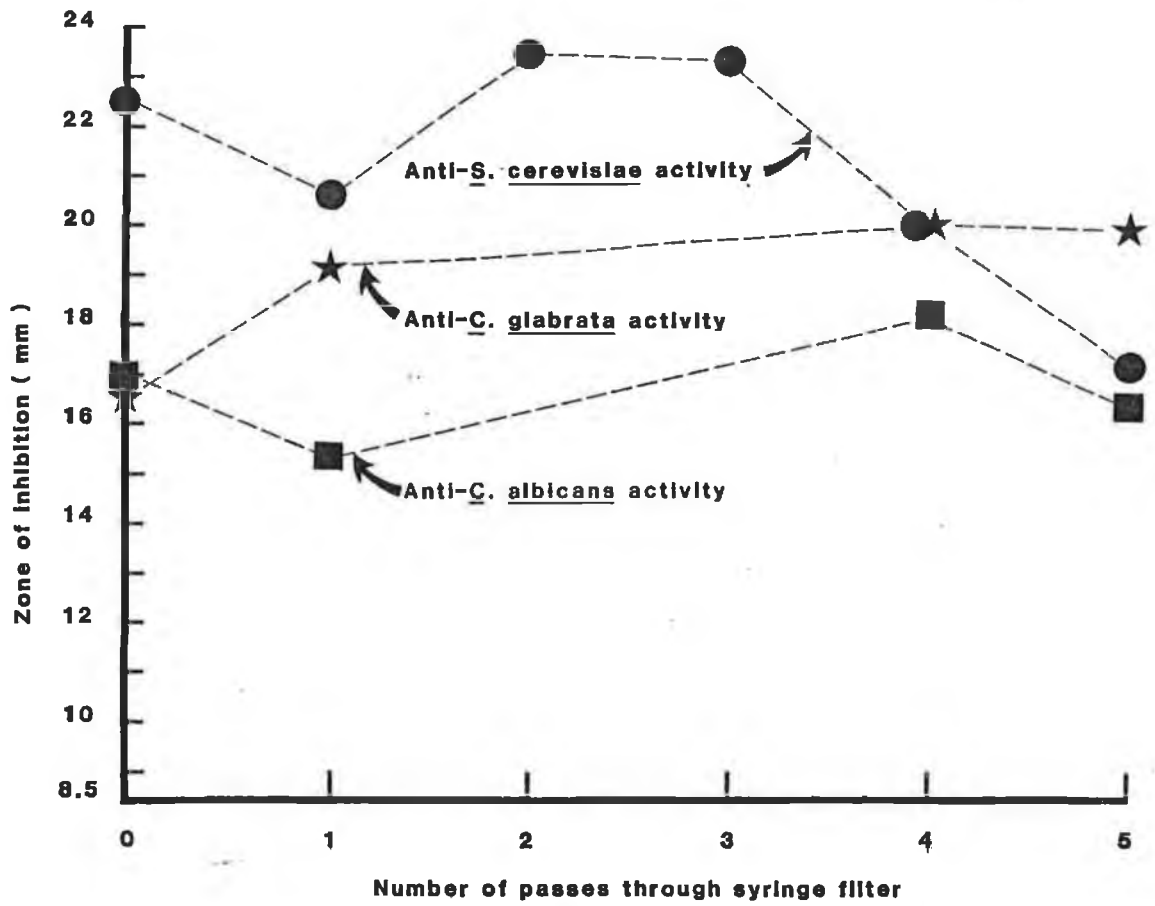


Fig 3.6.4 The effect of filter sterilization on toxin stability

SECTION 3.6.4.3 THERMAL STABILITY

Ashida et al. (1983) reported that the KII toxin from W. mrakii LKB 169 "was stable against heat (boiling for 3 min at pH 4) and in the pH range 4-11 at 25°C". In the present research, the KII fraction prepared from culture broth of W. mrakii LKB 169 (buffered at pH 4.5) was held in a boiling water bath for 3 minutes, or incubated at 55°C for 1 hour; in both experiments there was a total loss of activity as determined by the KG bioassay. However, when the KII fraction was buffered at pH 4.0, the killer toxin was thermostable. The KII fraction (2 ml, pH 4.0) was incubated in a sealed vial to minimize evaporation and took 2.5 minutes to reach approximately 98°C. Plate 6 shows that there was no significant loss of activity following incubation in a boiling waterbath for 10 minutes. Therefore the KII fraction, believed to contain the KG toxin (defined in Section 3.6.5) was thermostable at pH 4.0 but not at pH 4.5. Hence, pH 4.0 is the appropriate pH for storage and processing of this toxin.

SECTION 3.6.4.4 THE EFFECT OF PROTEASES ON THERMAL DECAY AT 37 °C

The stability of killer toxins under physiologically relevant conditions is important in determining practical applications for these novel therapeutic agents. In vitro evaluation of the therapeutic potential of killer toxin for the treatment of Candida vaginitis was determined by incubating cell-free culture broth from W. mrakii (buffered at pH 4.5) at 37°C and residual activity was bioassayed with time.

Proteolytic enzymes which have been used to characterize killer toxins are listed in Table 1.1.3; however, since evaluation of novel therapeutic agents for candidosis is one of the long-term objectives of this study, the secreted



PLATE 6 THE THERMOSTABLE KG TOXIN

The KII fraction, buffered at pH 4.0 was incubated at 100°C for 10 minutes. Toxin solutions were cooled to 4°C prior to bioassay with C. glabrata NCYC 388 at pH 4.6 and 25°C.

1 , Control

2 , Test Solution

acid proteinase from C. albicans was of special interest. The interaction of such an acid protease with toxins from W. mrakii was discussed fully in Section 3.5, where its role in limiting toxin action was considered.

In the present section, papain was found to show selective proteolysis of killer toxins and hence was deemed a useful biochemical tool to differentiate similar toxins. In addition, the effect of an acid proteinase from Aspergillus saitoi was examined but was found to have no effect on the biological activity of the KA, KG or KS toxins from W. mrakii KLB 169. Kinetic data for the decay of these putative toxins from LKB 169 at 37°C (pH 4.5) is presented in Figures 3.6.5, 3.6.6 and 3.6.7.

a) STABILITY OF THE PUTATIVE KA TOXIN

The kinetics of decay of the putative KA toxin at 37°C was determined by the KA bioassay at 25°C. This is an agar diffusion assay with the indicator strain, C. albicans A72, in a medium containing 10% glycerol. Figure 3.6.5 shows that the putative KA toxin was unstable at 37°C (pH 4.5). The anti-C. albicans activity from 24-hour culture broth of LKB 169, steadily decayed and was undetectable after 90 minutes' incubation. Dimethylsulfoxide (DMSO, 1%) accelerated the rate of decay.

Papain (1 mg/ml) rapidly eliminated biological activity of the putative KA toxin. No activity was detected after 10 minutes' incubation at 37°C. This indicates that the KA toxin had a proteinaceous nature. The possibility that KA is a glycoprotein might be investigated using a similar approach but has not been done here.

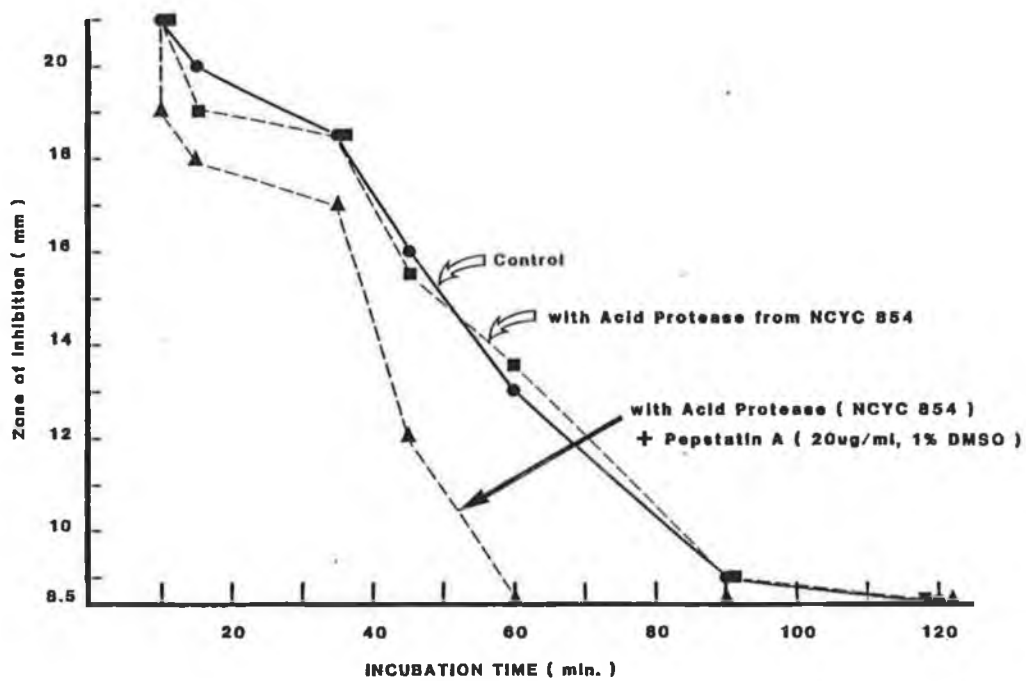


Fig 3.6.5 Stability of the KA toxin from *W. mrakii* LKB 169 at 37°C and pH 4.5

b) STABILITY OF THE PUTATIVE KG TOXIN

C. glabrata NCYC 388 was used as the test organism in the bioassay for the putative KG toxin. Figure 3.6.6 shows the kinetics of decay at 37°C of the anti-C. glabrata activity from 24 hour culture broth of LKB 169 which was pH 4.5. Activity remained detectable for over 120 minutes, in stark contrast to the stability of the toxin at pH 4.0 (see Plate 6).

With the proteolytic enzyme papain (1 mg/ml) there was an initial rapid loss of biological activity followed by a slow rate of decay. This biphasic decay curve suggests that the activity determined by the KG bioassay is heterogeneous. The initial loss maybe due to inactivation of KA or KS toxins or possibly an unidentified toxin. In view of this, the KG toxin is defined as being insensitive to papain.

DMSO (1%, v/v) accelerated the decay of activity but only to a limit reached at 60 minutes, which most likely reflects the decay of the KA toxin component.

The KG toxin which is stable to the action of this solvent, decays rather slowly both in the presence of papain and in the presence of DMSO; therefore, it appears to be fairly robust.

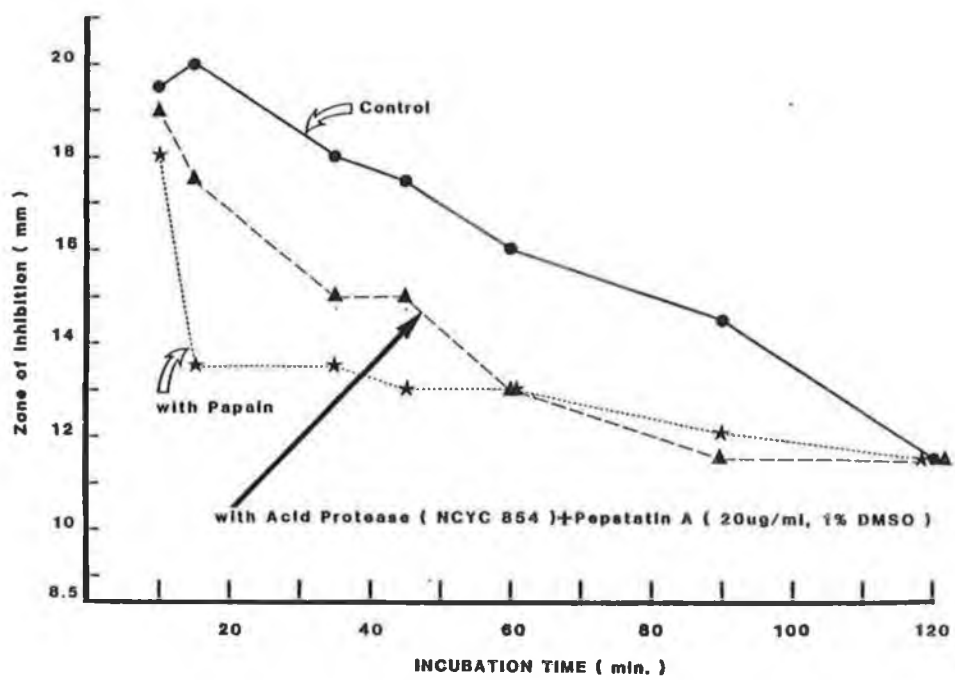


Fig 3.6.6 Stability of the KG toxin from *W. mrakii* LKB169 at 37°C and pH 4.5

c) STABILITY OF THE PUTATIVE KS TOXIN

S. cerevisiae was the indicator strain in the KS bioassay (see Plate 7). This bioassay was considered relatively selective for the KS component in cell-free culture broth of W. mrakii LKB 169. Figure 3.6.7 shows that KS activity was detected for over 120 minutes and the toxin was slightly more thermolabile than the KG toxin.

Biological activity of the KS toxin was destroyed within 10 minutes by papain (data not shown). In addition, DMSO (1%, v/v) accelerated the decay by more than 30 minutes (see Figure 3.6.7).



KS Toxin Bioassay

PLATE 7 THE EFFECT OF PROTEASES ON THE PUTATIVE KS TOXIN AFTER A 15-MINUTE CO-INCUBATION AT 37°C (pH 4.5)

1. Control (KS toxin activity from 24-hour culture of LKB169)
2. Acid proteinase from C. albicans NCYC854
3. As (2), plus 20 µg/ml pepstatin A (1% DMSO)
4. Acid proteinase from Aspergillus saitoi
5. Papain (1 mg/ml; reducing conditions)

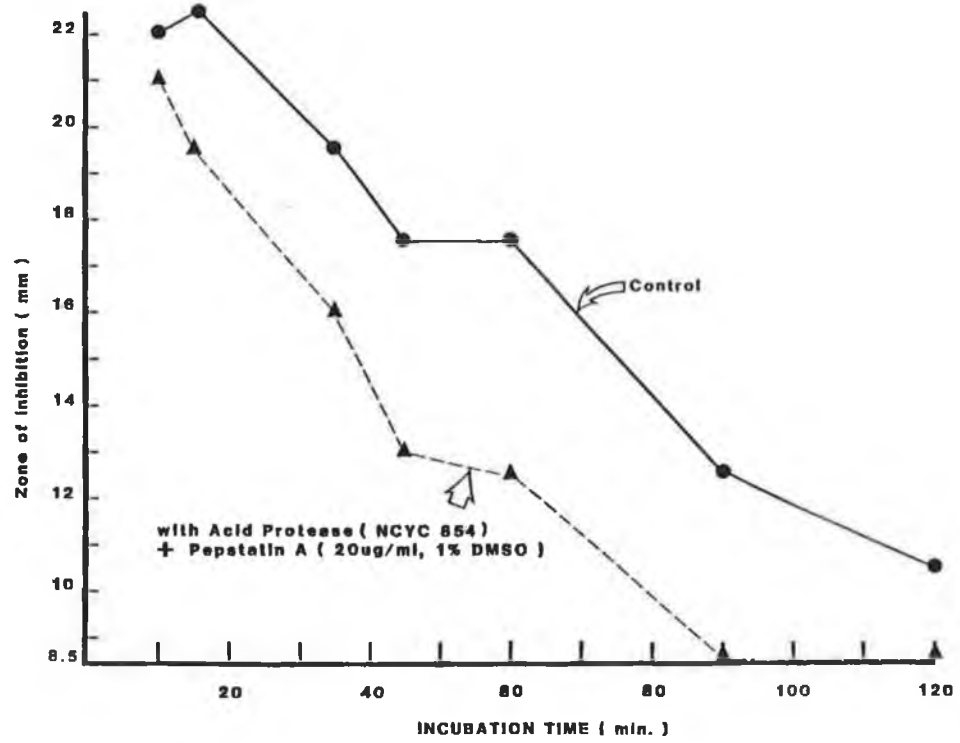


Fig 3.6.7 Stability of the KS toxin from *W. mrakij* LKB169 at 37°C and pH 4.5

TABLE 3.6.2 TOXIN MULTIPLICITY: 3 DISTINCT PROTEIN TOXINS
SECRETED BY W. MRAKII LKB 169

NOMENCLATURE	ANTIMICROBIAL SPECIFICITY*			RELATIVE STABILITY [†]	SENSITIVITY TO PAPAIN
	1	2	3		
<u>KA</u> toxin	+	+	n.d.	90 min	YES
<u>KG</u> toxin	-	+	+	120 min	NO
<u>KS</u> toxin	-	n.d.	+	120 min	YES

* Tested against: 1. C. albicans A 72
 2. C. glabrata NCYC 388
 3. S. cerevisiae NCYC 1006

† Time to which activity was detected when incubated at 37°C (pH 4.5)

SECTION 3.6.5 DESCRIPTION OF THE TOXINS PRODUCED BY W. MRAKII LKB 169

The distinguishing feature of three distinct protein toxins secreted by W. mrakii LKB 169 are summarized in Table 3.6.2.

a) KA TOXIN

Here, KA toxin is defined as a protein secreted by W. mrakii LKB 169, which is inhibitory to C. albicans and may be assayed with C. albicans strain A72 at pH 4.6. Kinetic data suggests this toxin is active against C. glabrata since a loss of the KA toxin titre produced a concomitant reduction in activity against C. glabrata (Section 3.4.5).

The KA toxin was unstable at 37°C (pH 4.5), with no detectable activity remaining after 90 minutes (Figure 3.6.5). The KA toxin is inactivated by papain; however, it is not affected by an acid proteinase from C. albicans NCYC 854.

b) KG TOXIN

The KG toxin is defined as a protein, secreted by W. mrakii LKB 169 which is strongly inhibitory to C. glabrata and may be the same as the "KII toxin" described by Ashida et al. (1983). It is assayed with C. glabrata NCYC 388 at pH 4.6. This toxin is not active against C. albicans but shows moderate activity against S. cerevisiae. (Ashida et al. (1983) have reported additional antimicrobial activity of their KII toxin against C. krusei and C. tropicalis which are also aetiological agents of candidosis).

At pH 4.5 this toxin is moderately stable: activity was detectable for over 2 hours when incubated at 37°C (see Figure 3.6.6). In addition, the KG toxin appears stable in the presence of DMSO (1%, v/v) at 37°C (pH 4.5). At pH 4.0 this protein was remarkably thermostable, being able to withstand 100°C for up to 10 minutes without loss of activity (see Plate 6, and Section 3.6.4.3).

The KG bioassay appeared to detect more than one inhibitory material; however, the KG toxin was defined as being papain resistant (see Section 3.6.4.4). This biochemical test distinguishes it from the KA and KS toxins. Extracellular Ca^{++} (Section 3.6.3) inhibited the action of the KG toxin in a dose dependent fashion (Figure 3.6.2) suggesting the KG toxin to be membrane active, in agreement with Ashida et al. (1983).

c) KS TOXIN

The KS toxin is defined as a protein secreted by W. mrakii LKB 169 which is strongly inhibitory to S. cerevisiae. This toxin is assayed with S. cerevisiae NCYC 1006 as the indicator strain. Activity against C. glabrata has not been established but this toxin is not effective against C. albicans.

The KS toxin is moderately stable at 37°C (pH 4.5); activity was detectable up to 120 minutes (see Figure 3.6.7). The decay in biological activity was accelerated by DMSO. Finally, this protein toxin was sensitive to papain under reducing conditions.

SECTION 3.6.6 CHARACTERIZATION OF TOXINS FROM W. MRAKII NCYC 500

The existence of three distinct toxin species from a different strain of W. mrakii (NCYC 500) was established from kinetic data of production using differential bioassays (see Figure 3.4.6). The nomenclature KA^1 , KG^1 and KS^1 will be used to distinguish the NCYC 500 toxins from similar activity from LKB 169. The ratio of activities in 24 hour culture broth from NCYC 500 was as follows: $KS^1 > KG^1 > KA^1$; this may, however, reflect different in intrinsic sensitivity of the indicator strains (see Figure 3.6.1).

Figure 3.6.8 shows the thermal decay of these putative toxins at 37°C (pH 4.5), together with the effect of co-incubation with two proteases. Firstly, it is clear that these three toxins show the same rate of decay, but are more thermostable than corresponding activities from LKB 169. Next, the proteolytic enzyme papain (1 mg/ml; reducing conditions) had no effect on any of these putative toxins. This contrasted with the KA and KS toxins from LKB 169 and was in variance with the report by Young & Yagiu (1978), who found the activity from NCYC 500 - as determined by S. cerevisiae NCYC 1006 - to be papain sensitive. Toxins from NCYC 500 cannot be differentiated by their sensitivity to papain and stability at 37°C, unlike those from LKB 169.

Finally, although the NCYC 500 toxins were insensitive to the acid proteinase from C. albicans NCYC 854; their activity was potentiated by the acid proteinase from Aspergillus saitoi. The unusual finding was most marked with the KG^1 toxin after 35 minutes' incubation (Figure 3.6.8), and may be due either to activation of the protein toxins, to destruction of an endogenous protease or to facilitation of toxin action via causing damage of the outer proteinaceous layer of the target yeast used in the bioassay.

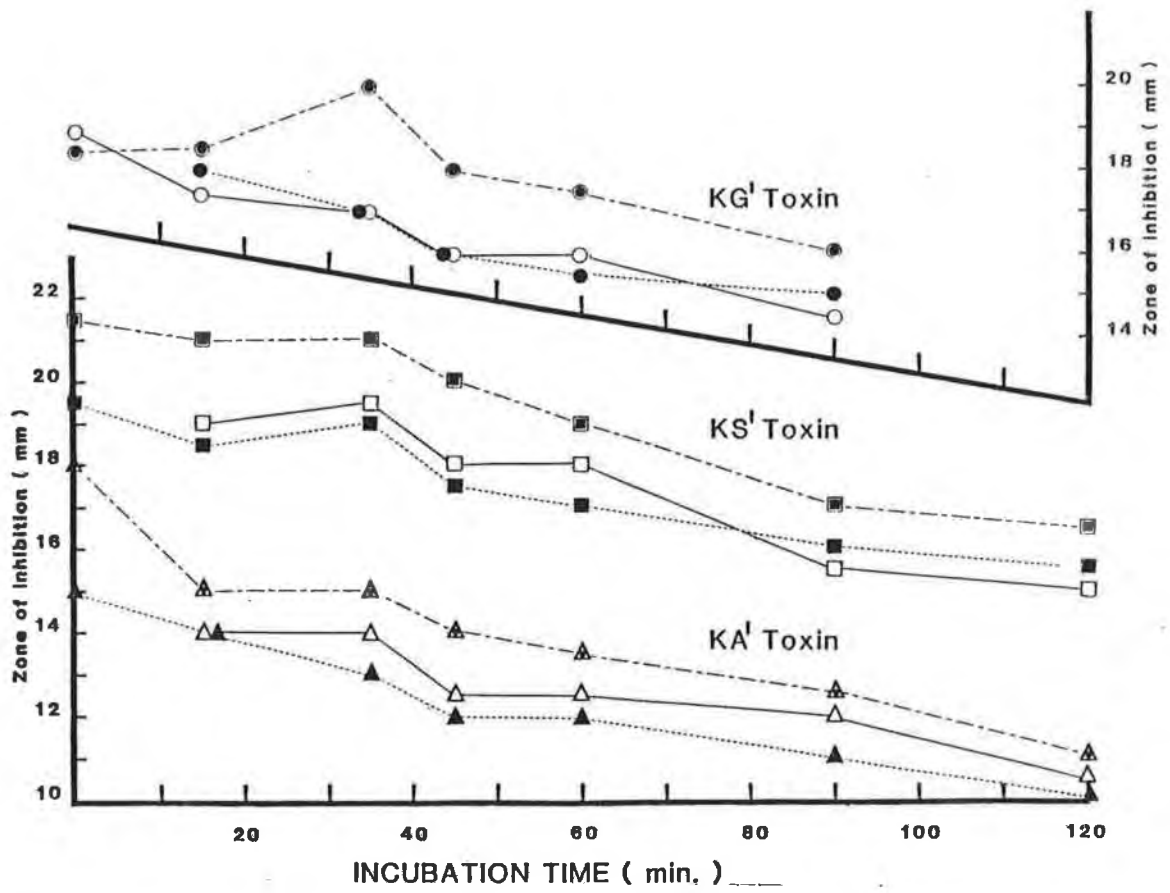
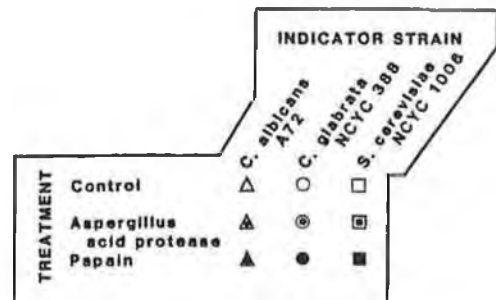


Fig 3.6.8 Decay of killer toxins from *W. mrakii* NCYC 500 at 37°C and pH 4.5



SECTION 3.6.7 SUMMARY

In the present section the heterogeneous nature of toxin activity in culture broth from W. mrakii was indicated by the dose-dependent inhibition of antimicrobial activity in the KII fraction by CaCl_2 , in contrast to whole broth, which was Ca^{++} -resistant. Three distinct protein toxins were described from culture broth of W. mrakii LKB 169 based on thermal stability, protease sensitivity and selective antimicrobial properties.

The relative stability of the putative toxins from W. mrakii LKB 169 was in the order; KG > KS >> KA; with the KA toxin being most unstable (pH 4.5, 37°C). None of these toxins were found to be affected by the acid proteases from Aspergillus saitoi or C. albicans. However, papain rapidly destroyed the KA and KS activity. Antimicrobial activity determined by the KG bioassay was found to be heterogeneous; the component insensitive to papain was defined as the KG toxin.

Resistance to destruction is an important factor influencing the effectiveness of an antimicrobial agent. Evidence was presented in Section 3.6.4.3 that showed the KG toxin to be stabilized at pH 4.0, so that it became resistant to boiling. Under physiological conditions which mimic the vaginal environment (pH 4.5, 37°C), the three putative toxins from W. mrakii LKB 169 were all unstable.

Three toxin species were also secreted by W. mrakii NCYC 500, designated KA¹, KG¹ and KS¹ which differ in antimicrobial specificity and kinetics of production. The NCYC 500 toxins are distinct from those produced by LKB 169 in being insensitive to papain and have a greater thermal stability. This latter difference may provide an explanation for the more potent expression of

killer activity shown by NCYC 500 (see Table 3.1.7).

SECTION 4 GENERAL DISCUSSION

SECTION 4.1 TOXIN MULTIPLICITY

The secretion of distinct toxin types by different yeast strains is well-documented. For example, the three killer specificities in strains of Ustilago maydis is due to secretion of distinct proteins (Kandel & Koltin, 1978). However, secretion of two or more distinct toxin species by one yeast has not yet been established.

Three reports with tentative evidence for ^umultiple toxins from one yeast have appeared, but as yet none of these have been substantiated. Bussey & Skipper (1975) partially purified the "pool efflux-stimulating toxins" from Torulopsis glabrata ATCC 15126 and reported finding three glycoproteins with activity against S. cerevisiae. However, these "bands" have not been further characterized. Ashida et al. (1983) separated toxin activity from W. mrakii LKB169 into two peaks by gel filtration (KI and KII); however, they both behaved in the same manner with regard to antimicrobial spectrum and physicochemical nature. Finally a gel filtration profile of toxin from a strain of W. mrakii was interpreted by Polonelli et al. (1985) as suggesting the presence of more than one active substance but the broad band of activity might solely refer to a failure to have performed the chromatography under conditions allowing sharp separation of toxin peaks.

This thesis is the first report which substantiates killer toxin multiplicity in yeast. Three distinct protein toxins have been identified in culture broth from W. mrakii; possibly more exist. The multiple toxins from W. mrakii LKB169 differ from those produced by W. mrakii NCYC500. An attempt

should be made to resolve these protein toxins by gel electrophoresis and correlate antimicrobial activity with discrete bands. The Williopsis toxins may be a mixture of similar polypeptides whose ratio differs depending on the growth conditions. In fact, a different toxin may predominate in cultures grown at neutral and low pH. A similar effect is known to occur in fermentations of Streptococcus lactis to produce the bacteriocin, nisin (Rayman & Hurst, 1984). This idea should be investigated further. Also it would be fruitful to examine toxin production from W. mrakii against a wider range of test organisms in order to further characterize the KA, KG and KS toxins and to find evidence for other toxin species. In such an investigation, the question of toxin synergism should be examined.

Killer toxin multiplicity has implications for killer yeast classification (Young & Yagiu, 1978) and also for "killer system" biotyping (Polonelli et al., 1983). It would be important to establish the identity of toxins produced by killer strains used in biotyping. This would avoid duplication, i.e., killer strains which secrete different quantities of the same toxin (Lehmann et al., 1987). It would aid in monitoring strain stability and would certainly lead to an update of killer yeast classification.

Killer system biotyping with live cultures (Polonelli et al., 1983) should be superceded by biotyping with purified toxin to eliminate dose-dependent and multiple toxin effects. In fact, Polonelli et al. (1985) found purified toxins to give improved performance in biotyping yeasts.

SECTION 4.2 STRAIN INSTABILITY

Although yeast cells are considered to be robust and tolerant of unfavourable conditions, certain maintenance methods can result in instability of properties. It was a surprising finding that W. mrakii LKB169 'mutated' with subculture within a month in the laboratory. In contrast, W. mrakii NCYC500 was serially subcultured for at least 12 months before altered properties were evident. As strain instability was of paramount importance in the present study it is recommended that in future studies made on W. mrakii, researchers should minimize subculturing since it promotes alteration in properties.

Serially subculturing may select for fast-growing mutants which may not have the desired characteristics (Dietz, 1982) - in this case toxin secretion. In addition future killer yeast researchers should aim to minimize instability by maintaining master cultures in liquid nitrogen or by lyophilization. Monitoring strain instability is particularly important in killer biotyping where loss of perhaps one of three secreted toxins could lead to anomalous results since such an occurrence could go unnoticed for some time.

SECTION 4.3 THERAPY OF CANDIDA VAGINITIS - A practical application for killer toxins from W. mrakii

Toxins from W. mrakii were shown to display antimicrobial activity against C. albicans and C. glabrata, the two most prevalent aetiological agents of vaginal candidosis. All three Williopsis toxins, KA, KG and KS, described in this study were found to be unstable at pH 4.5 with activity decaying over 1-2 hours at 37°C. However, when the pH was reduced to pH 4.0 the KG toxin was stable. Activity of the KG toxin was unaffected by boiling for 10 minutes at this pH. The activities of the Williopsis toxins were not affected by extracellular proteolytic enzymes from C. albicans.

For therapeutic use the narrow range of species "killed" by each of the Williopsis toxins presents a problem; a combination of toxins such as the KA and KG toxins or of toxins administered with an antifungal drug might be an appropriate therapy. These toxins could be formulated in a cream base buffered at pH 4.0 to preserve activity. Production of workable amounts of Williopsis toxin is required in order to initiate therapeutic studies in an animal model for vaginal candidosis.

In this study Debaryomyces vanriji NCYC577 was the only killer strain found to produce anti-Candida activity at 37°C and pH 4.5 in the screening assays. It is possible that a live culture of D. vanriji may be effective in treating vaginal candidosis much in the same way as a Lactobacillus preparation (Sandler, 1979). It is of interest that a related species D. hansenii produces an antimicrobial agent active against certain anaerobic clostridia (Fatichenti et al., 1983). The genus Debaromyces has potential as a source of antimicrobial substances and should be investigated further.

Killer-Candida for recurrent vaginal candidosis?

Possession of killer character may endow a yeast with selective advantage when in competition with another. C. albicans can be a harmless commensal in the gastrointestinal tract of man and little difference is seen between the adherence of a "commensal" strain and that of a "thrush" strain (Smith, 1985). If an attenuated strain of C. albicans is given the selective advantage of killer character it may reduce the symptoms of vaginal candidosis to a sub-clinical form. This novel therapy would be particularly valuable in recurrent vaginal candidosis. Such a "therapeutic strain" should be of weak pathogenic potential. For example, it should not secrete hydrolytic enzymes (acid proteinase or phospholipase) and should have a reduced ability to germinate. The primary aim in developing such a strain of C. albicans would be to displace a pathogenic strain of C. albicans in chronic vaginal candidosis where standard antimycotic therapies have been unsuccessful.

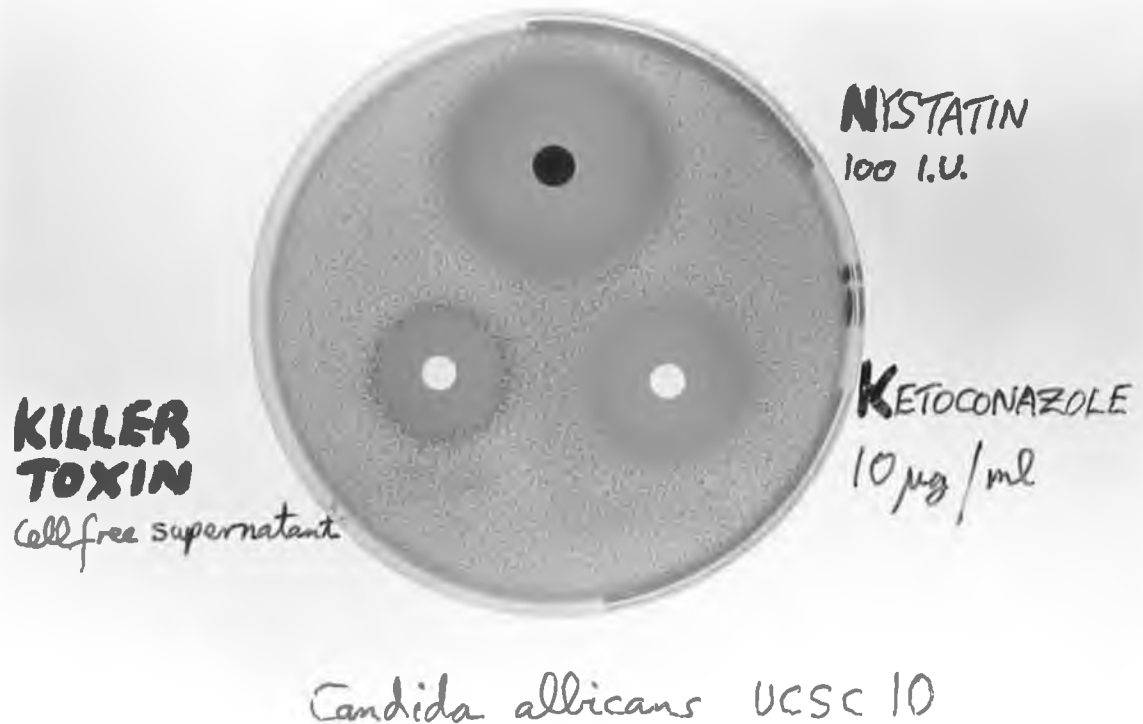


PLATE 8 KILLER TOXIN - A NOVEL ANTIFUNGAL AGENT

1. Agar diffusion assay with C. albicans UCSC10 (indicator organism) after 2 days' incubation at 25°C. Each well (8.5 mm diameter) contained 50 µl solution; 9 cm petri dish containing 15 ml agar (pH 4.5).
2. Killer toxin activity in cell-free broth from 24 hour cultures of W. mrakii NCYC500.
3. Ketoconazole, 10 µg/ml in DMSO; Neat DMSO had no effect.
4. Nystatin, OXOID NS 100 susceptibility disc (100 I.U. Nystatin).

SECTION 4.4 POTENTIAL THERAPEUTIC VALUE OF YEAST KILLER TOXINS IN CANDIDOSIS

Therapeutic exploitation of yeast killer toxins would face all the problems inherent to any protein or peptide in medical use. Proteins are usually antigenic and subject to proteolytic degradation. They are frequently unable to penetrate cells and tissues, due to their size and may have low activity at physiological pH.

Scepticism in the use of killer toxins for yeast infections was expressed by Middelbeek et al. (1980a) who drew attention to a major limitation - namely, lability at physiological pH and temperature. Antigenicity may also be a limitation (Bussey, 1981). However, there must be many more yeast toxins to be discovered; varying in their spectrum of activity and physio-chemical characteristics. Some of the toxins known today may find limited use if formulated appropriately.

Polonelli et al. (1983) found a certain C. glabrata killer yeast to be active only above pH 6.0. Ashida et al. (1983) and Ohta et al. (1984) described low molecular weight toxins from Wiliopsis strains which were remarkably stable to heat (see Section 1.1.4 or 1.4.1). The 8500 molecular weight toxin from W. saturnus, characterized by Ohta et al. (1984) had a broad antiyeast spectrum and was stable over a wide pH range.

Ashida et al. (1983) was optimistic that their 8900 molecular weight toxin from W. mrakii would have wide application as an antibiotic. Both of these toxins contained no carbohydrate residues. Therefore they are likely to be far less immunogenic than glycoprotein toxins.

Finally, the fact that a 3,500 molecular weight bacteriocin (nisin) is widely used as a food preservative (Stewart and Russell, 1986) lends some support to the proposed medical use of killer toxins. Additional support for the use of protein antifungals comes from the findings of Pope & Davies (1979) and Chalkley et al. (1985) that "mycolases" are effective antifungal agents in vivo with low toxicity.

ESTABLISHED PROTEIN THERAPEUTIC AGENTS

Valuable information on the performance of proteins as therapeutic agents comes from the enzymes and polypeptide antibiotics already in medical use. Enzymes have very specific actions; are active in very low concentrations and can easily be prepared in solutions that are compatible with blood (Chalkley et al., 1985). In general, the enzymes that have been used in therapeutics (see Table 4.1) were chosen because they are not very effective antigens. However, on occasion they do produce hypersensitivity reactions. The risk of allergic and anaphylactic reactions may be severe if they are given by injection.

In addition, the antifungal peptide saramycetin has had limited trials in man for the treatment of deep-seated fungal infections involving Blastomyces dermatidis, Coccidioides immitis, Histoplasma capsulatum and Sporothrix schenckii (D'Arcy & Scott, 1978). This polyacidic peptide of 14,000 molecular weight was administered subcutaneously at a daily dose of 4-8 mg/kg (Ryley et al., 1981).

TABLE 4.1 ENZYMES USED AS THERAPEUTIC AGENTS
(from Bowman & Rand, 1980)

ENZYME	MEDICAL USE
L-ASPARAGINASE	Cancer chemotherapy
CHYMOTRYPSIN	Facilitates the surgical removal of cataracts from lens of eye
DEOXYRIBONUCLEASE	To reduce viscosity
HYALURONIDASE	To increase rate of absorption of drugs given by intramuscular injection
PENICILLINASE	Antidote to anaphylactic reaction to penicillin
PLASMIN (Streptokinase; urokinase)	Treatment of thrombotic disease
STREPTODORNASE	Promotes wound healing
TRYPSIN	Promotes wound healing

SECTION 4.5 THE PHARMACOLOGY & TOXICOLOGY OF PEPSTATIN A

Pepstatin A is a specific inhibitor of carboxyl proteinases (E.C. 3.4.23) such as the secreted acid proteinase from C. albicans (MacDonald & Odds, 1980; Schonborn et al., 1985). It is a pentapeptide of 686 molecular weight obtained from several species of Streptomyces (Reynolds & Prasad, 1982). Pepstatin has remarkably low toxicity. LD₅₀ value in mice is 1090 mg/kg i.p. and in rats 875 mg/kg i.p.; the oral LD₅₀ value exceeds 2g/kg for all species (Windholz et al., 1983).

This enzyme inhibitor was discovered in a screening program looking for inhibitors of pepsin which might be useful in the treatment of gastric and duodenal ulcers (Umezawa, 1982). However, a double blind randomized clinical trial showed no efficacy for ulcer healing (Bonnievie et al., 1979). Pepstatin (100 mg) was administered orally, seven times a day for six weeks. Pepstatin is also known to have an antihypertensive effect due to inhibition of renin - a key enzyme in the renin-angiotensin system for blood pressure control (Oldham et al., 1984). Pepsin and renin are two human acid proteinases and pepstatin A is a competitive inhibitor of such enzymes (Umezawa, 1982).

Secreted acid proteinases play an important role in the pathogenicity of C. albicans and C. tropicalis (Ruchel, 1986). These enzymes cause tissue damage in vivo (MacDonald & Odds, 1980; Ruchel, 1986) and contribute to the persistence of the opportunistic pathogen in mucous membranes by cleaving immunoglobulins (Ruchel et al., 1982; Ruchel, 1986).

Ruchel (1986) proposed the therapeutic use of pepstatin A in candidosis based on the results of two important experiments. Firstly, pepstatin A protected "macrophage-like cells" against the cytotoxic effects of a

proteolytic strain of C. albicans. Secondly, intravenous pepstatin A lowered the number of yeasts recovered from mouse kidneys in an experimental infection of two weeks duration.

Topical therapy of *Candida* vulvo-vaginitis would be a practical application for pepstatin A. It may have a synergistic effect in combination with antimycotic drugs. Since pepstatin inhibits the secretory acid proteinase from *Candida* it could reduce tissue damage and hence the morbidity of candidosis. In particular, the extracellular *Candida* proteases cleave the secretory immunoglobulin A (Ruchel et al., 1982) which allows the pathogen to persist on the vaginal mucosa. In theory, pepstatin circumvents this determinant of pathogenicity so that the body may be better able to rid itself of the pathogen. Therefore pepstatin A might be used for *Candida* vulvo-vaginitis as a therapeutic agent in its own right or in combination with antifungal drugs. In combination with killer toxins it should protect these antifungal agents from carboxylproteinases.

SECTION 4.6 CONCLUDING REMARKS

Use of methylene blue to estimate C. albicans was found to be questionable. Consequently the method does not reliably differentiate lethal action from inhibitory action. The primary function of methylene blue is to differentiate dead yeast colonies by selectively staining them blue (Lindgren, 1949). In addition it also provides contrast to aid in the interpretation of the assay. It seems a reasonable assumption that omission of methylene blue could not have an adverse effect on the assay. However, preliminary studies failed to demonstrate killer toxin action in liquid broth in the absence of methylene blue. In contrast antimicrobial activity was readily demonstrated in agar containing methylene blue. Although methylene blue was not tested in broth, it is possible that methylene blue may be pharmacologically active. This conclusion is corroborated by the findings of Bilinski et al., 1985 who found the presence of methylene blue to be a prerequisite for antibacterial activity by two yeast strains. Additional support comes from the lack of anti-C. albicans activity found in the screening program of Kandel & Stern (1979) without methylene blue in contrast to the high frequency observed by Middelbeek et al. (1980a) using methylene blue.

Among the difficulties in detecting killer activity is the narrow "pH window" for activity and the growth dependence of toxin production. Both of these limitations could be eliminated by using a modification of the pH-gradient plate method of Wimpenny & Waters (1984). A single streak-plate assay would be expected to show the best activity in those areas where the pH favours growth of the killer yeast and the activity of the toxin. This technique would minimize the chance of missing killer activity in a screening program since each plate could contain a stable pH gradient from pH 4 to pH 8.

Finally, inhibition of morphogenesis in C. albicans would make an interesting prospective study. Plate 9 shows a magnified view of a streak-plate assay of the killer yeast W. saturnus NCYC 22 over YEPD agar (pH 4.5) seeded with C. albicans NCYC854. After 5 days' incubation at 25°C, mature colonies of NCYC854 produced aerial hyphae and other filamentous outgrowths. The killer yeast appears to have inhibited filamentation in this *Candida* species without inhibiting smooth colony development. There was progressive inhibition of filamentation approaching the W. saturnus streak. This striking yeast-yeast interaction provokes notions of selective toxicity for mycelium or inhibition of the dimorphic transition in C. albicans. A prospective study could rule out non-specific effects like nutrient depletion and secretion of organic acids. This would be a timely study, with the recent publication of a methodology to induce germ-tubes in C. albicans at low pH (Pollack & Hashimoto, 1987).

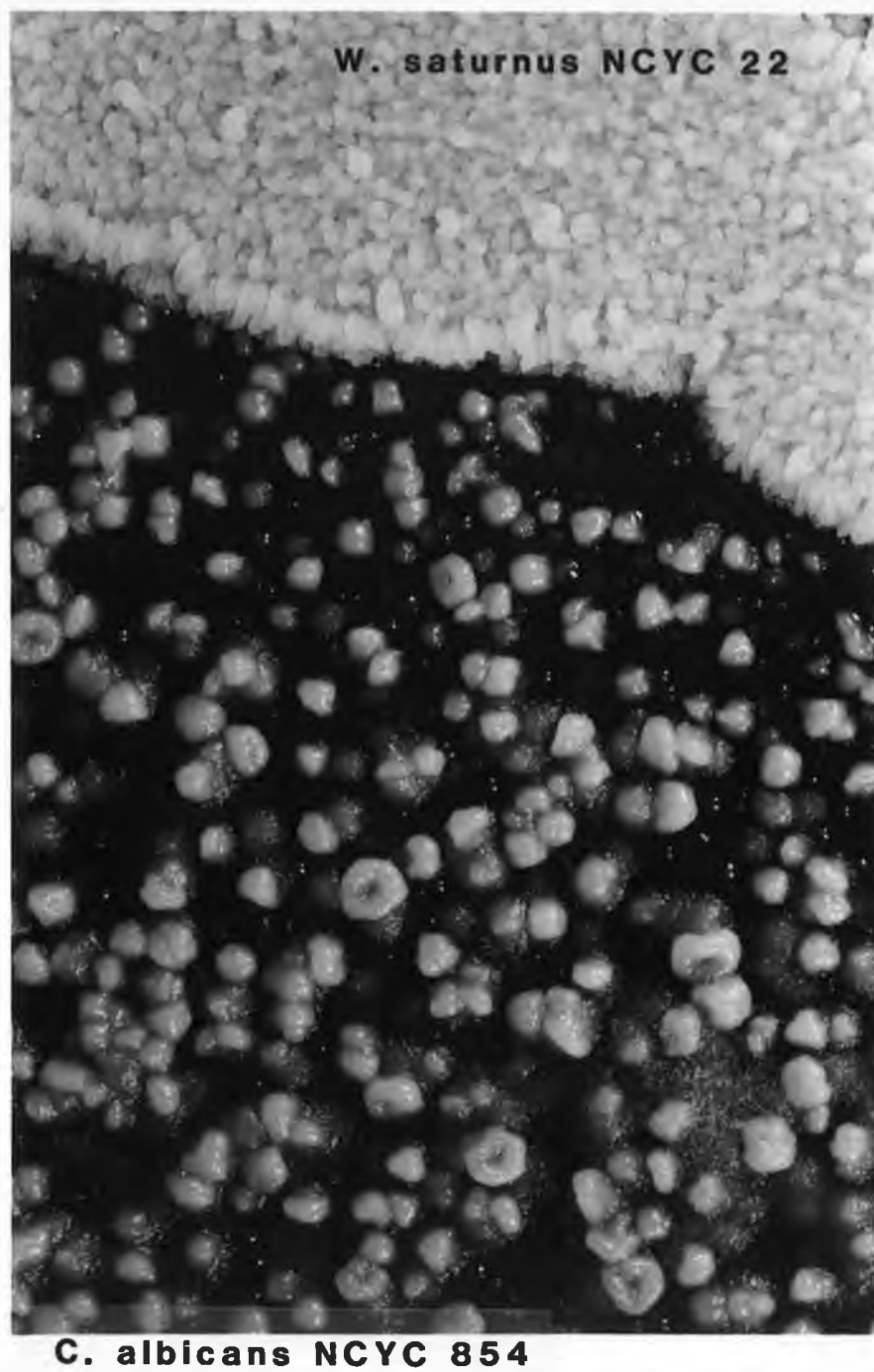


PLATE 9

Magnified view of *W. saturnus* NCYC22 streaked on the surface of seeded (*C. albicans* NCYC854) YEPD agar (pH 4.5), following incubation (5d, 25°C)

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