DIMORPHISM IN FUNGI

A THESIS

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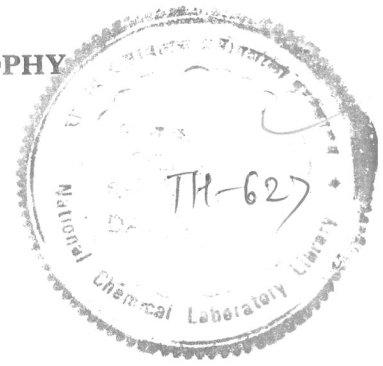

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DECLARATION

Certified that the work incorporated in the thesis *'Dimorphism in Fungi'* submitted by Smt. Ameeta Khale was carried out by her under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Dr. M.C. Srinivasan
(Research Guide)

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LIST OF ABBREVIATIONS

α-KG	α-ketoglutarate
ADH	Alcohol dehydrogenase
ADP	Adenosine 5′ diphosphate
3′AMP	Adenosine 3′ monophosphate
5′AMP	Adenosine 5′ monophosphate
ATP	Adenosine 5′ diphosphate
C:N ratio	Carbon:nitrogen ratio
cAMP	Adenosine 3′,5′-cyclic monophosphate
cGMP	Guanosine 3′,5′-cyclic monophosphate
CMC	Carboxymethyl cellulose
dbcAMP	N^6,O^2-dibutyryladenosine 3′,5′-cyclic monophosphate
DMSO	Dimethyl sulfoxide
DNSA	Dinitrosalicylic acid
EDTA	Ethylenediaminetetraacetic acid
GA	Glucosoamine
GC/GLC	Gas-liquid chromatography
GDH	Glutamate dehydrogenase
GDP-man	Guanosine-5′-diphosphate mannose
GOGAT	Glutamate synthase
GS	Glutamine synthetase
h, hrs	Hour(s)
H_2O_2	Hydrogen peroxide
HNO_2	Nitrous acid
HPLC	High pressure liquid chromatography
M-phase	Mycelial phase
mg	Milligram
min	Minute(s)
MSX	Methionine sulphoximine

NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NaF	Sodium fluoride
NAG, GlcNAC	N-acetylglucosamine
$NaNO_2$	Sodium nitrite
NTG	N-methyl-N′-nitro-N-nitrosoguanidine
ODC	Ornithine decarboxylase
PMSF	Phenylmethane sulphonyl fluoride
POPOP	1,4-di-2-(5-phenyloxazoly)-benzene
PPO	2,5-diphenyloxazole
SEM	Scanning electron microscopy
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
UDP-Glc	Uridine-5′-diphosphate glucose
UDP-GlcNAc	Uridine-5′-diphosphate-N-acetylglucosamine
µg	Microgram
µl	Microliter
µmol	Micromole
w/v	Weight-to-volume ratio
Y-phase	Yeast phase
=	Equals
>	Greater than
<	Less than

ABSTRACT

Dimorphism, i.e., the ability of cells to grow either as yeast-like or mycelium-like is a reversible phenomenon observed among various genera of fungi e.g., *Mucor, Mycotypha* and pathogens like *Candida, Endomycopsis* and *Histoplasma*. These transitions between dimorphic states can be controlled experimentally by environmental and culture conditions. Temperature or nutrition, or both, are usually the causative factors responsible for triggering a change in the fungal morphology. Studies in fungal morphogenesis have focussed their attention on the biochemical changes that occur during dimorphic transition. Examples of various parameters affecting morphogenesis are: carbon and nitrogen metabolism, rates of protein and RNA synthesis, intracellular levels of cAMP etc. However, the correlation of these parameters with morphogenesis is still unclear.

Fungal morphogenesis has intrigued biologists and biochemists for a long time. The primary reasons for this are :

(1) The prospects for the better understanding of pathogenesis of clinical infections caused by dimorphic fungi like *Histoplasma* and *Candida*.

(2) It serves as a model in eukaryotic differentiation, since in eukaryotes, the more complex developmental processes such as embryogenesis are unidirectional, whereas dimorphism is reversible.

One of the ways of understanding the phenomena of dimorphism is to undertake biochemical analysis of monomorphic mutants defective in the dimorphic transition process so that the alterations associated with morphogenesis can be compared with those of the parent strain.

The present investigation, therefore, aims to gain an insight into the phenomenon of dimorphism in *Benjaminiella poitrasii*, a rare Mucoralean fungus exhibiting dimorphic behaviour, depending on the environmental conditions. An attempt to compare the transitional events with its monomorphic mutants of *B. poitrasii* has also been undertaken.

The work is presented in six Chapters and a brief outline of the studies carried out and the significant results obtained are discussed below.

Chapter I: General introduction

This part comprises of literature survey pertaining to dimorphism in different genera of fungi and their morphological and biochemical characterizations.

Chapter II: Materials and methods

In this Chapter the experimental and analytical methods used for the study have been elucidated.

Chapter III: Dimorphism in *Benjaminiella poitrasii*: Isolation and biochemical studies of morphological mutants

Studies on transition behaviour, isolation and characterization of mutants and factors triggering dimorphism in *B. poitrasii* have been discussed.

The dimorphic cycle of *B. poitrasii* can be divided into 3 stages :

Spore germination

Vegetative growth [mycelial phase (M) and yeast phase cells (Y)]

Reproductive phase (either by sporangiospore and/or zygospore formation or budding)

Transition studies: Alterations in environmental conditions may give rise to transition from yeasts to hyphae or *vice-versa*. Studies carried out on parent strain revealed that Y→M temperature transition (37°C → 28°C) was a rapid process, and occurs within 2-3h. In contrast the reverse M→Y morphogenesis (28°C → 37°C) was a slower process taking 8-10 h. Thus, it is apparent that growth polarization is more easily gained than lost.

Isolation and characterization of mutants: To fully understand the phenomena underlying dimorphism in *B. poitrasii* the isolation of morphological mutants and its biochemical characterization was undertaken. We were able to isolate yeast-phase mutants (growing in Y-form at both 28°C and 37°C) after NTG treatment. This morphology was maintained under all kinds of growth conditions. The mutants were seen to have a typical unicellular yeast-form and reproduced by budding. Mutants of fungi belonging to other

Mucorales (e.g., *M. rouxii*), lost their stability after 2-3 subcultures. In contrast, the mutants of *B. poitrasii* were seen to be stable and have been maintained without loss of morphological form for 3 to 4 years. This feature allows the biochemical properties with respect to dimorphism of the mutants to be well characterized.

Factors which could affect the morphological expression studied are:

1. Temperature
2. Environment of cultivation
3. Nutritional composition of growth medium

Temperature: In *B. poitrasii* parent strain, temperature was found to be one of the agents triggering dimorphism. At 28°C, M-phase developed while at 37°C, Y-phase developed on YPG medium.

Environment of cultivation: In *Mucor* sp., induction of yeast development occurs in the presence of carbon dioxide (CO_2) and absence of air, while, anaerobic incubation under nitrogen (N_2) resulted in the development of mycelial form. It was therefore concluded that CO_2 tension is more important than anaerobiosis. Similar studies carried out on *B. poitrasii* showed that anaerobiosis rather than CO_2 tension is more important for yeast morphology. This result is based on the fact that the yeast form developed both in CO_2 atmosphere as well as under N_2.

Nutritional factors: A key factor in *B. poitrasii* dimorphism is the carbon source. Depending on the glucose concentration in shake flasks, *B. poitrasii* could develop hyphal or yeast growth. When glucose concentration is below 0.5%, the fungus displays predominantly hyphal growth, while at concentrations 5.0% and above, the growth was only yeast type. The fungus grew as mycelial type on a variety of carbon sources including mannose, sucrose, lactose, starch and carboxymethyl cellulose. Nitrogen source also seems to play an important role in the dimorphic transition. In general, yeast-form requires a complex nitrogen source along with glucose, while hyphal growth could occur on minimal media. The results suggest that a high C:N ratio in the medium could induce yeast morphology, whereas, with a low C:N ratio mycelial form developed.

Chapter IV : **Cell wall analysis of the yeast and mycelial phases of *Benjaminiella poitrasii* and its morphological mutants**

The shape of a fungal cell being dictated by the cell wall, studies on the chemical composition and cross-linking of cell wall components are reported in this Chapter.

Cell walls of *B. poitrasii* contain chitosan as the structural polysaccharide with chitin present in relatively less amount. The cell wall also contains mannan, protein, glucans, lipids and small amounts of phosphate. In both the yeast and mycelial cell walls hardly any qualitative differences in the composition of these components have been found. However, quantitative differences exist. The major differences are between mannose (48.1%) in Y-phase and (26.6%) in M-phase and glucosamine (35.6%) in M-phase and (20.5%) in Y-phase. Proteins were solubilized from cell wall and separated by PAGE and stained for proteins. About 30 bands could be detected by coomasie blue staining for both the phases. The results of cross-linking studies between the above various components suggest that most of the protein is complexed with mannose while glucan and chitin are linked together.

Chapter V: **Dimorphism in *Benjaminiella poitrasii*: Significance of NADP:NAD-glutamate dehydrogenase ratio in transition of parent strain and morphological mutants**

The more stringent growth requirements of yeast cells as compared to mycelial cells, suggest that some key enzymes of carbon and nitrogen metabolism might be differentially expressed in the two morphological forms. Therefore, studies were carried out on pathways which could link carbon and nitrogen metabolism to cell wall synthesis with particular emphasis on chitin.

Glutamate dehydrogenase (GDH)/ Glutamine synthetase (GS)/ Glutamate synthase (GOGAT): Studies on glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamate synthase (GOGAT) were carried out as a function of nutritional conditions and morphological state in *B. poitrasii*. These studies showed that while GS/GOGAT system maybe involved in ammonia assimilation, NADP:NAD-GDH ratio (i.e., GDH ratio) appears to play an important role in the morphological transition of the organism. Exogenous addition of α-ketoglutarate (1.0 mM) to YPG medium at 28°C, during yeast-to-mycelium transition

led to an increase in GDH ratio, which resulted in the maintenance of the yeast form. On the other hand, glutamate (1.0 mM) lowered the GDH ratio, leading to the induction of the germ tube. Mutants Y-2 and Y-5 (which do not undergo transition) showed a high GDH ratio thereby substantiating the above observations. Exogenous addition of cycloheximide and specific enzyme inhibitors also led to the conclusion that a high GDH ratio is essential for maintenance of yeast form. Addition of adenine, adenosine and cyclic AMP at 5.0 mM concentration to YPG medium led to delayed germ tube appearance. Based on the above observations and *in vitro* phosphorylation/dephosphorylation studies on NAD- and NADP-GDH, GDH ratio seems to play a role in the dimorphic behaviour of *B. poitrasii.*

CHAPTER VI: Dimorphism in *B. poitrasii*: Comparison of chitin synthetase and chitinase in yeast, mycelial forms and morphological mutants

In *B. poitrasii* parent strain and its morphological mutants, the trypsinized total specific activity of chitin synthetase was greater in yeast-phase and mutants than in the mycelial-phase. In contrast, the native activity was ~ 3 fold greater in mycelial phase than in yeast phase. The results indicate that while the enzyme was present in an active state in the mycelial form, it occurred as a zymogen in yeast form and mutant Y-2. Chitinase activity showed a slight variation. The activity in descending order was Y-phase > M-phase > Y-2 mutant. Thus, the overall turnover of chitin is greater in the yeast cells, and is reflected in the lower chitin content in cell walls of yeast.

Mycelial phase was found to be more sensitive to nikkomycin (a chitin synthetase inhibitor) than yeast phase or Y-2 mutant. The observation of bursting of hyphal tips was noticed within 1-5 min, at 0.5 mM while, in the latter no such effect was observed till 4 hr. This could either be due to the inability of nikkomycin to permeate the cell wall of Y-phase and Y-2 cells, or due to the existence of the enzyme in zymogenic (latent) form in the latter. These results seem to suggest a correlation between wall composition and dimorphism in *B. poitrasii.* Levels of chitin and the bursting of hyphal walls also convey that chitin is an essential component of the cell wall in *B. poitrasii.* Also, since nikkomycin brought about a change in the pattern of morphogenesis, a decisive factor in dimorphic development of the organism could be the overall rate or extent of chitin synthesis in the cell wall.

GENERAL INTRODUCTION

CHAPTER I

CHAPTER I

1.1 INTRODUCTION

The phenomenon of fungal dimorphism is expressed in cellular terms as the ability of cells of a species or strain to grow predominantly either as hyphal (exhibiting linear or apical growth) or as yeast-like (isotropic growth or spherical growth). At the colonial level, the effect brings about the transformation of rough filamentous colonies of the mycelial (or hyphal) stage to smooth, uniform colonies of the yeast type.

The term dimorphism generally evokes the existence of two exclusive states, i.e., yeast and mycelium, although in certain dimorphic fungi such as *Wangiella* (Geis and Jacobs, 1985), *Phialophora* (Oujezdsky *et al.*, 1973) and *Cladosporium* (Hardcastle and Szaniszlo, 1974), more than two cell types can exist. Dimorphism is also exhibited by different groups of fungi occurring throughout the taxonomic classification (Table 1.1).

In practical terms, an understanding of dimorphism is important for many reasons:

(i) It serves as an useful model to examine the basis of morphogenesis and cellular differentiation in eukaryotes. Developmental studies in microorganisms have been limited to the production of spores which is usually in response to nutritional deprivation, while the more complex developmental processes, such as embryogenesis, are unidirectional. In contrast, yeast-mycelial dimorphism is growth dependent as well as freely reversible (Sypherd *et al.*, 1978).

(ii) Because of the prevalence of dimorphism among clinically important fungi (Table 1.1), the understanding of this phenomenon may provide clues for an important group of fuangi which afflict man and animals (Rippon, 1980).

(iii) Relevance of dimorphism to the industrial production of single cell protein, since the capacity to form pellicles or mats by yeasts used in biomass production may alleviate difficulties inherent in harvesting individual cells from large quantities of culture fluid (Stewart and Rogers, 1978).

Table 1.1: Fungi exhibiting dimorphic phenomena.

Fungus	Significance	Taxonomic position[a]	Morphological characteristics	Triggering factors for Y → M transition	References
A. *Pathogenic fungi*					
Ajellomyces (Blastomyces) dermatitidis	Causes infection leading to lesions of skin and bones in humans	Ascomycetes	*Saprophytic phase* : Septate hyphae; cleistothecia and ascospores in sexual state.	Temperature (37°C → 31°C)	Domer (1985)
			Parasitic phase : Budding yeast cells		
Emmonsiella capsulata (Histoplasma capsulatum)	Causes infection of blood cells, intestines and respiratory tract in humans	Ascomycetes	*Saprophytic phase* : Septate hyphae; ascospores in sexual state	Temperature (37°C → 25°C)	Kobayashi *et al.* (1985)
			Parasitic phase : Budding yeast cells	Cysteine or cystine (1mM) - Y form maintained	Scherr (1957)
				Zinc (10 mg/100ml media) - Y form maintained	Pine and Peacock (1958)
Chrysosporium parvum	Causes mycosis which affects rodents	Hyphomycete (Deuteromycetes)	*Saprophytic phase* : Septate hyphae; sexual state unknown	Temperature (40°C → 30°C)	Hejtmánek (1985)
			Parasitic phase : Adiaspores		

Table 1.1 contd...

Fungus	Significance	Taxonomic position[a]	Morphological characteristics	Triggering factors for Y → M transition	References
Candida albicans	Agent of candidiasis in man	Deuteromycetes	*Saprophytic phase :* Budding yeast cells; sexual state unknown *Parasitic phase :* Mixtures of true hyphae, pseudohyphae and yeasts	Temperature (25°C → 37°C) GlcNAc (2.5mM) Zinc (9μM) inhibits Y → M pH (4.5→6.7)	Soll and Bidell (1978) Chaffin and Sogin (1976) Simonetti *et al.* (1974) Shepherd *et al.* (1980) Yamaguchi (1975) Buffo *et al.* (1984)
Exophiala (Cladosporuim) werenekii	Agent of tinea nigris, an infection of skin in humans	Deuteromycetes	*Saprophytic phase :* Yeasts in young cultures; septate hyphae in older cultures; sexual state unknown *Parasitic phase :* Mixtures of hyphae and yeasts	Temperature (37°C → 25°C) Nutrition (Czapek-Don yeast extract → Czapek-Dox) medium	Hardcastle and Szanislo (1974)
Paracoccidiodes brasiliensis	Causes infection of skin, mucous membrane and lymph nodes in humans	Deuteromycetes	*Saprophytic phase :* Septate hyphae; sexual state unknown *Parasitic phase :* Budding yeast cells	Temperature (37°C → 25°C)	Nickerson and Edwards (1949) San-Blas and San-Blas (1985)

Table 1.1 contd...

Fungus	Significance	Taxonomic position[a]	Morphological characteristics	Triggering factors for Y → M transition	References
Sporothrix schenkii	Causes infection of skin and lymph nodes in humans	Deuteromycetes	*Saprophytic phase :* Septate hyphae; sexual state unknown	Temperature (37°C → 25°C)	Travassos (1985)
				$CO_2 \rightarrow N_2$ or air	Travassos (1985), Romano (1966)
			Parasitic phase : Budding yeast cells	Nutrition (glucose-cysteine blood agar → simple media	Travassos (1985)
Coccidiodes immitis	Causes infection of respiratory tract skin and bones in humans	Unknown, probably Ascomycetes	*Saprophytic phase :* Septate hyphae that yield arthoconodia on fragmentation; sexual state unknown	Transition is from M → Y Mycelia grown on glucose-yeast extract medium for 9-16 days formed arthroconodia	Cole and Sun (1985)
			Parasitic phase : Spherules which are swollen arthroconidia		
B. *Non-pathogenic fungi*					
Candida tropicalis	Saphrophyte	Deuteromycetes	Mycelium septate, not extensive; mostly covered with buds. Budding yeast or yeast-type cells	Ethanol (7.1% w/v)	Tani *et al.* (1980)

Table 1.1 contd...

Fungus	Significance	Taxonomic position[a]	Morphological characteristics	Triggering factors for Y → M transition	References
Mucor sp. (*M. rouxii*, *M. bacilliformis, and M. racemosus*)	Saprophytes, rarely causing disease.	Zygomycetes	Coenocytic hyphae; zygospore formation; multipolar budding yeast cells present.	Carbon dioxide tension (< 30% CO_2) or $CO_2 \rightarrow N_2$ or air	Bartnicki-Garcia and Nickerson (1962)
				Glucose concentration (< 1.0 %)	Bartnicki-Garcia (1968)
				Complex nitrogen source → ammonium salts	Sypherd *et al.* (1978)
Mycotypha sp. (*M. microspora* and *M. africana*)	Saprophytes found in air and soil	Zygomycetes	Coenocytic hyphae; zygospore formation; budding yeast cells.	pH (5.8-6.5 → 4.5 or above 7.4)	
				$CO_2 \rightarrow N_2$ or air	Schulz *et al.* (1974)
				Glucose concentration (10% w/v → 1.0% w/v)	

a Obtained from Ainsworth and Bisby's Dictionary of Fungi by Ainsworth G.C. (1968); Ainsworth, G.C., F.K. Sparrow and A.S. Sussman (eds.), *The Fungi, An Advanced Treatise*, Vol. IV A (1973), Academic Press, New York; Kreger-van Rij N.J.W. (1984), *The Yeasts: A Taxonomic Study*.

Dimorphic fungi such as *Mucor*, *Mycotypha* and pathogens like *Candida*, *Endomycopsis*,*Blastomyces*, *Paracoccidiodes* and *Histoplasma* have been studied in detail. The transitions between their dimorphic states can be controlled by environmental and culture conditions or both. Studies on the above organisms have dealt with biochemical changes which occur during dimorphic transitions and the various parameters which have been measured are: changes in carbon and nitrogen metabolism, rates of protein and RNA synthesis, intracellular levels of cAMP etc. The correlation of these parameters with dimorphism is however, still not clear. In molecular and ultrastructural terms, an understanding of fungal dimorphism requires an explanation for restricted growth at the hyphal tip in mycelial phase, whereas growth is generalised throughout the wall surface in yeast phase (Stewart and Rogers, 1983).

1.2 DIMORPHISM AT THE CELLULAR LEVEL

The change in cell shape (and colonial morphology as a consequence) is the most evident feature of dimorphism. The vegetative growth in dimorphic fungi generally comprise of hyphal growth, yeast growth and dimorphic transitions.

1.2.1 *Hyphal growth*

Hyphal growth, is characterized by apical growth of the cell wall (Bartnicki-Garcia and Lippman, 1969; Bartnicki-Garcia, 1973). The length, width and degree of branching vary among organisms as well as the culture conditions. During hyphal growth, it is common for arthrospores to be produced by fragmentation or septation of hyphae. These arthospores are often mistaken for yeast cells since they are spherical. However, arthrospores do not form buds.

1.2.2 *Yeast growth*

Yeast growth is characterized by spherical uni- or multipolar budding cells with cell wall synthesis distributed evenly over the cell surface (Bartnicki-Garcia and Lippman, 1969). These buds may originate at any location on the mother cell.

1.2.3 *Dimorphic transition*

Dimorphic transition, is freely reversible and morphological shifts in either direction (i.e., Y↔M) can occur. Yeast phase cells convert to hyphal by germ tube formation

and subsequent elongation. Hyphal cells may convert to yeast phase by one of the three basic mechanisms: by lateral budding e.g., *Mucor* (Sypherd *et al.*, 1978), by terminal budding as in *Paracoccidiodes brasiliensis* and *Sporothrix schenkii* (Garrison, 1985) or by arthrospore formation as in *Coccidiodes immitis* (Garrison, 1985).

1.2.4 *Polymorphism*

Some microorganisms like *Wangiella* or *Philaphora* consist of more than one morphology like yeast cells, hyphae, pseudohyphae, spherules and sclerotic cells.

1.3 FACTORS INFLUENCING DIMORPHISM

Temperature, atmospheric condition or nutritional factors singly or in a combination are usually the agents which trigger a change in fungal morphology (Romano, 1967).

1.3.1 *Temperature*

Ricketts (1901) and Hamburger (1907) were the first to observe the importance of temperature in dimorphism. In most of the temperature dependent dimorphic fungi, it was seen that yeast phase growth occurred between 35-37°C, while the mycelial phase growth occurred between 20-30°C (Table 1.1). In *B. dermatitidis* for instance, irrespective of the growth medium, it was observed that temperature was the key factor in determining form, with 35-37°C being optimum for yeast phase and 31-33°C for the mycelial phase (Domer, 1985). Similarly, in *Histoplasma capsulatum*, mycelial formation occurred at 25°C, while the yeast phase grew at 37°C. In *H. capsulatum* although temperature is a key factor, in inducing transition, it is not the sole governing factor as in addition to 37°C, sulfhydryl-containing compounds must be present in the medium for yeast development (Kobayashi *et al.*, 1985). The dimorphic transition in *P. brasiliensis* depends exclusively on temperature of incubation (Nickerson and Edwards, 1949) and is independent of the composition of culture media and other external parameters (Inlow, 1979; San-Blas and Centeno, 1977). At 37°C, growth of the cells is in yeast form, while the mycelial form grows slowly at 22°C. In *Candida albicans* also temperature has been used along with pH to regulate the morphological forms.

Cells in a defined medium at 25°C grow in yeast form while at 37°C, blastospore formation can occur (Soll, 1985). In *Mucor* sp., dimorphism was not found to be regulated by temperature.

1.3.2 *Atmospheric conditions*

The influence of carbon dioxide (CO_2) tension in complementation with hexose concentration in the growth medium is well documented in the morphogenesis of *Mucor* (Bartnicki-Garcia, 1963, 1968b). A high partial pressure of carbon dioxide (pCO_2) is needed for complete yeast development at low glucose concentration (< 0.1%) and *vice versa*, while, at low p CO_2 a higher glucose concentration (> 0.1%) is sufficient to give complete yeast development.(Bartnicki-Garcia and Nickerson, 1962; Bartnicki-Garcia, 1968b). In the absence of CO_2, the growth of the fungus is typically mycelial. Exogeneous CO_2 is not an absolute necessity for yeast development as *M. rouxii* grew in yeast form in a 100% nitrogen atmosphere (provided 8.0% glucose concentration was present in the growth medium). Lower glucose concentrations under nitrogen atmosphere yielded a hyphal morphology (Bartnicki-Garcia, 1968b). The stimulatory effect of carbon dioxide on yeast development can be overridden by the presence of oxygen or nitrogen and mycelial development occurred (Bartnicki-Garcia, 1962).

1.3.3 *Nutritional factors*

The kind and concentration of hexoses in the growth medium is an important factor in inducing dimorphism (Table 1.1). In *Mucor* sp., it is observed that, the two morphological forms are obtained under the same atmosphere (30% CO_2) by using different hexose concentrations in the medium (at 0.05% glucose, mycelial phase develops while at 5.0% glucose, yeast phase occurs). Other hexoses, such as fructose, mannose and galactose, in decreasing order of efficiency are also known to stimulate yeast development in *M. rouxii* under the conditions mentioned above (Bartnicki-Garcia, 1963).

The induction of morphological transition in fungi by amino acids has been reported. Proline, leucine, cysteine or cystine have been the most frequently cited amino acids influencing dimorphism in *C. albicans* (Dabrowa *et al.*, 1976; Wain *et al.*, 1975), *H. capsulatum* (Gupta and Howard, 1971; Scherr, 1957; Maresca and Kobayashi, 1989) and *B.*

dermatitidis (Bawdon and Garrison, 1974). Studies on the effect of amino acids on *M. rouxii* (Leija *et al.*, 1986) have also been carried out, where,using amino acids such as leucine, methionine, argnine and histidine as a nitrogen source, the spores differentiated only into hyphae.

The effect of sulfhydryl groups on dimorphism of *H. capsulatum* has been well documented (Maresca and Kobayashi, 1989; Scherr, (1957) demonstrated that yeast cells maintained their morphology at 25°C, provided cysteine (1.0 mM) was present in the culture medium. This effect was attributed to a role for cysteine in lowering oxidation-reduction (O/R) potential of the medium. Rippon (1968) conclusively proved that a reduced O/R potential was responsible for M → Y transition, and that a reversion to the mycelial form occurred as the O/R potential was restored towards a more oxidizing environment. Garrison *et al.* (1970) related the rate of respiration in yeast and mycelial forms with the uptake of cysteine and cystine. Yeasts incubated at 37°C had a higher level of respiration than mycelia incubated at 25°C. Also, the addition of cysteine or cystine to the medium stimulated the respiration of yeasts but not that of mycelia (Maresca *et al.*, 1977; Maresca and Kobayashi,1989).

1.3.4 *pH*

Schulz *et al.* (1974) found that morphological transition in *Mycotypha africana* and *Mycotypha microspora* was regulated by pH. Maximal yeast growth stimulation (yeast form > 90%) occurred within the pH range 5.8 to 6.5, while mycelial development was found below pH 4.5 and above pH 7.4 (Table 1.1). In *C. albicans*, cells grew in yeast form at 25°C in a defined medium. On attaining stationary phase, if these cells were released into fresh medium at 37°C and pH 4.5, they grew as yeast cells. If released at pH 6.7 at the same temperature (37°C) hyphal formation occurred (Soll, 1985).

1.3.5 *Effect of inhibitors uncoupling respiration*

Evidence has accumulated suggesting that mitochondrial functions are needed for hyphal formation. For example, mutants of *Mucor* which were unable to transform to mycelial phase were found to be devoid of cytochrome oxidase activity (Storck and Morill,

1971). The presence of chemical agents which impair or uncouple respiration such as phenethyl alcohol (PEA), do so by stimulating alcoholic fermentation and inhibiting oxidative phosphorylation, thereby inducing formation of yeast cells (Terenzi and Storck, 1969). Similarly, the inhibition of cytochrome oxidase activity by chloramphenicol in aerobically germinating spores of *M. rouxii* stimulated yeast cell formation (Zorzopulos *et al.*, 1973). In *Mycotypha* too, chloramphenicol was found to enhance yeast formation. Inhibition of oxidative phosphorylation by oligomycin was also found to have strong yeast-inducing effects. Therefore, the influence of these factors on dimorphism of *Mycotypha* can be interpreted on the basis of a coupling between fermentation and yeast growth, or, respiration and mycelial growth (Schulz *et al.*, 1974). Similarly, studies with *C. albicans* suggest that transfer of electrons from flavoprotein was required for maintenance of yeast morphology, while a buildup of reduced flavoprotein favoured mycelium formation (Land *et al.*, 1975a, 1975b). The relationship between respiration/fermentation and dimorphism has been a subject of considerable analysis. Respiratory metabolism, was at one time thought to be an essential correlate of hyphal growth. This was later shown not to be the case (Rogers *et al.*, 1974; Paznakos and Sypherd, 1975; Stewart and Rogers, 1978; Sypherd *et al.*, 1978).

1.3.6 *Metal ions*

Ions have also been reported as participants in the dimorphic process, although their mechanisms of action have not been studied in detail. The addition of Cu^{2+} to cultures of *C. albicans* consistently maintains the organism in the yeast phase (Vaughn *et al.*, 1978). In *H. capsulatum* (Pine and Peacock, 1958) and *C. albicans* (Widra, 1964), Zn^{2+} reverses the filamentous growth. This effect in *C. albicans* is based on the fact that RNA contents fall in zinc deficient cultures, thereby suggesting a role for zinc in the synthesis of RNA or its degradation (Yamaguchi, 1975; Bedell and Soll, 1979).

1.4 CARBON METABOLISM ASSOCIATED WITH DIMORPHISM

The requirement of hexoses for yeast development implied that a relationship exists between hexose catabolism, fermentation rates and yeast morphology. In an attempt to determine whether a change in the major pathways for dissimilation of glucose occurred as a consequence of morphogenesis in *Mucor*, the distribution of carbon catabolized in yeast and mycelium was studied. It was found, that while 14.0% of glucose was catabolized *via*

pentose phosphate pathway (PPP) in yeast cells, 28.0% is processed *via* PPP in mycelial cells. Thus, a shift in the route by which glucose was catabolized occurred, depended upon the morphological form of the organism (Inderlied and Sypherd, 1978). In view of these differences, major physiological differences on the expression of some key enzymes between the yeast and mycelial forms were thought to exist and three forms of pyruvate kinase were detected in *M. rouxii* (Friedenthal *et al.*, 1973). Paznokas and Sypherd (1977) examined a variety of cultural conditions to determine if a relationship between the pyruvate kinase isozymes and morphological form occurred in *Mucor*. Their results indicated that pyruvate kinase does not appear to be involved in morphogenetic process.

The catabolism of glucose in *H. capsulatum* (Mahvi, 1965) and *P. brasiliansis* (Kanetsuna *et al.*, 1966) occurs through the Embden-Meyerhof pathway (EMP) in both yeast and mycelial forms. All the enzymes involved in this pathway are present in both forms of these fungi, although higher activities are found in the yeast phase. In *C. albicans*, a greater percentage of glucose proceeds *via* the hexose monophosphate shunt in yeasts than in mycelial cells (Schwartz *et al.*, 1982).

1.5 NITROGEN METABOLISM ASSOCIATED WITH DIMORPHISM

The growth requirements were more stringent in yeast phase than in mycelial phase for most dimorphic fungi especially with respect to complex organic nitrogen source. The pattern of activities of several enzymes of nitrogen metabolism have been studied (San-Blas and San-Blas, 1983). In *M. rouxii*, the NAD-dependent glutamate dehydrogenase (GDH) activity is greater in mycelial cells than in yeast cells when grown in the same medium. During Y→M transition, the increase in NAD-dependent GDH activity precedes the appearance of mycelial cells both under aerobic and anaerobic conditions. The cells could be maintained in yeast form (thereby suppressing morphological differentiation) by providing exogenous dibutyryl cAMP (cAMP analog) which prevented the increase in GDH activity (Peters and Sypherd, 1979).

Changes in the cellular concentration of polyamines have been related to a number of morphological and developmental changes in eukaryotic cells (Stewart and Rogers, 1983). Ornithine decarboxylase (ODC) is the initial enzyme in the biosynthetic pathway for

polyamine synthesis. An increase in the ODC activity during Y→M transition has been reported in *M. racemosus*. Exogenous putrescine prevents the enzyme increase but not the morphic change. The ODC activity increase is dependent on protein synthesis, suggesting synthesis of new enzyme (Inderlied *et al.*, 1980). Thus, although polyamines are known to be associated with changes in growth rates in a number of cells (Whitney and Moris, 1978), the increase in putrescine during Y → M transition in *M. racemosus* may be dependent directly on growth rate rather than being directly related to morphogenesis.

1.6 SYNTHESIS OF MACROMOLECULES

It is expected that in dimorphic organisms, conversion between morphological phases may be closely associated to the turnover of new macromolecules. This is especially the case in those dimorphic fungi in which synthesis of new macromolecules is necessary to initiate the dimorphic process. Alternatively, some of the macromolecules needed for the above process may already be present, although in an inactive form.

1.6.1 *RNA synthesis*

The role of RNA in dimorphism of *M. rouxii* was first demonstrated by Haidlee and Storck (1966). During Y→M transition, RNA precursors were incorporated into RNA in a discontinous fashion and it was preceeded by a burst of protein synthesis prior to germ tube appearance (Orlowski and Sypherd, 1977, 1978). Stewart and Rogers (1983) have suggested a simple regulatory mechanism for synthesis of proteins from morphic mRNAs which is based on polyamine stimulation of certain mRNA species. Selective activation of RNA polymerase (Abraham and Pihl, 1981) may also provide a means for differential genetic expression during morphogenesis. In *H. capsulatum*, new RNA synthesis was measured by the amount of radioactive guanine incorporated into the cells (Cheung *et al.*, 1974).

Little is known about the synthesis of macromolecules during the transformation process in *P. brasiliensis* Ramirez-Martinez (1970) suggested that nucleic acid content in the fungus reached maximum values at the end of the initial growth phase. The synthesis of RNA during the dimorphic process has also been followed using radioactive uridine as a marker (San-Blas *et al.*, 1980).

1.6.2 *Protein synthesis and "Morphic" proteins*

In *M. racemosus*, it has been suggested that protein synthesis is a function simply of growth rate and not of development (Orlowski and Ross, 1981), on similar lines to that in baker's yeast (Boehlke and Friesen, 1975; Bonven and Gullov, 1979). Thus, the data indicate that translational control of gene expression (by changing the rate of initiation of translation) as proposed by Lodish (1976) cannot by itself account for dimorphism. Larsen and Sypherd (1979, 1980) have suggested that the phosphorylation of S_6 ribosomal protein in *M. racemosus* is correlated with higher rates of protein synthesis but not with morphogenesis. In *P. brasiliensis*, San-Blas *et al.*, (1980) have suggested that the machinery for the early steps in the transition of yeast to mycelial form may already be present in the yeast form although in an inactive form, the activation of which could be initiated by the temperature change.

Hiatt *et al.* (1980) analyzed the cellular proteins of *M. racemosus* during Y→M transition using 2-dimensional polyacrylamide gel electrophoresis(2-D PAGE). Of the 400 of 500 polypeptides resolved by electrophoresis, relatively few were specific to one or the other morphological form. The rate of synthesis of many peptides appeared to alter, but this could have been a consequence of the stimulation of growth rate (and protein synthesis) accompanying the Y→M change. None of these phase-specific peptides have been functionally identified. Similar studies were undertaken by Manning and Mitchell (1980 a,b) with cytosolic proteins of *C. albicans*. They concluded that few phenotype-specific polypeptides exist and the differences observed in 2-D PAGE may be arising due to protein modification and not due to synthesis of new polypeptides. An example of a phase-specific protein was described by Boguslawski *et al.* (1975): a 24,000 dalton protein capable of inhibiting RNA polymerase activity, present only in the mycelial phase cells of *H. capsulatum*. However, no further studies on this aspect have been reported.

1.7 CYCLIC NUCLEOTIDES

1.7.1 *Cyclic AMP (cAMP)*

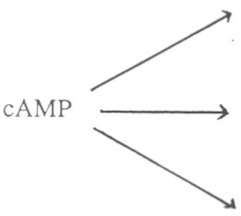
Cyclic nucleotides are know to play a key role in the control of cellular processes. In most of the cases cyclic nucleotides function as secondary messengers to signals that are received by the cell surface. However, in certain cases, as in the differentiation process in

Dictyostelium discoideum (Van Driel, 1982) it has been known to play a role as a primary messenger. A clear relationship exists between intracellular cAMP concentration and cell morphology (Sypherd *et al.*, 1978) as discussed below.

1.7.1.1 cAMP in yeast and mycelial forms

Cyclic AMP was first implicated as an effector of dimorphism in *Mucor* by Larsen and Sypherd (1974) who showed that high intracellular levels of cAMP are associated with yeast cells and low levels are characteristic of hyphal cells. Also, it was observed that the addition of dibutyryl cAMP (a lipophilic cAMP derivative) to yeast phase cultures of *M. racemosus* inhibited transformation to hyphae. Endogenous cAMP levels in the yeast form cells declined about 4-fold prior to appearance of hyphal cells. The above observations were also reported for *M. rouxii* (Paveto *et al.*, 1975). Although, the above result was obtained only if the medium contained sufficient glucose. Therefore, cAMP may not have an exclusive role to play in controlling morphogenesis (Larsen and Sypherd, 1974; Paveto *et al.*, 1975; Paznokas and Sypherd, 1975).

Similar morphological findings have been reported for both *C. albicans* and *H. capsulatum*, although in these cases the relationship between morphology and intracellular cAMP concentration was not the same as that for *Mucor*. In *C. albicans* a rise in cAMP levels accompanied germ tube formation (Niimi *et al.*, 1980). Exposure of yeast cultures to dbcAMP or to theophylline (a cAMP phosphodiesterase inhibitor) induces transition to the mycelial form at the non-permissive temperature (Chattaway *et al.*, 1981). Likewise, in *H.capsulatum* development of yeast-like forms was observed with a decrease in cAMP levels (Maresca *et al.*, 1977). The Y→M transition is accompanied by an increase in intracellular cAMP levels (Medoff *et al.*, 1981; Sacco *et al.*, 1981). At 37°C, Y→M transition could be induced by cAMP and agents that raise the intracellular levels of cAMP (Sacco *et al.*, 1981). The possible roles for cyclic AMP in the regulation of morphogenesis in dimorphic fungi is suggested below:



1) cAMP regulates gene transcription; gene products directly or indirectly affect cell wall synthesis and morphogenesis.

2) cAMP activates cAMP-dependent protein kinases that are concerned with cell wall biosynthesis.

3) cAMP interacts with microtubules and microfilaments; may therefore regulate the supply of precursors (e.g., NAG) to the site of cell wall synthesis.

Thus, in the studies of exogenous addition of cAMP, it is important to demonstrate that any response specific for cAMP is not produced by non-cyclic adenine nucleotides, adenine or adenosine, although such controls have not been reported (Pall, 1981).

1.7.1.2 Adenylate cyclase and cAMP phosphodiesterase

Cyclic AMP is synthesized by the reaction catalyzed by adenylate cyclase, and is hydrolyzed by cAMP phosphodiesterase. Any alteration in the amount of activity of either of these two enzymes could affect intracellular cAMP concentration. Paveto *et al.* (1975) showed that in *M. rouxii*, the decline in intracellular cAMP levels during yeast to mycelium transformation corresponded to a rapid increase in phosphodiesterase activity while the levels of adenylate cyclase remained steady. Attempts have also been made to establish correlations between the activities of the two enzymes, cAMP concentrations and fungal morphology, (Cantore *et al.*, 1980; Orlowski, 1980).

1.7.1.3 cAMP-dependent protein kinases

Moreno *et al.*, (1977) demonstrated the presence of two protein kinase in *M. rouxii*, one cAMP-independent particulate enzyme and a second cAMP-dependent soluble kinases. Since cAMP-dependent protein kinase have been shown to have an important role in mediating the effect of cAMP, it may be hypothesised that the drop in level of cAMP just before the appearance of germ tubes could promote inactivation of cAMP-dependent protein kinases. As a consequence, the concentration of a particulate phosphoprotein responsible for inhibiting apical growth might be reduced, allowing hyphal development to proceed (Moreno and Passeron, 1980).

The target substrate of the *M. rouxii* protein kinase is not yet known, although, Galvagno *et al.* (1979) have proposed that a cAMP-dependent protein kinase may regulate the levels of cAMP-phosphodiesterase in *M. rouxii*.

1.7.1.4 cAMP and nitrogen metabolism

In the experiments which established a link between cAMP and dimorphism in Mucorales, a complex medium was used. Paznokas and coworkers (Wertman and Paznokas, 1981; Trupp and Paznokas, 1981 a,b) developed a minimal medium which supported the germination of *M. racemosus* and also the vegetative growth of both hyphal and yeast form. This has permitted the effects of exogenous addition of cAMP on the two to be evaluated.

M. racemosus possesses NAD-dependent glutamate dehydrogenase and NADPH-dependent glutamate dehydrogenase (Peters and Sypherd, 1979). In *M. racemosus* ammonia fixation occurs most likely via the glutamine synthetase/ glutamate synthase pathway (Sypherd *et al.*, 1978). A delicate balance between glutamate catabolism and assimilation must exist. Peters and Sypherd (1979) have reported that NAD-dependent glutamate dehydrogenase, the catabolic enzyme, was under negative cAMP control, possibly mediated by a cAMP-dependent phosphorylation as reported for *C. utilis* (Hemmings, 1978) and *S. cerevisiae* (Hemmings, 1980). In the presence of high dbcAMP, NAD-glutamate dehydrogenase activity would be decreased, possibly leading to a decrease in α-ketoglutarate concentration.

1.7.2 *Cyclic GMP*

The presence of cyclic GMP in *M. racemosus* has been demonstrated (Orlowski and Sypherd, 1976). Ungerminated spores, yeast cells, hyphal and cells undergoing Y→M transitions all have equivalent amounts of cGMP. The addition of exogenous cGMP or its dibutyryl derivative did not influence cell morphology in any phase, eliminating a possible implication of cGMP in dimorphism of *M. racemosus*. Similarly, in *C. albicans* germ tube formation was not accompanied by changes in cGMP content (Niimi *et al.*, 1980). Therefore, cGMP does not seem to play an important role in dimorphism.

1.8 CELL WALL

1.8.1 *Chemical structure*

It is considered that the shape of a fungal cell is determined by the formation of its cell wall. From this concept, it follows that morphogenetic changes in dimorphic fungi should accompany either changes in cell wall structure and biosynthesis, or changes in the arrangement of the same structures to generate different shapes. Polysaccharides, which represent about 80% of the dry matter of fungal cell walls, are composed of amino sugars, hexoses and hexuronic acids (Farkas, 1979). Glucose and N-acetylglucosamine (GlcNAc) usually represent the chemical elements of skeletal wall polysaccharides such as chitin, β-glucans and α-glucans. The other sugars are present mainly in the form of homo- and heteropolysaccharides, often in chemical complexes with proteins.

1.8.2 *Cell wall and morphogenesis*

Cell wall of *M. rouxii* has been extensively studied in the yeast and mycelial phases (Table 1.2). The cell wall contains chitin as the structural polymer and is characterized by the presence of the deacetylated polymer, chitosan. The cell walls also contain mannan, polyuronides, protein and polyphosphate, but are lacking in glucans. The major quantitative difference between the yeast and hyphal forms is in the amount of mannose, which is 8 times higher in yeast cell wall (Bartnicki-Garcia and Nickerson, 1962). Dow and Rubery (1977) have also confirmed the higher content of mannose in the walls of the yeast form of *M. rouxii*.

The chemical composition of cell walls from *C. albicans* blastospores and mycelia are similar, both containing chitin, alkali-insoluble glucan and alkali soluble mannan. Differences are quantitative, particularly in terms of glucosamine, glucose, mannose and protein content (Chattaway *et al.*, 1968).

Several differences exist between the yeast and mycelial forms in *Sporothrix schenkii*. Yeast walls have a higher carbohydrate and lower lipid and protein content than mycelial phase. Although the chemical composition of cell walls differs in both the phases in *S. schenkii*, no model has been put forward for phase transition. It seems that no single

Table 1.2: Percentage of cell wall components in some dimorphic fungi.

Organism	Glucose	Mannose	Rhamnose	Galactose	Fucose	Gluconic acid	Chitin	Chitosan	Protein	Lipid	Phosphate	References
Blastomyces dermatitidis												
Yeast	36.0	Tr	-	Tr	-	-	37.0	-	7.8	5.5	0.14	Kanetsuna and Carbonell (1971)
Hyphae	44.0	8.8	-	4.4	-	-	22.8	-	10.9	9.6	0.2	
Candida albicans												
Blastospores	25.3	17.4	-	-	-	-	1.5	-	25.5	ND	0.5	Chattaway *et al.* (1968)
Hyphae	30.4	17.0	-	-	-	-	6.5	-	15.8	ND	0.7	
Histoplasma capsulatum												
Yeast	21.1	1.2	-	-	-	-	25.0	-	5.0	6.8	ND	Domer (1971)
Hyphae	5.5	5.1	-	-	-	-	4.0	-	10.0	6.2	ND	
Mucor rouxii												
Yeast	-	8.9	-	1.1	3.2	12.2	8.4	27.9	10.3	5.7	22.1	Sypherd *et al.* (1978)
Hyphae.	-	1.6	-	1.6	3.8	11.8	9.4	32.7	6.3	7.8	23.3	
Paracoccidiodes brasiliensis												
Yeast	33.5	Tr	-	Tr	-	-	43.4	-	10.1	11.0	0.2	Kanetsuna *et al.* (1969)
Hyphae	34.0	Tr	-	Tr	-	-	13.3	-	32.9	7.9	0.6	
Sporothrix schenkii												
Yeast	48.1	36.0	15.7	Tr	-	-	7.0	-	14.4	18.0	0.7	Previato *et al.* (1979)
Hyphae	54.9	28.8	16.2	Tr	-	-	7.0	-	21.7	26.0	1.2	

Tr Traces.

ND Not determined.

polymer is responsible for the alterations in the physicochemical properties of *S. schenkii* cell wall. Soluble and insoluble glucans that may have a role in transformation have very similar chemical structures in *S. schenkii*, yeast and mycelial walls (Previato *et al.*, 1979).

The basic composition of the cell wall of *B. dermatitidis*, regardless of form, was glucose, mannose, galactose, N-acetylglucosamine and amino acids (Domer, 1985). The predominant neutral sugar was found to be glucose while GlcNAc was the only amino sugar present. The yeast form contains more total amino sugar than the mycelial form, with glucose being present as either α- or β-glucan (Kanetsuna and Carbonell, 1971; Domer, 1971; Davis *et al.*, 1977). The glucan linkages, in fact, provide one of the striking differences between the yeast and mycelial walls. In the yeast wall, 95% of glucan is α-1,3 linked, whereas only 60% of the mycelial wall has it (Kanetsuna and Carbonell, 1971; Davis *et al.*, 1977). The remaining glucans are β-1,3 linked.

H. capsulatum, may be divided into chemotypes I and II, according to differences in their cell walls, chemotype II wall having an α-1,3 glucan which is absent in chemotype I. Chemotype I however, has a greater abundance of chitin.

Biochemical studies on the yeast and mycelial phases of *P. brasiliensis* were carried out by Kanetsuna *et al.* (1969, 1970, 1972). Protein levels were found to be consistently higher in mycelial preparations than in yeast preparations. No significant differences are observed in the total hexose content in each phase (Table 1.2), although the nature of the polysaccharide is not the same in both the forms (β-1,3 glucan occurred in mycelial form, while α-1,3 glucan occurred in yeast form). These difference led Kanetsuna *et al.* (1972) to propose a hypothesis to explain the morphological changes observed during transition from yeast to mycelium which will be discussed in Section 1.11.

1.8.3 *Biosynthesis of cell wall components*

Since in many dimorphic fungi, the yeast and mycelial walls are qualitatively but not quantitatively similar, researchers believe that differences must exist in the enzymatic machinery leading to cell wall construction. Therefore, various studies have been carried out on these pathways.

1.8.3.1 Glucans

Until recently, there have been few reports on the biosynthesis of fungal cell wall glucans in a cell free system. The most abundant glucans of fungal cell walls are those with the β-configuration, while a relatively smaller group of fungi contain in their walls glucose polymers linked by α-glucosidic bonds. The donor of glucosyl units in these reactions is uridine-5′-diphosphate glucose, UDP-Glc (Wang and Bartnicki-Garcia, 1976; Lopez-Romero and Ruiz-Herrera, 1978; San-Blas, 1979) and the overall polymerisation reacting is:

$$\text{UDP-Glc} + (\beta\text{-Glc})_n \rightarrow \beta\text{-Glc}_{n+1} + \text{UDP}$$

The reaction does not seem to require the presence of an acceptor or participation of a lipid intermediate for glucosyl transfer. The yeast enzyme β-1,3 glucan synthetase was found to be localised on the plasma membrane (Shematek *et al.*, 1980).

UDP-glucose is the only glucosyl donor in *C. albicans* (Orlean, 1982) and in *P. brasiliensis* (San-Blas, 1979; San-Blas and San-Blas, 1982). In yeast and mycelial forms of *C. albicans* β-glucan synthase activities are similar and are activated by ATP and GTP, suggesting a phosphorylation step in the enzymatic reaction (Orlean, 1982). In *P. brasiliensis*, the glucan synthetase obtained from yeast preparations can synthesise glucan from UDP-glucose more efficiently at 37°C than at 25°C, while reverse holds true for the mycelia.

1.8.3.2 Protein-polysaccharides (Mannan)

The structural complexity of the mannan molecule suggests that its formation would require the participation of a multienzyme system. It has been estimated that at least ten mannosyltransferases, each of them catalyzing the formation of a specific glycosidic bond, are involved in the biosynthesis of the mannoprotein molecule (Ballou, 1976). The transfer of mannosyl moieties from GDP-mannose occurs *via* a lipid intermediate, dolicol-phosphate (Farkas, 1985). The formation of O-glycosidic linked and N-glycosidic linked manno-oligosaccharides in yeast mannan involve different reactions sequences. The molecular mechanisms of mannoprotein synthesis in different dimorphic fungi has not been extensively studied. In *M. rouxii* the analysis of the level of mannosyltransferases in both

the forms (Y and M) as well as during transition has been carried out (Gutierrez and Ruiz-Herrera, 1979). According to them the activity was higher in hyphal, although the amount of mannose was greater in yeast cell wall. Therefore, it was concluded that mannosyl transferase levels in *M. rouxii* are not directly correlated to morphogenesis.

1.8.3.3 Chitin

Chitin, a β (1 → 4) polymer of N-acetylglucosamine is a major cell wall component of most filamentous fungi. Deacetylation of chitin results in the formation of chitosan, which is a major component of cell walls in Mucorales. In *M. rouxii* the amount of chitosan exceeds chitin more than three fold (Bartnicki-Garcia, 1968a). Chitin is cross-linked to proteins and other polysaccharides (chitosan, glucans, and mannans) and through these, the microfibrils associate laterally and are embedded in the amorphous matrix of the cell wall (Stewart and Rogers, 1983).

It was observed that N-acetylglucosamine (GlcNAc) can induce germ tube formation in *C. albicans* at either 37°C or 28°C (Simonetti *et al.*, 1974; Mattia and Cassone, 1979). GluNAc utilization by the cell involves partitioning of GluNAc-6-phosphate between the catabolic pathway to fructose-6-phosphate and the anabolic pathway to chitin and glycoprotein (Gopal *et al.*, 1982). The first step in this pathway is the phosphorylation of GluNAc which is carried out by GluNAc kinase, an inducible enzyme in *C. albicans* (Singh and Datta, 1979). Its pattern of induction was examined and it was concluded that this enzyme is not a control point for the dimorphic development (Shepherd *et al.*, 1980). Two other enzymes, GluNAc-6-phosphate deacetylase and glucosamine-6-phosphate deaminase, were also studied (Gopal *et al.*, 1982) and the above mentioned enzymes were also found not to play any critical role in the dimorphic process.

The enzyme leading to the formation of UDP-N-acetylglucosamine (needed for chitin and glycoprotein biosynthesis) is glutamine-fructose-6-phosphate aminotransferase (EC 2.6.1.16) and catalyzes the reaction:

Glutamine + Fructose-6-phosphate → Glucosamine-6-P + Glutamine

This enzyme has been studied in *Blastocladiella emersonii* (Norrman *et al.*, 1975) and *C. albicans* (Chattaway *et al.*, 1973), and occurs in the soluble cytosolic fraction. In *Neurospora crassa* (Endo *et al.*, 1970 a,b) and in *B. emersonii* (Norrman *et al.*, 1975), the enzyme is subject to feed-back inhibition by UDP-GluNAc. In *C. albicans*, the enzyme was found to be competitive with respect to L-glutamine while it was uncompetitive with respect to fructose-6-phosphate (Gooday, 1990).

The enzyme catalyzing the transfer of GlcNAc from UDP-GlcNAc to a a growing chain of N-acetylaminosugar residues is chitin synthetase (Fig. 1). Chitin synthetase (EC 2.4.1.16), in *S. cerevisae* exists as an inactive zymogen whose activity can be elicited on treatment by protease. Activation by protease appears to be a general mechanism for regulation of chitin synthetases (Cabib, 1987), although the possibility of physiological activation by some other protein modification has not been discarded (Cabib *et al.*, 1990). The protease can be inhibited by its specific inhibitor or by compartmentalization under physiological conditions. Activation of chitin synthetase zymogen and stoichiometry of catalyzed reaction (Cabib *et al.*, 1990) is as follows:

Chitin synthetase zymogen

↓ Protease

Active chitin synthetase

↓

$$n\ UDP\text{-}GlcNAc \rightarrow n\ UDP + [\beta\ (1 \rightarrow 4)\ GlcNAc]_n$$

The bulk of chitin synthetase is found in plasma membrane and in cytoplasmic vesicles (chitosomes), in both cases being activatible by protease (Bartinicki-Garcia *et al.*, 1979; Cabib *et al.*, 1979). The active enzyme requires phospholipids for its activity. Primer requirement is uncertain. A divalent cation is required, generally Mg^{2+}. The enzyme is activated by GlcNAc and chitin oligomers while it is inhibited by its product, UDP (Gooday, 1990).

In *M. rouxii*, the regulation of chitin synthetase appears to be different from *S. cerevisiae*. No specific inhibitor of protease which would activate the synthetase zymogen has been identified. Instead, an endogenous inhibitor competes with UDP-GlcNAc to inhibit the enzyme directly. This inhibitor has no effect on the proteolytic activation of the zymogen (Lopez-Romero *et al.*, 1978). In yeast phase of *M. rouxii*, the zymogen form predominates. In hyphal, the predominant form is the active one. Both cell types, however, have the same total activity when the zymogen is fully activated (Ruiz-Herrera and Bartnicki-Garcia, 1976). The extent of zymogen activation (by trypsin) in both morphic forms is ~ 7 fold (Braun and Caldrone, 1978). In *C. albicans*, Chiew *et al.*, (1980) suggested that chitin synthesis is regulated by chitin synthetase since the enzyme is activated 4-5 fold during germ tube formation.

Sburlati and Cabib (1986) isolated a second chitin synthetase (Chs 2) present in low levels from *S. cerevisiae* strains that harbored a disrupted CHS 1 gene and were therefore devoid of Chs 1 activity. Chs 2 shows a higher stimulation by Co^{2+} than by Mg^{2+}, whereas Chs 1 requires Mg^{2+} but is not affected by Co^{2+}. Furthermore, the pH optimum of Chs 2 is between 7 and 8 whereas that of Chs 1 is between 6 and 6.5, and Chs 2 is more resistant to inhibition by polyoxin D and NaCl than Chs 1. Conversely, Chs 1 and Chs 2 share certain common properties: both enzymes are attached to the plasma membrane and both are stimulated by proteolytic treatment (Sburlati and Cabib, 1986). According to Cabib *et al.* (1990), their results have indicated the role of Chs 2 in cell division while the role of Chs 1 remains obscure. The presence of Chs 2 in dimorphic fungi has not been demonstrated and it is of interest whether two such chitin synthetases exist in these fungi.

Chitinases (3.2.1.14) have been suggested to play a morphogenetic role during branching of hyphae or budding of chitinous yeast cells (Gooday, 1990). Bartnicki-Garcia (1973) presents the unitary model of cell wall growth in which lytic enzymes play a vital role in maintaining a balance between wall synthesis and wall lysis during hyphal apical growth, maintaining the apex in a plastic state and allowing insertion of new chitin into the wall. According to Gooday (1990) chitinases could have a more positive role in cell wall growth: 1) they could regulate the formation of crystalline-chitin in its fully hydrogen bonded form. Vermeulen and Wessels (1984, 1986) have shown that synthesis and crystallization of chitin

chains are two distinct processes, separated in time and space 2) they could be involved in the modelling of the resultant microfibrils so that they take the form characteristic of the particular wall construction. Thus, Gow and Gooday (1983) have shown that *C. albicans* has short stubby chitin microfibrils in their hyphal walls 3) they could also be involved in the cross-linking of chitin to other wall components such as glucans. Evidence for association of chitinases with chitin synthetase has been observed in *M. mucedo* (Gooday *et al.*, 1986), *M. rouxii* (Gooday, 1990) and *C. albicans* (Barrett-Bee and Hamilton, 1984).

1.8.4 *Inhibitors of cell wall polymer synthesis*

A large number of factors can affect hyphal growth especially those whose mechanism can be directly ascribed to effects on the rates of cell wall polymer synthesis which in turn may affect hyphal extension (Gooday, 1978). A tool to study biochemical processes is the use of antibiotics that block a particular reaction. Inhibitors of polymer synthesis have been used to prove the developmental role of polysaccharide synthesis. Polyoxin D is a structural analog of UDP-N-acetylglucosamine, the substrate for chitin synthetase (Endo *et al.*, 1970 a). It is a competitive inhibitor binding more tightly to the enzyme (Ki ~ 1μM) than the substrate (Km ~ 500 μM). When added to intact cells or hyphae, inhibition of chitin synthetase by polyoxin D caused apical weakening and bursting or ballooning in cultures of *M. rouxii* hyphae (Bartnicki-Garcia and Lippman, 1973). A number of other polyoxins and related analog, nikkomycin have been characterised (Gooday, 1978, 1990). All either inhibit hyphal elongation or cause ballooning in a number of fungi. Other inhibitors include kitizin (Maeda *et al.*, 1970) and tunicamycin, which inhibits protein glycosylation. However, the broad specificity of tunicamycins makes it difficult to interpret the results, because they inhibit glycosylation of proteins as well as mannan synthesis (Kuo and Lampen, 1976), and although they are analogs of UDP-GlcNAc, they appear to have little effect on chitin synthesis *in vivo* (Kuo and Lampen, 1976; Kato *et al.*, 1978). Papulacandin B, an inhibitor of glucan synthesis, induces cell lysis in *C. albicans* (Traxler *et al.*, 1977). In the M-form of *P. brasiliensis*, this antibiotic leads to the production of yeast like structures (San-Blas and San-Blas, 1985) under *in vivo* conditions. However, this antibiotic for unknown reasons is not able to block glucan synthesis under *in vitro* conditions in *P. brasiliensis*. Incorporation of polysaccharides into walls of regenerating protoplasts of *C.*

albicans was followed in the presence of papulacandin B, tunicamycin and nikkomycin, (Elorza *et al.*, 1987). Their results showed that regeneration of walls by protoplasts occurs in two steps: Firstly, a chitin skeleton is formed, and in a latter step glucan and mannoproteins were added to the growing structure.

1.9 CELL MEMBRANES

In order to understand host responses against fungal infections, studies dealing with the characterization of antigens, which are invariably present in the cell membranes have been carried out (Kumar *et al.*, 1985; Edwards *et al.*, 1986). Therefore, the preparation of antifungal vaccines by using specific membrane proteins makes it important to obtain pure membranes in order to characterize membrane antigens (Medoff *et al.*, 1986). Studies indicate a role for plasma membrane in biological processes such as morphogenesis and recognition of external messages (Quinn, 1976). Enzymes involved in the pathway leading to cell wall synthesis e.g., chitin synthetase are plasma membrane bound. So also is adenylate cyclase, which leads to the synthesis of cAMP, an important molecule in the morphogenetic process.

To date plasma membranes from mycelial and yeast phases of *H. capsulatum* have been isolated and purified using discontinous sucrose density gradient (Kumar *et al.*, 1988). A comparison of the two fractions revealed quantitative and qualitative differences in the expression of several membrane specific enzymatic activities as well as physical differences upon observation by electron microscopy. The differences may arise from different lipid or carbohydrate composition of the two membrane preparations.

1.10 GENETIC APPROACHES TO THE STUDY OF DIMORPHISM

It can be anticipated that a full understanding of the phenomena that underlie dimorphism will require isolation of morphological mutants and their biochemical and genetic characterization. These goals are hampered by the problems involved in recombinational analysis due to long period of dormancy of zygospores in *Mucor*, or by the lack of a known sexual phase as in *Candida*.

In *Mucor*, for the above reason, attempts to isolate mutants have been only partially successful. Peters and Sypherd (1978) have described procedures for selecting nutritional and morphological mutants of *M. racemosus* by freeze-thawing enrichment

process. In *M. rouxii*, the morphological mutants obtained were extremely unstable and lost after 2-3 subcultures (Ruiz-Herrera, 1985). However, stable yeast-like mutants from *M. bacilliformis* were obtained after nitrosoguanidine treatment of the mycelium (Ruiz-Herrera *et al.*, 1983). Biochemical analysis were included in the studies of the mutants and various factors have been suggested to be involved in *Mucor* dimorphism. The data show that (1) the mutants were unable to grow on non-fermentable carbon sources; (2) they respire with glucose as substrate; and (3) respiration is resistant to cyanide (4) some of mutants were found to have low levels of NAD-dependent glutamate dehydrogenase (5) three of the fourteen mutants were found to have high levels of cAMP (6) the mutants had low levels of ornithine decarboxylase activity. However, the exact correlation of these events with morphogenesis is yet to be figured out.

Heterokaryon formation by fusion of protoplasts of auxotrophic strains of *M. racemosus* and analysis of the prototrophic recombinants have been described by Genther and Borgia (1978). It becomes possible to select heterokayons directly by morphological crieteria and thus to measure complementation between morphological mutants (Lasker and Borgia, 1980; Stewart and Rogers, 1983).

In *C. albicans*, Olaiya and Sogin (1979) reported the DNA content of the organism to be equivalent to the diploid *S. cerevisiae*. Olaiya *et al.* (1980) found that some strains of *C. albicans* unable to form germ tubes had half the DNA content of the dimorphic strains. Hybridization of *C. albicans* spheroplasts by fusion has also been carried out (Poulter *et al.*, 1981; Saracheck *et al.*, 1981) which would permit genetic analysis. However, so far, the genetic tools being developed for *C. albicans* have not applied to problems of dimorphism.

1.11 MODELS FOR FUNGAL DIMORPHISM

An implied assumption in studies of fungal morphology is that cell shape is determined by the cell wall, therefore structural studies of fungal dimorphism appear to be currently directed at testing a model which has the following salient features. Vesicles containing the necessary polysaccharide synthetases and precursors are assembled at or near

the Golgi apparatus and/or the endoplasmic reticulum, and moved from internal tracts of the cytoplasm to sites of wall growth. Lysins, to weaken or cleave pre-existing wall polymers, are also positioned at growth sites and may be transported in other vesicles.

At the walls growth site, vesicles fuse with or are transferred across the cell membrane and the synthetases begin or continue the synthesis of major microfibrillar polysaccharides found in dimorphic fungi. The secretion and formation of new microfibrils is promoted by the lysins and synthetases, and cross-linking proteins or matrix polysaccharides consolidate the new microfibrils. A "growth unit" (Bartnicki-Garcia, 1973) thus functions. Therefore, it is the triumvariate cytoplasm $\leftrightarrow$ membrane $\leftrightarrow$ wall which is involved in the morphogenesis of the fungal cell. With chitin synthesis for example, the enzyme chitin synthetase is present in *S. cerevisiae* plasma membrane as latent zymogen (Cabib *et al.*, 1974). It can be activated by the action of a protease which is present in the vesicles in the cytoplasm. Thus, the site and timing of initiation of chitin deposition may be controlled by the selective activation of zymogen molecules in the membrane by release of the protease from the vesicles at that point (Gooday, 1978). It was also been shown that the protease while rapidly activating the chitin synthetase zymogen, will also slowly inactivate the resultant chitin synthetase thus providing a mechanism for termination of chitin synthesis (Hasilik, 1974). Chitin synthetase in *Mucor* is also controlled in a similar manner; some of the chitin synthetase in the cell homogenates is in an inactive latent form and can be activated by proteolysis; this protease is inactivated by an inhibitor and the active chitin synthetase is inactivated by further incubation with the protease (McMurrough and Bartnicki-Garcia, 1973; Ruiz-Herrera and Bartnicki-Garcia, 1976). In *M. rouxii* chitin synthetase as mentioned in Section 1.9.3 has different properties in the yeast and mycelial cells. The yeast cells have a synthetase that is predominantly latent, have low protease activity, and a high inhibitor level, and *vice versa* in mycelial cells. They rationalize these differences with the two different growth forms: the apical extension of hypha is maintained by a sharp gradient of active chitin synthetase which is rapidly inactivated during transition from tip to lateral wall, while the isodiametric expansion of the yeast cell does not require critical control of inactivation of the enzyme.

However, the aspects of the model which are uncertain are:

1. Selection of growth sites within the cells

2. How are the vericles containing the precursors and enzymes transported from sites of synthesis to sites of wall growth

3. How the external or environmental signals are transmitted to the controlling elements in (1) and (2).

Growth site selecting and coupling of external signals to wall synthesis may be closely related phenomena. This coupling process is being studied in terms of membrane potential and cyclic nucleotides (Terenzi and Storck, 1969; Stewart and Rogers, 1983).

Several possibilities exist as to how the vesicles may find their way from Golgi apparatus or the endoplasmic reticulum to specific sites in the cell wall. Cytoplasmic streaming could deliver vesicles rapidly to particular points in the cell. However, these streams themselves need to be specifically directed, although random collision between vesicles and sites of deposition would occur. In such a case specific recognition sites on vesicles and plasma membrane or the target site are important (Stewart and Rogers, 1978). The possibility of microtubules and microfilaments influencing cell shape by positioning and stabilizing the cell wall synthesizing enzymes could occur. Microtubules could also specify sites of access of vesicles to the plasma membrane either by presenting a barrier or by generating a guiding structure, thus regulating the supply of precursors for cell wall synthesis.

In *P. brasiliensis*, β-1,3 glucan occurred in mycelial form, while α-1,3 glucan occurred in yeast form. These differences led Kanetsuna *et al.* (1972) to propose a hypothesis to explain the morphological changes observed during transition from yeast to mycelium. Since the α-1,3 glucan shapes the outer layers and chitin the inner layer in the yeast cell wall (Carbonell, 1969), these authors suggested that the low amounts of β-glucan may be localized in discrete islets in the yeast cell wall. Through an unknown mechanism that may involve the participation of β-glucanase and disulfide reductase, the yeast wall would soften around the β-glucan islets, allowing the formation of a bud. At 37°C, the synthesis of α-glucan and chitin is more active than that of β-glucan, and the high activity of disulfide reductase limits the frequency of disulfide links in the yeast-wall proteins. Both processes result in the formation of a daughter cell with the round shape characteristic of yeasts. Conversely, at 22°C,

the synthesis of α-glucan decreases (probably due to inhibition/regulation of α-glucanase) and formation β-glucan occurs in budding places. The inhibition of α-glucan results in the formation of a tunnel through which the M form extends, into an elongated shape characteristic of hyphae (Kanetsuna, 1981).

The more recent model by San-Blas and San-Blas (1985), gives relevance to the transition stages, not considered in the earlier model (Kanetsuna, *et al.*, 1972, 1981). The new model is also based on the same assumption as that of Kanetsuna *et al.* (1972). During Y → M transition, the synthesis of new RNA and proteins, as well as growth, stops for 8h; α-1,3 glucan synthesis decreases and buds are produced by the yeast cells. At this early stage of transition, no synthesis of β-1,3 glucan occurs. The β-1,3 glucan present in the yeast cell may exist as islets in the cell wall. Through the actions of β-glucanase and protein disulfide reductase a loss in cell wall rigidity may occur. The decrease in the rate of synthesis of α-1,3 glucan and the internal pressure on the softened cell wall may then initiate the bud, the cell wall of which would at this initial stage of dimorphism still be made of α-1,3 glucan and chitin. It is assumed that at this stage there would be no apical growth and the elongation of the mycelial bud would still be under the control of Y cell. Later on, when synthesis of new proteins resumed, β-1,3 glucan would be syntheized and apical growth resulting in the building of hyphal would proceed as suggested by Kanetsuna *et al.* (1972).

1.12 SUMMARY

While morphogensis in living organisms is usually a definite part of their life cycle and essential to growth and maturation, fungal dimorphism is not essential to the fungal life cycle but rather a response to a change in the habitat where it develops (San-Blas and San-Blas, 1983). Although, work has been carried out on the effect of stimulus (temperature or nutrition), and insight has been gained in the process affected (cell wall synthesis, cAMP influence etc.), the phenomenon is not yet clearly understood. To fully understand the process of dimorphism, research needs to be carried out in all events which oscillate between gene activation at one end and morphological changes in the other (San-Blas and San-Blas, 1983).

Determinants in the process of morphogenesis are the biochemical pathways responsible for the cell metabolism. The data available, tends to suggest that despite superficial similarities, dimorphic fungi differ amongst themselves in underlying mechanisms, making it difficult for any generalisation from a given set of results.

The cell wall has been considered as the main structure affected by morphogenetic changes. This line of research has received constant attention, but the results are disappointing since morphological variations in fungi do not always parallel important changes in their cell wall structures. The synthesis and degradation of the cell wall components has also been a subject of intensive research. Again, no general rules can be drawn from the results obtained so far. In this respect, isolation of morphological mutants and protoplasts will provide a better understanding of the role of fungal cell wall and also cell membrane in the process of dimorphism.

Since dimorphism is an important feature of most pathogenic fungi, it is expected that research will continue on the above and other lines to fully understand this phenomenon.

1.13 PRESENT INVESTIGATIONS

Based on the above literature survey, studies on *Benjaminiella poitrasii* (a dimorphic fungus isolated in our laboratory) were carried out as listed below:

1) The signals triggering the morphological transitions.

2) The isolation of morphological mutants which is a pre-requisite to understand the phenomena of dimorphism.

3) The isolation of cell wall, their chemical composition and cross-linking of wall components.

4) The significance of glutamate dehydrogenase, glutamate synthase and glutamine synthetase in the dimorphic phenomena.

5) The enzymes involved in synthesis and degradation of chitin i.e., chitin synthetase and chitinase.

MATERIALS AND METHODS

CHAPTER II

CHAPTER II

2.1 MATERIALS

The materials and chemicals used in studies were purchased from the suppliers shown in Table 2.1.

2.2 METHODS

2.2.1 *Reagents and apparatus*

All reagents and buffers were prepared in glass distilled water. The operations for cell wall isolation and enzymatic studies were performed at 0-4°C. Centrifugations were carried out in Sorvall (Model RC-5) centrifuges. Measurements of absorbance in ultraviolet and visible range were carried out using Beckman (Model 25) or Shimadzu spectrophotometer (Model uv 240) in cuvettes of 3 ml or 1 ml capacity with 1 cm light path.

2.2.2 *Organism*

The parent strain *Benjaminiella poitrasii*, which was isolated from a soil sample and identified by Dr. M.C. Srinivasan, NCL, Pune, has been used in the studies. The morphological mutants Y-2 and Y-5 were isolated from this strain.

2.2.3 *Media and culture conditions*

Stock cultures were maintained by subculturing weekly on YPG agar slants (Table 2.2). The different types of media used in the course of this study are also presented in Table 2.2.

2.2.4 *Spore suspension*

Parent strain spores were obtained from 7-day old cultures grown at 28°C on YPG agar media. Spores were harvested, washed and resuspended in sterile distilled water, and counted on a haemocytometer grid. Approximately, 10^6-10^7 spores were inoculated per 50 ml of culture media.

2.2.5 *Separation of phases and estimation of growth*

Physical separation of the yeast and mycelial phase was done by filteration through a glass filter (G 1, Jensil, India). Accordingly, yeast phase (Y) cells were obtained

Table 2.1: Source of the chemicals and materials

Chemicals, Materials	Suppliers
Ammonium chloride, sodium tetraborate, p-dimethylaminobenzaldehyde, anthrone, sodium nitrite	British Drug House Ltd., India
Malt extract, Bacto yeast extract, Bacto peptone, Bactoagar	Difco Laboratories, USA
N,N,N′,N′ tetramethyl ethylene diamine (TEMED)	Eastman Kodak Co., Rochester, NY, USA.
Sodium arsenate, silver nitrate	E. Merck Ltd, India
N-Methyl-N′-nitro-N-nitrosoguanidine (NTG), 2-mercaptoethanol, carbazole, ethanol	Fluka Chemie AG
UDP-[U 14-C] GlcNAc, 2,5-diphenyl oxazole (PPO), 1,4-di-2 (5-phenyloxazoly)-benzene, 4-methyl-umbelliferyl-$(NAG)_4$	Gifted by Prof. G.W. Gooday Aberdeen, U.K.
Isophthalic acid	Gifted by Dr. S. Ponrathnam, NCL, Pune, India
Dextrose (AR grade)	Glaxo Laboratories Ltd., India
Glucose oxidase kit	Kabi Diagnostica, Sweden
Glucosamine. HCl	Koch-light Laboratories, U.K.
Tris Buffer	Loba-Chemie Indoaustranol Co., India
Hydrogen peroxide (H_2O_2)	Qualigenes Pvt. Ltd., India
Hydroxylamine.HCl	Rudipont-industria chimica, S.P.A-Torino, Italy.
Nystatin, chloramphenicol, tetracycline	Sarabhai M Chemicals, India
Glacial acetic acid, acetone, acetic anhydride, chloroform, methanol (all AR grade)	S.D. Fine Chemical Co., India

Table 2.1 contd...

Chemicals, Materials	Suppliers
Cycloheximide, α-ketoglutarate NADH, NADPH, L-glutamine, N-acetyl-glucosamine, UDP-N-acetylglucosamine, glucuronolactone, azaserine, methionine sulfoximine, acrylamide, N,N′-methylene-bisacrylamide, Coomasie brilliant blue, adenosine triphosphate, adenosine diphosphate, cyclic AMP, 5′-AMP, 3′-AMP, adenine, adenosine, para-nitrophenyl-β-D-galactopyranoside, carboxymethyl cellulose	Sigma Chemical Company, USA
Trichloroacetic acid (TCA), Folin-phenol reagent	SRL Laboratories Pvt. Ltd., India
Rest of the chemicals	Analytical Grade (AR)

Table 2.2: Composition of the complex media[a]

Composition[b]	YP	YPG	MYP	MYPG
Yeast extract	3.0	3.0	3.0	3.0
Malt extract	-	-	3.0	3.0
Bacto peptone	5.0	5.0	5.0	5.0
Glucose[c]	-	10.0	-	10.0

a The media composition is given in gm per 1000 ml of distilled water and the final pH adjusted to 6.5.

b Bactoagar (2.0% , w/v) was used for preparation of agar plates and slants.

c Generally, the concentration of glucose used was 1.0% (w/v), unless otherwise mentioned.

in the filterate, while mycelial phase (M) cells remained on the filter. Cells were examined microscopically for their morphology. Growth of the separated phases was determined on dry weight basis. The cells were dried in an incubator (80°C) till constant weight was obtained.

2.2.6 *Determination of morphology*

Morphology was determined according to Chattaway *et al* (1973) and Peters and Sypherd (1979). Aliquots were taken from each sample just before harvesting and examined microscopically on a haemocytometer grid. Single or budding cells were counted as one yeast morphological unit; cells with one or more germ tubes were counted as one hyphal morphological unit. In mycelium-to-yeast transition studies, mycelial strands were counted once for each haemocytometer square in which they appeared. During this transition, mycelial strands form beaded structures. Since a single hyphal cell can give rise to a number of yeast cells, the percentage of hyphal cells not forming the above beaded structures were expressed as a percentage of total cell count.

2.2.7 *Transition studies*

For these studies, mycelial phase cell and yeast phase cell inocula were prepared from spore suspensions by incubating the flasks under shaking conditions (200 rpm) for 48h at 28°C and 37°C, respectively. For, yeast-to-mycelium (Y → M) transition, the yeast cells (8×10^6 cells/50 ml media) were used to inoculate YPG medium and incubated at 28°C under shaking conditions. On the other hand, in mycelium-to-yeast (M → Y) transition, studies were carried out at 37°C using mycelial phase cells as an inoculum.

2.2.8 *Mutagenesis and selection of morphological mutants*

N-Methyl-N′-nitro-N-nitrosoguanidine (NTG, Lot No. 222293 681) was used for the mutation studies. 10^6 spores/ml from a 7-day old *B. poitrasii* parent strain slant were harvested in sterile distilled water and after centrifugation resuspended in 5.0 ml sterile 0.05 M citrate-phosphate buffer, pH 6.6, containing 4.0 mg/ml NTG for 10-20 min under shaking conditions at 28°C. The mutagen treated spores were centrifuged, washed 2-3 times with sterile distilled water and plated on MYPG agar medium, containing 1.0% glucose. Plates were incubated for 5-10 days at 28°C to obtain discrete colonies.

Survival curves were done with a constant time of exposure (10 min) and varying the doses of NTG (2.0-6.0 mg/ml) as well as with a constant concentration of NTG (4.0 mg/ml) and different times of exposure (10-30 min). With 4 mg/ml NTG treatment for 15 min it was possible to obtain 4.0% of the survivors as morphological (yeast form) mutants. Colonies with a characteristic yeast morphology were selected by visual screening. The mutants were maintained on MYPG agar slants at 28°C.

2.2.9 *Growth on various carbon sources*

The parent and mutant strains were grown at 28°C for 48h under shaking conditions in a defined medium, pH 6.5 (Table 2.3) as well as in a complex medium (MYPG liquid medium, pH 6.5). Glucose was replaced with other carbon source as indicated in Table 2.3.

2.2.10 *Cell free extract preparation*

Cell free extracts were prepared as described by Ferguson and Sims (1971). Cells were collected on Whatman filter papers, washed with ice-cold distilled water, followed by potassium phosphate buffer (5 mM, pH 7.2 containing 0.25mM EDTA, 50 mM K_2SO_4) and broken in a Braun's homogenizer for 60s (2 cycles of 30 s each). The extract was centrifuged at 12,500 x g for 15 min and the supernatant used for the estimation of enzyme activities. β-Mercaptoethanol to a final concentration of 1.0 mM was added to the extracts for glutamate dehydrogenase and glutamate synthase activity determinations, after aliquots had been withdrawn for the other enzyme activities and protein determination.

2.2.11 *Enzyme assays*

Cellulase (EC 3.2.1.4): Extracellular endoglucanase activity was determined by estimating the reducing sugars liberated following the hydrolysis of carboxymethyl cellulose (CMC) by DNSA method (Miller, 1969). The reaction mixture (1.0 ml) containing 0.5 ml of CMC (1.0%) in acetate buffer (50 mM, pH 4.5) and the appropriately diluted enzyme was incubated at 50°C for 30 min. The reaction was terminated by the addition of 1.0 ml DNSA followed by heating in a boiling water bath for 5 min. After cooling, 10.0 ml of distilled water was added to the mixture and the colour was read at 540 nm.

Table 2.3: Composition of the defined medium[a]

Components	%
KH_2PO_4	0.2
$(NH_4)_2HPO_4$	0.7
$MgSO_4 . 7H_2O$	0.03
$CaCl_2 . 2H_2O$	0.03
Glucose (or other carbon sources as indicated)[b]	1.0
	mg/ml
$FeSO_4 . 7H_2O$	0.5
$MnSO_4$	0.16
$ZnSO_4$	0.033
$CoCl_2$	0.2

a The final pH of the medium was adjusted to pH 6.5.

b Carbon sources used: Xylose, mannose, sucrose, lactose, starch and carboxymethyl cellulose.

One unit (U) of cellulase activity was defined as the amount of enzyme required to release 1.0 µmol of glucose/min/ml under the given assay conditions.

α-Amylase (EC 3.2.1.1): Extracellular α-amylase activity determination was carried out according to Worthington Enzyme Manual (1977). The assay mixture (1.0 ml) containing 0.5 ml of soluble starch solution (1.0%) in sodium phosphate buffer (0.02M, pH 6.9) and the appropriately diluted enzyme was incubated for 30 min at 30°C. DNSA reagent (1.0 ml) was added followed by heating in a boiling water bath for 5 min. After cooling, 10.0 ml distilled water was added and the colour was read at 540 nm.

One unit (U) of activity is defined as the amount of enzyme required to release from soluble starch 1.0 µ mol of maltose/min/ml under the given assay conditions.

β-galactosidase (EC 3.2.1.23): The extra- and intracellular enzyme activities were measured according to Worthington Enzyme Manual by measuring the increase in absorbancy at 405 nm resulting from the hydrolysis of p-nitrophenyl-β-D-galactopyranoside.

One unit (U) causes the hydrolysis of 1.0 µmol of p-nitrophenyl-β-D-galactopyranoside /min/ml under the given assay conditions.

Alcohol dehydrogenase (ADH, EC 1.1.1.1): The method used for its estimation was according to the Worthington Enzyme Manual in which the rate of absorbance at 340 nm resulting from reduction of NAD by ethanol is measured. The reaction mixture contained 20-50µl crude extract, sodium pyrophosphate buffer (0.03M, pH 8.8), ethanol (2.0 M). The reaction was initiated by the addition of NAD (0.025M) to the cuvette and the change in A_{340} was recorded for 4 min. A control was run for each assay in which ethanol was omitted from the reaction mixture.

One unit (U) of enzyme activity was defined as the amount of enzyme causing reduction of 1.0 µmol of NAD/min/mg protein.

Glutamate dehydrogenase (GDH): Intracellular NAD- and NADP-dependent GDH (EC 1.4.1.2 and EC 1.4.1.4, respectively) were assayed according to Peters and Sypherd (1979). Activity was measured in the reductive amination of α-ketoglutarate by following the decrease in absorbance of NAD(P)H at 340 nm. The reaction mixture (1.0 ml) contained 50-100 µl crude extract, NH_4Cl (200 mM) and α-ketoglutarate (30 mM) in potassium

phosphate buffer (100 mM, pH 8.0). The reaction was initiated by the addition of NADH or NADPH (0.125 mM) to the sample cuvette. A control was run for each assay in which α-ketoglutarate was omitted from the reaction mixture.

Glutamate synthase (GOGAT, EC 2.6.1.53): GOGAT was measured according to Roon *et al.* (1974) by following NADH and NADPH oxidation at 340 nm The reaction mixture (1.0 ml) contained 50 µl crude extract, α-ketoglutarate (10 mM), L-glutamine (10 mM), freshly prepared, in potassium phosphate buffer (100 mM, pH 7.8). The reaction was started by the addition of NADH or NADPH (0.2 mM) to the sample cuvette. Controls were run for each assay in which L-glutamine was omitted from the reaction mixture.

One unit (U) of GDH or GOGAT activity was defined as the amount of enzyme causing oxidation of 1.0 nmol of NAD(P)H/min/mg protein.

Glutamine synthetase (GS, EC 6.3.1.2): GS was measured according to Bender *et al.* (1977). A fresh concentrated assay mixture was prepared, containing hydroxylamine.HCl (18 mM), $MnCl_2$ (0.27 mM), sodium arsenate (25 mM), ADP (0.36 mM), Tris-HCl buffer (135 mM, pH 7.2). The pH was adjusted to 7.2 at room temperature with 2.0 M NaOH or 1.0 M HCl. The reaction mixture contained 0.4 ml of the above assay mixture, 0.2 ml crude extract and 0.35 ml glass distilled water. The reaction mixture was equilibrated for 5 min at 37°C. Reaction was initiated by the addition of 0.05 ml of 0.2 M L-glutamine (final concentration 20 mM). After incubation at 37°C for 15 min, the reaction was terminated by the addition of 1.0 ml stop mixture (55 g $FeCl_3.6H_2O$, 20 g Trichloroacetic acid and 21.0 ml concentrated HCl in a final volume of 1.0 litre). Samples were centrifuged for 10 min to remove precipitate. The absorbance was read at 540 nm.

One unit (U) of enzyme activity was defined as the amount of enzyme producing 1.0 nmol of γ-glutamylhydroxymate/min/mg protein.

Chitin synthetase (EC 2.4.1.16): The enzyme assays were carried out using the filter assay for chitin synthetase (Gooday, personal communication). This method is based on the fact that newly-synthesized chitin microfibrils adsorb onto the glass-fibre matrix of Whatman GF/C filters while the unincorporated radioactive substrate passes straight through. The standard assay volume was 25µl containing 5µl sample and final concentrations of

Tris-HCl (50 mM, pH 8.5), $MgCl_2$ (10 mM), EDTA sodium salt (1 mM) and N-acetylglucosamine (GlcNAc, 25 mM) and uridine diphosphate N-acetylglucosamine (UDPGlcNAc, 1 mM) containing 12.5 nCi UDP-[U 14-C]-GlcNAc. The assay mixture was incubated at 37°C for 30 min and terminated by the addition of 10% (w/v) trichloroacetic acid (TCA). The reaction mixtures were filtered through glass microfibre filters (Whatman GF/C, 2.5 cm) on a Millipore manifold system.

The filters were presoaked in 5% (w/v) TCA in sodium pyrophosphate ($Na_4P_2O_7$, 20 mM) solution and rinsed with distilled water to prevent substrate adsorption. The reaction tubes were washed thrice with 100µl Triton-X-100 (1.0 %, v/v) and once with distilled water. The filters were transferred into scintillation vials and dried at 80°C for 8h prior to radioactive counting.

Counting of radioactivity: Each vial was filled with 4.0 ml scintillation fluid. The scintillation fluid contained 4.0 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-di-2-(5-phenyloxazoly)-benzene (POPOP) per litre of toluene. Radioactivity was counted for 5 min in a Packard 300-C liquid scintillation counter.

Method of conversion to Katals: Katals is defined as the activity expressed as moles of substrate converted per second, in this case being the conversion of UDP-N-acetylglucosamine to the chitin polymer.

Each sample contained 20µl of assay reagent containing 1.0 mM UDP-N-acetylglucosamine as substrate. This is equivalent to 20 nmol of substrate which gives a count of 22069 CPM (counts per min) when placed in the scintillation counter. Therefore, 1.0 nmol is equivalent to 1103 CPM, which allows us to calculate the nmol converted after the incubation hour. This value is then converted to give a value per second on dividing by 3600 (Gooday, personal communication).

Chitinase (EC 3.2.1.14): The chitinase activity was measured by using a modified method of Yang and Hamaguchi (1980). The activity of the whole cells was measured using phorogenic substrate, 4-methyl-umbelliferyl-$(NAG)_4$, 0.8 mM in 50% ethanol (Gooday, personal communication). The assay containing 50 µl substrate and 50µl of the whole cells were incubated at 37°C for 30 min. Appropriate blanks, which contained

the same concentrations of substrate but no enzyme, were also incubated. The amount of 4-methylumbelliferone was then determined fluorometrically by measuring fluorescence intensities at 440nm with excitation at 360nm. Chitinase activity was also determined using acid-swollen chitin as a substrate. Phosphoric-acid swollen chitin was prepared as described earlier (Vyas and Deshpande, 1989). The reaction mixture containing 1.0 ml of 0.7% acid-swollen chitin, 1.0 ml of acetate buffer (0.05 M, pH 5.0) and 1.0 ml of crude extract was incubated at 50°C for 1h. The amount of reducing sugars produced was determined by the Nelson method, using NAG as standard.

One unit was defined as the amount of enzyme producing 1.0 µmol of product/min/mg protein.

2.2.12 *Protein determination*

Samples were precipitated with ethanol for 16h at 4°C. The sample was centrifuged at 12,500 x g for 10 min. The pellet was then resuspended in glass distilled water and protein estimated according to Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard. Protein was also determined according to Bradford (1976), wherever mentioned.

2.2.13 *Isolation of cell walls*

All operations were conducted between 0-4°C to prevent endogenous degradation. The cells were harvested on Whatman filter papers, and the check for purity of samples was carried out using light microscopy. Cells were washed and resuspended in cold distilled water. The cells were broken by mechanical shaking with glass beads (0.45-0.5 mm) in a Braun homogenizer. Breakage of cells was determined by light microscopy and the minimum cell breakage was found to be 95%. The homogenate was freed from glass beads by decantation. The cell walls were sedimented by centrifugation at 1,500 x g for 10 min. The pellet was washed in cold distilled water (5-6 times) until the supernatant became clear. The pellet was then resuspended in distilled water and the cytoplasmic material and membranes adhering to the cell walls was eliminated by the following two steps: the resuspended pellet was sonicated twice for 30s each in an ice bath. After centrifugation at 1,500 x g for 10 min,

the cell wall fragments were washed in cold sodium chloride (NaCl) with decreased concentrations (5.0%, 2.0% and 1.0%) successively, twice each, followed by washings (10-12 times) in cold distilled water. The purity of the preparation was determined by examination under light microscopy and by measuring the absorbance at 260 nm and 280 nm at each step. The purified samples were lyophilized and stored at -10°C.

2.2.14 *Cell wall hydrolysates and fractionation*

Cell wall hydrolysis: For cell wall analysis studies, two types of hydrolyzates were prepared: (a) for the analysis of sugars, cell walls were hydrolyzed in sealed tubes in 1N HCl at 100°C for 6h, according to Moreno *et al.* (1969). The HCl was evaporated *in vacuo* over NaOH pellets and the residue suspended in a small amount of glass distilled water. The samples were centrifuged and passed through a column (0.5 cm x 7.0 cm) of Dowex-1 (OH^- form) and Dowex-50 (H^+ form). The desalted solution was lyophilized and used for the estimation of sugars; and (b) for the analysis of aminosugars and amino acids, the cell walls were hydrolyzed in sealed tubes under nitrogen with 6N HCl at 105°C for 8h and 16h, respectively. The HCl was evaporated *in vacuo*, the residue dissolved in glass distilled water and the insoluble material removed by centrifugation.

Cell wall fractionation: Studies on cell wall fractions in order to determine the cross-links between the various cell wall components was determined according to Siestma and Wessels (1981). The cell walls were extracted twice with 1M KOH under nitrogen at 60°C for 20 min. The residue was washed with water, then dried and treated with dimethyl sulfoxide (DMSO) at room temperature for 16h under shaking conditions. The material remaining after DMSO extraction was washed with water and treated with 40% (w/v) NaOH under nitrogen at 100°C for 1h. The residue was then treated with nitrous acid (HNO_2) and finally extracted with 1M KOH at 60°C for 10 min. After each extraction procedure a sample was taken from the washed insoluble residue to determine the total amount of carbohydrate, glucan and glucosamine.

Discrimination between chitin and chitosan in cell wall fractions: This was carried out according to Briza *et al.* (1988). Cell walls were suspended in 1.0 ml glass distilled water and 1.5 ml of 2 M $NaNO_2$ (sodium nitrite) and 0.5 ml of 2M HCl was added. After 4h

at room temperature, the residual wall fragments were removed by centrifugation and hydrolyzed in 6 M HCl at 100°C for 8h. After removal of HCl *in vacuo*, glucosamine and N-acetylglucosamine were determined as described in Section 2.2.15. As controls, walls were acetylated with acetic anhydride or deacetylated with 40% NaOH at 100°C and treated with HNO_2 as described above.

2.2.15 *Methods of analysis*

Estimation of total hexoses: The carbohydrate content was estimated by the anthrone test according to Stewart (1975) using glucose as standard. The difference between anthrone values and glucose values as determined in the hydrolysates by the glucose oxidase-peroxidase method (Bergmeyer *et al.* 1983) was used as a measure of the amount of mannose in the samples, taking into account the difference in molar extinction coefficients of glucose and mannose in the anthrone reaction.

Estimation of hexosamines: Aminosugars were determined using the method of Good and Bessman (1964) for total glucosamine using glucosamine.HCl as a standard. The method of Ressig *et al.* (1955) was used for acetylglucosamine determination, using N-acetylglucosamine as standard.

Estimation of protein and aminoacids: Protein was determined spectrophotometrically by the method of Lowry *et al.* (1951), following digestion of cell walls in 1 M NaOH for 10 min at 100°C and centrifugation to remove insoluble material. Crystalline bovine serum albumin was used as a standard and treated in the same manner. Amino acids in the hydrolysates were analyzed in an automated amino acid analyzer.

Estimation of uronic acids: Uronic acid were determined by the carbazole procedure as modified by Bitter and Muir (1962), using glucuronolactone as a standard. Cell walls were hydrolyzed with 1.0% HCl at 100°C for 3h according to Claviere-Martin *et al.* (1988).

Lipid extraction and determination: Lipid content in the isolated cell walls was determined by the method of Claviere-Martin *et al.* (1988). Readily extractable or free lipids

were obtained by extracting the cell walls for 18h at room temperature with chloroform/methanol (2:1, v/v) at 45°C for 3h. Bound lipids were extracted from the insoluble material with 2M HCl/chloroform/methanol (1:16:8, v/v at 45°C for 3h. The extracts were then evaporated to dryness and weighed.

Others: For ash determination, cell wall samples were heated to 600°C until constant weights were obtained. For total phosphate determination cell wall material was digested for 60 min with 10 N H_2SO_4 at 130°C, cooled and 30% H_2O_2 added and again heated at 130°C for 10 min (Chattaway *et al.*, 1968). On cooling, distilled water was added and after heating at 100°C for 30 min, the phosphate in the hydrolysates was determined by the method of Ames (1966).

Amino acid analysis: Cell wall samples were hydrolyzed in 6 N HCl at 110°C for 16h in evacuated sealed tubes as described previously. The residue obtained after removal of HCl was dissolved in citrate buffer, pH 2.2 and aliquots were analyzed using Beckman automatic amino acid analyzer (Model 120B) by the method of Spackman *et al.*, (1958). A standard mixture of amino acids obtained from Sigma was run prior to the samples.

Gas-liquid chromatography: Quantitative analysis of total fatty acids was performed by gas liquid chromatography on Shimadzu GC R1A. Chloroform/methanol extracted samples were injected into a column of 10% diethyleneglycol succinate (DEGS) on chromosorb W/HP 80-100 mesh at a column temperature of 185°C.

Paper chromatography: For the detection of sugars in hydrolysates, paper chromatography was performed on Whatman No.1 filter paper irrigated with ethylacetate-pyridine- water (8:2:1, v/v). Spots were developed with alkaline silver nitrate (Trevelyan *et al.* 1950). For quantitative measurements, unsprayed portions of the chromatogram containing the suspected sugars were cut out, eluted with hot distilled water and the sugar estimated by the anthrone method.

Polyacrylamide gel electrophoresis: Polyacrylamide slab gel electrophoresis of proteins was performed as described by Laemmli (1970). Constant protein was loaded for each sample to determine protein by staining with Coomassie blue as described by Reisner *et al.*(1975). Protein contents in samples were estimated according to Bradford (1976).

Photomicrographs: Light microscopy was carried out on 'DOCUVAL' microscope. For photography, ORWO DK-5 (35 mm black and white, 25 ASA) film was used. For scanning electron microscopy (SEM), the intact cells were prepared by fixing them in glutaraldehyde (prepared with 0.05 M cacodylate buffer, pH 7.0) at 4°C for 1h. The material was washed in distilled water and dehydrated in graded acetone series. The dehydrated cells were dried in a Polaron E 3000 critical point apparatus using liquid carbon dioxide. The samples were mounted onto specimen holders, coated with gold in a Polaron sputter coater and examined in a Jeol JXA-50 scanning electron microscope.

DIMORPHISM IN *Benjaminiella poitrasii*: ISOLATION AND BIOCHEMICAL STUDIES OF MORPHOLOGICAL MUTANTS

CHAPTER III

CHAPTER III

3.1 INTRODUCTION

Dimorphism, the ability of cells to grow either as yeast-like or mycelium-like (the terms mycelium, hyphal and filamentous are used equivalently), is a reversible phenomena observed among various taxonomic groups of fungi, e.g., *Mucor, Mycotypha* and pathogens like *Candida, Sporothrix* and *Histoplasma* (Bartnicki-Garcia, 1963; Sypherd *et al.* 1978; Schulz *et al*, 1974; Maresca *et al.* 1989; Travassos, 1985; Odds, 1979). The transitions between dimorphic states can be controlled experimentally by environmental and culture conditions. Temperature or nutritional factors or both are usually the causative factors responsible for triggering a change in the fungal morphology. Most of the studies on fungal morphogenesis have dealt with biochemical changes which occur during dimorphic transitions. The nature of dimorphic change and its regulation have been the subject of much experimentation and speculation. In this regard various parameters have been measured; among these are studies on carbon and nitrogen metabolism, rates of protein and RNA synthesis; intracellular levels of cAMP and respiratory activity (Stewart and Rogers, 1983; Shepherd *et al.*, 1985). However, the correlation of these parameters with morphogenesis has still not been clearly understood due to strain dependent variations in stimuli inducing such transitions. Furthermore, it is difficult to separate the causes of dimorphism from its effects.

Biochemical analysis of monomorphic mutants, defective in dimorphic transition would be an useful approach towards the better understanding of the phenomenon, especially by comparing with the 'wild type' parent strain. Employing this approach Ruiz-Herrera *et al.* (1983) studied monomorphic mutants of *Mucor bacilliformis* to distinguish alterations associated with morphogenesis. Cannon (1986) isolated mutant MM2002 from *Candida albicans* by physical separation technique. This technique relies upon the incomplete transition of a culture from the yeast to the mycelial morphology. Cells failing to undergo the mycelial transition were isolated by differential filteration. Nombela *et al.* (1987) also used a similar approach for isolating mutants of *Candida* in order to understand the regulatory mechanisms involved in dimorphism.

The existence of specific genes whose expression would control morphogenesis of hyphae from yeast phase cells has been envisaged as a mechanism for dimorphic regulation. It has also been suggested that the major determinants of cell shape could be subject to metabolic regulation rather than gene expression (Odds, 1975). Thus, present efforts have been directed towards the isolation of mutants altered in functions that regulate dimorphism.

The strain of a rare Mucoralean fungus *Benjaminiella poitrasii* isolated in our laboratory exhibits dimorphic behaviour. To gain a better insight into the regulatory aspects underlying transformations from yeast-like to mycelial-like states, investigations were carried out and results obtained from them are reported here.

3.2 RESULTS

Benjaminiella poitrasii was found to grow in yeast form at 37 ℃ while it grew as mycelia at 28 ℃ on MYPG (1.0% glucose) agar media (Fig. 3.1)

3.2.1 *Life cycle*

The life cycle of *Benjaminiella poitrasii* can be divided into three stages:

A. Spore germination

B. Vegetative growth

C. Sporophore and zygospore formation

A. *Spore germination*

Spores of *B.poitrasii* are ellipsoidal giving rise to either vegetative yeast cells (Fig. 3.2a) or mycelium (Fig. 3.2b) depending on environmental conditions during germination. Irrespective of the ultimate morphology, spore germination can be divided into three phases:

1. Growth of the spore into a larger spherical cell

2. Multiplication of the spherical cell to form a small yeast-like colony

3. Emergence of germ tubes from the above yeast-like cells (in the case of mycelium development) or continuation of colony formation by budding (in case of yeast development).

Phase-1: The spore becomes enlarged 5-10 times its original size and appears as a large spherical cell. This occurs within 6-8 h of transfer to a fresh agar medium and incubation at 28°C or 37°C. This phase represents true growth in that protein content and cell mass (as dry weight) increases several fold during this time (Fig.

Fig. 3.1: Colonial morphology of *Benjaminiella poitrasii* on MYPG agar.
a: M-phase
b: Y-phase
c: Y-2
d: Y-5

Fig.3.1

Fig. 3.2: Scanning electron microscopy of *Benjaminiella poitrasii*.
a: Yeast phase cells
b: Mycelial phase cells

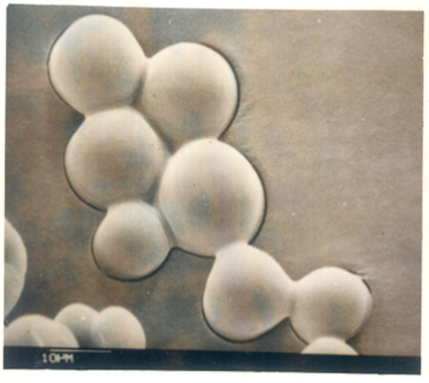


Fig.3.2a

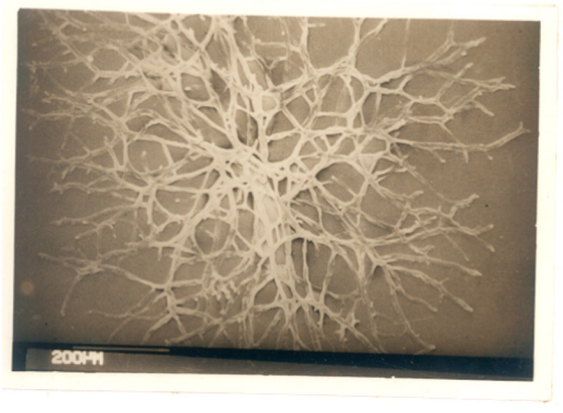


Fig.3.2b

3.3). This phase was found to be common to both yeast and mycelial phases and hardly any difference was found with respect to percent increase in protein content and dry weight. The cells were harvested at varying time intervals and the contents filtered onto Whatman paper No. 1 (size 4.0 cm) of known weight. Spores were washed with water and the dry weight and protein determined as mentioned under Materials and Methods. A considerable increase in cell mass and protein content was observed within 8h (Fig. 3.3).

Phase-2: During the second phase (9-14 h) yeast cells are budded off leading to the formation of an yeast-like colony becoming visible to the naked eye as pin points. The events till this phase in terms of morphology and colony growth behaviour are similar at both the temperatures i.e., 28°C and 37°C.

Phase-3: Further development involves the formation of filamentous mycelial growth at 28°C, which emerge as tubular outgrowths from the yeast-like cells, rapidly branching and leading to the establishment of a rapidly growing mycelial colony. By 48h the development of the asexual sporangiophores, sporangioles, spores and even the initiation of zygospore formation through conjugation of gametangia are observed at 28°C.

At 37°C, however, the mycelial phase of development and the accompanying differentiation of sporangiophores and zygospores is totally suppressed and the colonies continue to grow as yeast cells. Thus, mycelial phase (M) occurs at 28°C while at 37°C, the yeast (Y) phase predominates. The sequence of spore germination as seen under the light microscope is shown in Fig. 3.4.

B. *Vegetative growth*

Parent strain, *B. poitrasii* grew rapidly as mycelium (Fig. 3.5a) in YPG and MYPG at 28°C. Colony size was 0.5 cm at 48h while at 72h it was 0.7 cm. By 72h sporulation had begun. Mycelia are long branched 5-25μM in diameter and aseptate, becoming septate in older cultures. In addition to mycelial-phase (M-phase), *B. poitrasii* produces a large number of yeast-phase cells (Y-phase) cells on YPG and MYPG at 37°C (Fig. 3.5b), the colony size being 0.2 cm at 48 h and 0.3 cm at 72 h. The yeast cells flocculate immediately on standing and growth is characterised by spherical multipolar budding cells. The buds may originate at any location on the mother cell. Other members of Mucorales, like *Mucor rouxii* and *Mycotypha* sp. also form yeast-like budding cells in liquid culture, induced by reduced

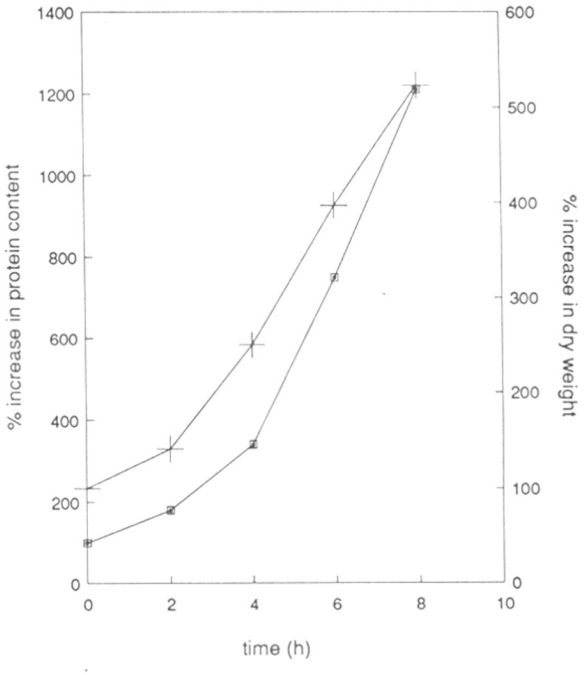


Fig. 3.3: Changes in dry weight and protein content during phase 1 of spore germination in *B. poitrasii.*

protein content
dry weight

Fig. 3.4: Light microscopy of spore germination at 28°C and 37°C in *Benjaminiella poitrasii*.

a-c: Stages common to both phases

d,e: Development of yeast phase growth at 37°C

f-h: Development of mycelial phase growth at 28°C

Fig.3.4a

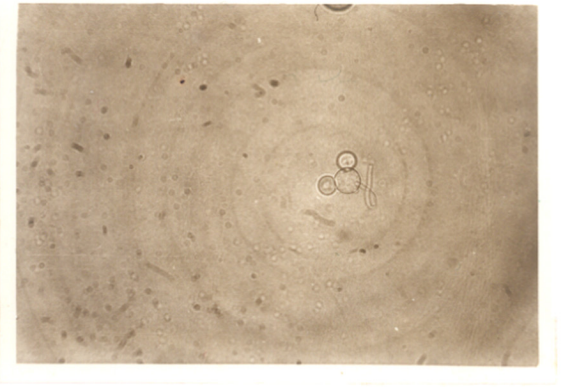


Fig.3.4b

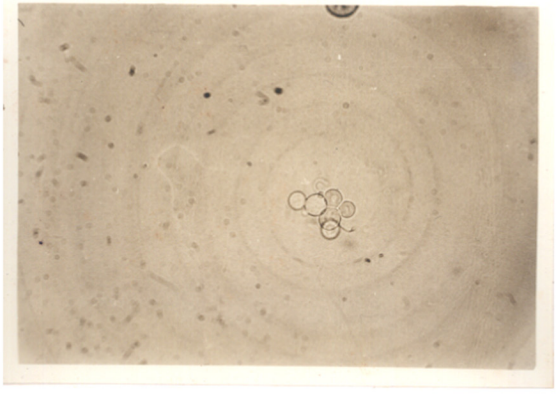


Fig.3.4c

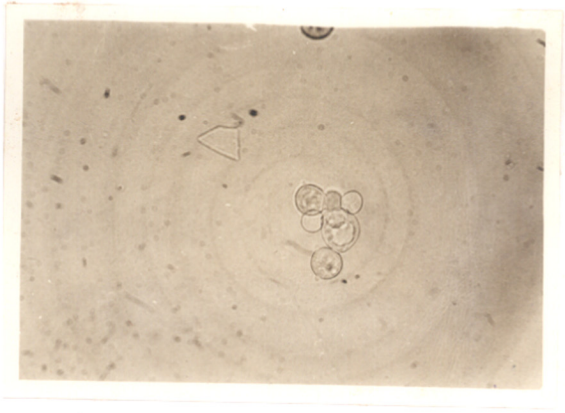


Fig.3.4d

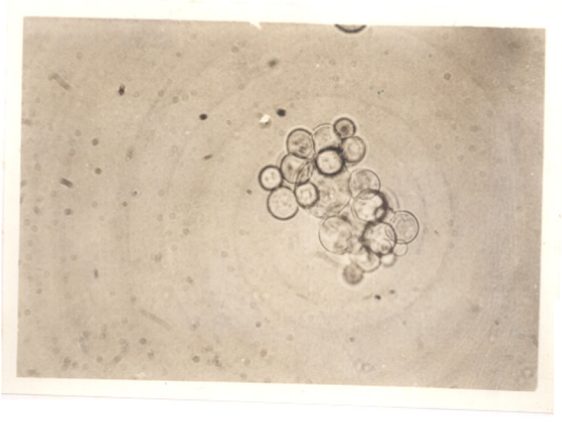


Fig.3.4e

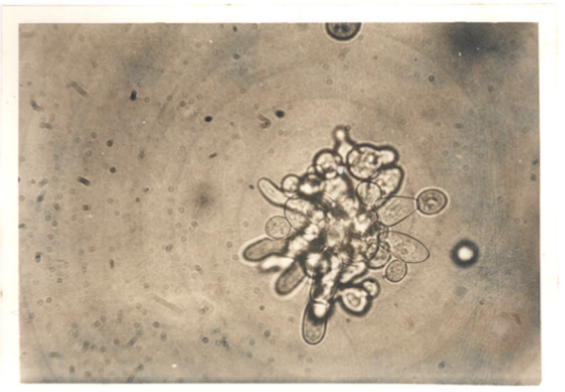


Fig.3.4f

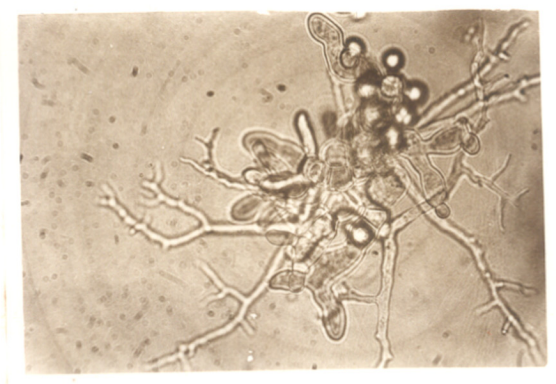


Fig.3.4g

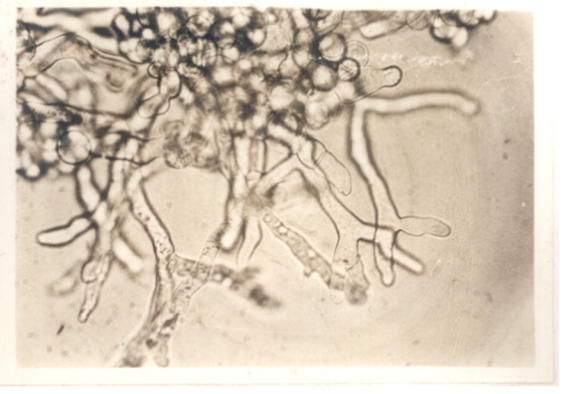


Fig.3.4h

Fig. 3.5: Light microscopy of *Benjaminiella poitrasii* parent strain.
a: Mycelial cells
b: Yeast cells
c: Sporophores
d: Zygospores

Fig.3.5a

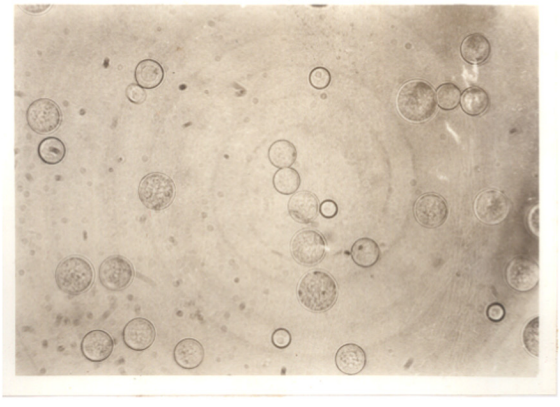


Fig.3.5b

Fig.3.5c

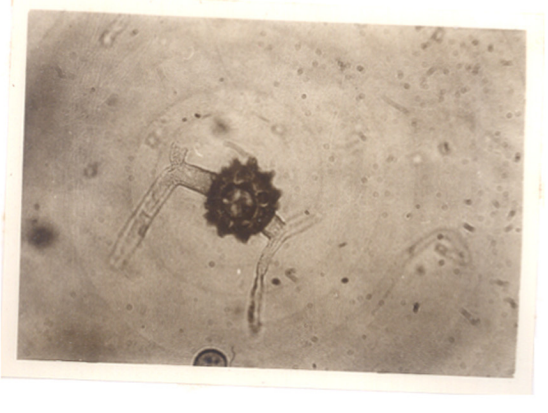


Fig.3.5d

oxygen and/or increased carbon-dioxide levels or pH changes (Sypherd *et al.*, 1978; Schulz *et al.*, 1974). However, *Benjaminiella poitrasii* can produce yeast-phase cells on the surface of solid nutrient rich culture media. This appears to indicate that anaerobic conditions are not required to induce the production of yeast-like cells in this organism.

Dimorphism in *B. poitrasii* is freely reversible and morphological shifts in either direction maybe performed. Transition studies, Y → M (Fig. 3.6) were carried out in MYPG and YPG (1.0%, w/v glucose) at 28°C under shaking conditions. Hardly any difference with respect to percent germ tube formation was found between YPG and MYPG media. It was observed, that by 72 h 45% of the cells were able to form hyphae in 1.0% glucose concentration, while almost all the cells had undergone transition at low glucose concentrations (0.1%). Transition was a rapid process starting at 3h (Table 3.1) irrespective of the glucose concentration. In the case of M → Y transition, spherical cells bud from sides and tips as well as the formation of some beaded structures could be seen (Fig. 3.7). Morphology was similar in the case of both MYPG and YPG media and the transition event was a slow process taking 8-10h and the percentage of Y-phase cells seen was ~15% in 0.1% glucose, while ~21% of the cells had undergone transition in 1.0% glucose concentration (Table 3.1). During morphological shifts in this direction, some hyphal elements always remain in the culture even after long periods, thereby making quantification difficult. It was thus easier and more accurate to quantify percent hyphal cells remaining than percent yeast-cells formed, since one hyphal unit could give rise to a large number of yeast cells). Thus, similar to the observations in *Mucor*, the transition studies show that polarization seems to be more easily gained than lost in *B. poitrasii* also.

C. *Sporophore and zygospore formation*

B. poitrasii at 28°C on MYPG and YPG sporulates within 2-3 days. Sporophores (Fig. 3.5c) arise from substrate hyphae, more or less erect, unbranched, becoming light gray to black in color. Sporophores are 5-10 μm in diameter and forms globose, apical fertile vesicles 25-75μm in diameter. These vesicles give rise over their entire surface, to a large number of pedicels of various lengths, having a single unispored sporangiolum (10μm x 7μm) which are ovoid. Multisporous sporangia are absent (Benny *et al*, 1985). Scanning Electron Microscopy studies (SEM, Fig. 3.8) show that sporophores arise from surface hyphae and grow outwards. During extension of the sporophore initial, the tips swell and give rise to numerous slender outgrowths. Each initial reaches a definitive size prior to elongation of

Table 3.1: Transition studies in *B. poitrasii* parent strain in MYPG under shaking conditions[a].

Time (h)	Y → M[b]		M → Y[b]	
	Germ tube (%)		Hyphal cells (%)	
	A	B	A	B
0	0	0	100	100
3	19	7	100	100
6	78	13	100	100
9	89	14	84	79
12	100	15	78	66
15	100	18	70	58
18	100	20	64	51
24	100	22	60	46
36	-	25	56	38
48	-	29	52	35
72	-	45	46	-
96	-	56	38	-

a The experiments were carried out in MYP media containing 0.1% glucose (A) and 1.0% glucose (B).

b Transitions were carried out as mentioned in Materials and Methods.

- Further observations were not included.

Fig. 3.6: Light microscopy of yeast-to-mycelial (Y→M) transition in *Benjaminiella poitrasii*.

a: 0h
b: 2h
c: 3h
d: 4h
e: 24h

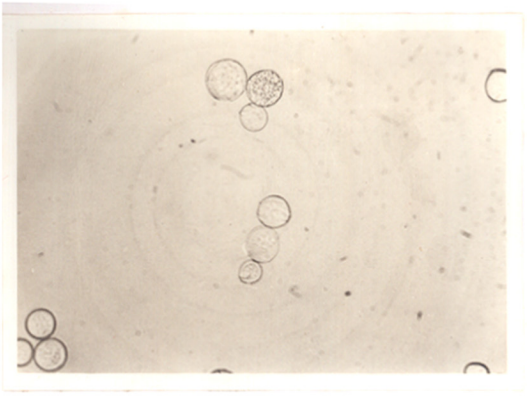


Fig.3.6a

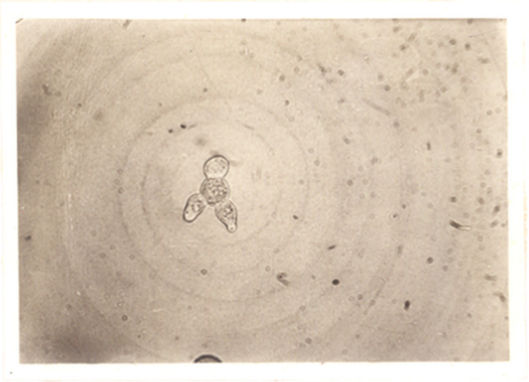
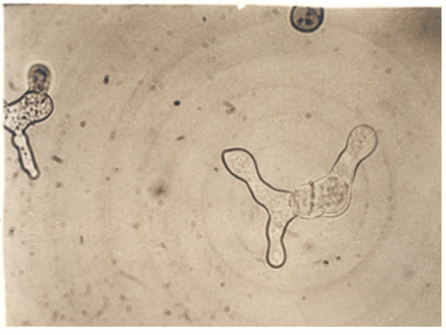


Fig.3.6b



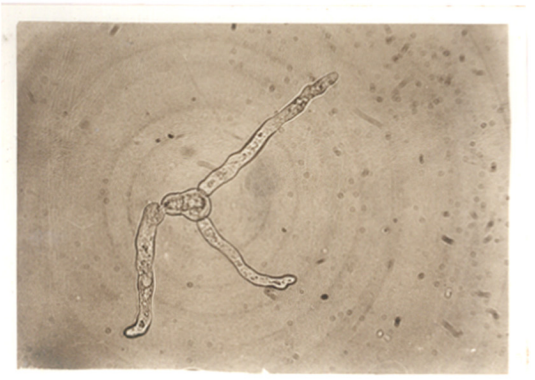


Fig.3.6d

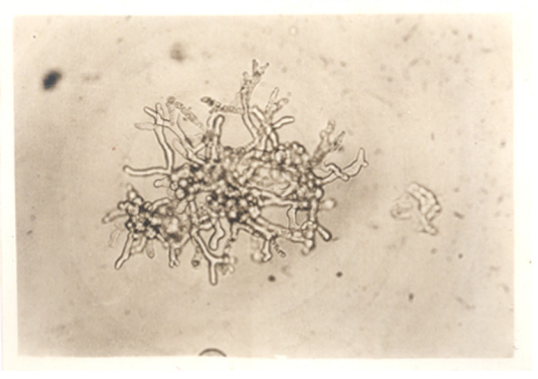


Fig.3.6e

Fig. 3.7: Light microscopy of mycelial-to-yeast (M→Y) transition in *Benjaminiella poitrasii*.

a: 0h
b: 10h
c: 14h (beaded structure formation)
d: 14h-16h (by lateral budding)
e: 24h

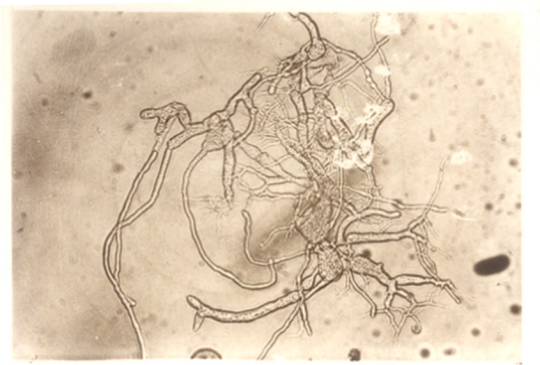


Fig.3.7a

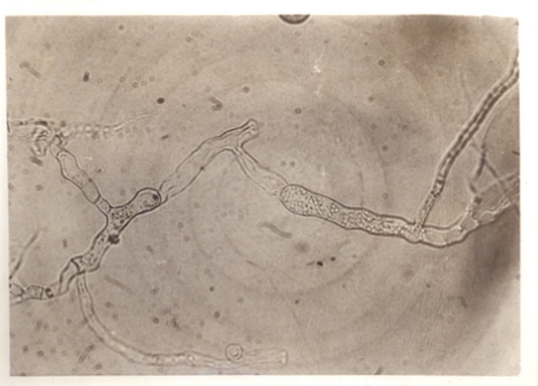


Fig.3.7b

Fig.3.7c

Fig.3.7d

Fig.3.7e

Fig. 3.8: Different stages of development in *Benjaminiella poitrasii*.

a: Sporophore initial
b: Swollen initial
c-f: Various stages in elongation of pedicels
g: Mature sporophore
h: Unispored sporangiola

Fig.3.8a

Fig.3.8b

Fig.3.8c

Fig.3.8d

Fig.3.8e

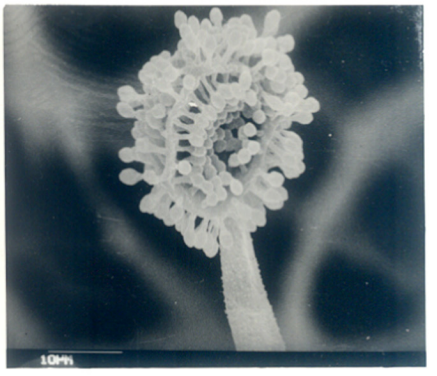


Fig.3.8f

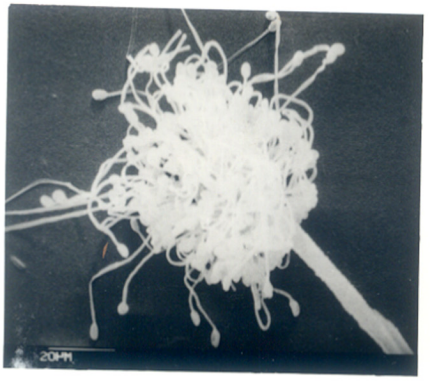


Fig.3.8g

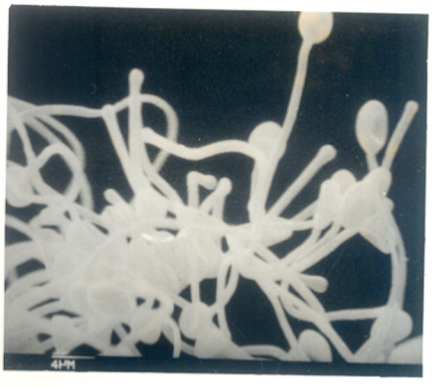


Fig.3.8h

the pedicel (Benjamin, 1960). The outgrowth immediately become enlarged distally to form sporangiola initial. Each sporangiole arises as a terminal swelling of the pedicel. Further, elongation of the pedicel occurs, the length varying. The sporangiole contains a single spore at its end. Sporangiola with their attached pedicels are readily detached from the subtending vesicles. Sporangiola are gray-black in colour.

Zygospores (Fig. 3.5d) are also produced in great numbers at 28°C on MYPG and YPG agar media. They are formed between opposed, smooth-walled suspensors arising from aerial hyphae near the surface of substrate. Zygospores are globose, blackish-brown (~50μm in diameter) and are covered with coarse conical or pointed projections over their surface.

Benny and Benjamin (1976) transferred *Cokeromyces poitrasii* R.K. Benjamin (1960) to *Mycotypha* because it showed closer affinities to *Mycotypha microspora* and *Mycotypha africana* than to the type species of *Cokeromyces*, *C.recurvatus* Poitras (Shanor *et al*, 1950). Benny *et al* (1985) have placed *Benjaminella poitrasii* under order Mucorales, and genera Mycotyphaceae based on the following morphological observations:

Key to the classification of B. poitrasii

Formation of zygospores	:	Mucorales
Formation of yeast-like cells; stolons absent; sporangiola dehiscing by a circumscissile fracture formed at the junction of denticle and pedicel	:	Mycotyphaceae
Fertile vesicle globose or ovoid; pedicels relatively long and twisted; sporangiola and denticles monomorphic	:	*Benjaminiella*
Sporangiola unispored; sporophores unbranched; zygospores formed near the surface of the substrate	:	*Benjaminiella poitrasii* (= *Cokeromyces poitrasii* = *Mycotypha poitrasii*)

3.2.2 *Isolation and characterization of mutants*

Morphological or monomorphic mutants (i.e., mutants able to grow in one form only at both 28°C and 37°C) were isolated after NTG mutagenesis (see under Materials and Methods) of parent strain spores. Hyphal or mycelium development is characterized by apical growth, while yeast-growth is isotropic (growth is equal all over the surface). Therefore, a number of colonies showing yeast growth on MYPG plates (1.0% , w/v, glucose) at 28°C

were picked up and transferred onto fresh MYPG plates. Most of the colonies reverted to the parental phenotype after successive transfers during a period of few subcultures. However, only two colonies Y-2 and Y-5, with characteristic yeast morphology (Fig. 3.1) were selected as they maintained a stable yeast form on MYPG at 28°C after repeated transfers. The colonies were compact, circular, off-white, elevated with a uniform edge. The visual difference between Y-2 and Y-5 was in their appearance. Y-5 was dry and had a wrinkled appearance than the moist, smoother-surfaced Y-2. Growth in liquid MYPG and YPG media of both the mutant strains was characterized by yeast-like cells, which on standing, flocculated and settled rapidly (1-2 mins.), similar to wild-type yeast-cells, leaving a clear supernatant. The morphologies were maintained under all kinds of growth conditions (liquid and solid media) and even under conditions which would normally induce mycelium formation. Thus, colonial morphology of the mutants was maintained along with their cellular morphology. The mutants are stable and have now been maintained without change for over 3-4 years. Light microscopy (LM) of Y-2 and Y-5 indicate that the mutants have a typical unicellular form and reproduce by budding (Fig. 3.9).

3.2.3 *Factors affecting morphological expression*

In other well studied dimorphic systems such as *Mucor*, *Histoplasma*, *Candida* a number of factors have been known to affect morphological expressions, namely:

A. Temperature of incubation

B. Atmosphere of incubation

C. Nutritional conditions

D. Effect of inoculum

E. Effect of antibiotics

Therefore, it was decided to study the effect of the above parameters with respect to morphological expression in *B. poitrasii*.

A. *Temperature of incubation*

In *B. poitrasii* parent strain, temperature was found to be one of the agents triggering dimorphism. A temperature range from 20°C to 45°C was studied in the parent strain, the results of which are tabulated in Table 3.2. In general, it was seen that mycelial-phase developed on MYPG and YPG (1.0% , w/v, glucose concentration) at 28°C while yeast-phase developed at 37°C on the same media. The mutants however, exhibited yeast-phase morphology at both 28°C and 37°C on the same media.

Table 3.2: Effect of temperature on the morphology of *B. poitrasii* parent strain[a].

Temperature of Incubation (°C)	Morphology
20	M
25	M
30	M = Y
35	Y(M)
40	Y
45	Y[b]

a The experiment was carried out in 1.0% (w/v) glucose for 48h under shaking conditions.

b Less growth obtained

Y(M) Most of the cells grew in yeast-phase (Y), but a small percent of cells were present as mycelia.

Fig. 3.9: Yeast form of morphological mutants Y-2 and Y-5 as seen under light microscope.

a: Y-2

b: Y-5

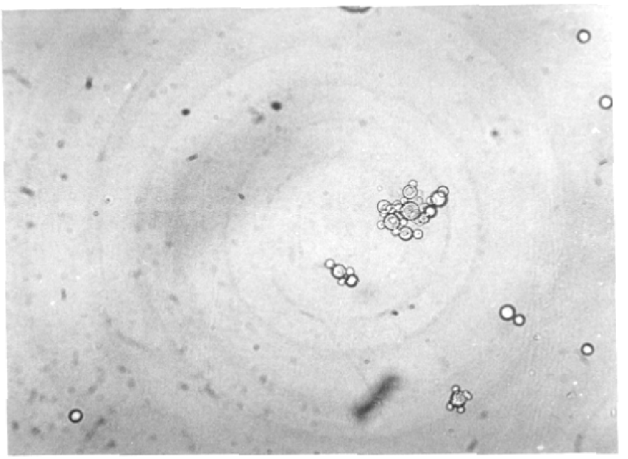


Fig.3.9a

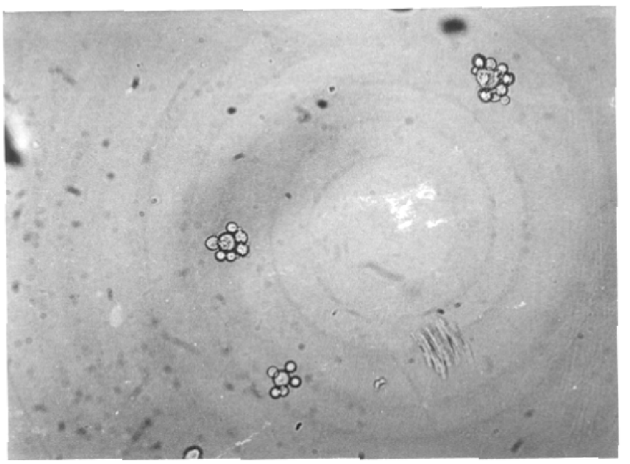


Fig.3.9b

B. *Atmosphere of incubation*

From the studies on *Mucor*, it became evident that the atmosphere of growth is of primary importance for morphogenesis. Previous observations (Sypherd *et al.*, 1978) showed that *Mucor* grew as hyphae under aerobiosis and as yeast under anaerobic conditions. Bartnicki-Garcia and Nickerson (1962) concluded that induction of yeast-like development involves two gaseous factors: presence of carbon-dioxide (CO_2) and absence of oxygen (O_2). It was also seen that anaerobic incubation of *Mucor* under an atmosphere of nitrogen (N_2) resulted in development of filamentous form. Therefore, it was concluded that CO_2 tension rather than anaerobiosis was more important for yeast morphology in *Mucor*.

Similar studies were carried out on *B. poitrasii* parent strain in YP and YPG media (1.0%, w/v, glucose) at 28°C and 37°, the results of which are tabulated in Table 3.3. Anaerobic conditions in liquid YPG resulted in abundant yeast like growth, although, at 37°C in CO_2 environment, growth was relatively poor. Growth on YP media in general was poor but the tendency to form yeast-phase at 28°C increased under N_2 and CO_2 atmosphere. Therefore, in contrast to *Mucor*, anaerobiosis was found to be important for yeast growth rather than CO_2 tension, as yeast morphology could also be obtained under N_2 atmosphere. Thus, the general conclusion that a minimal hexose concentration is needed for yeast morphology does not seem to hold in this case, since yeast-phase cells were formed at 28°C on YP media under CO_2 and N_2.

C. *Effect of nutritional conditions*

A key factor in *B. poitrasii* dimorphism is the carbon source. The effect of glucose concentration and relative aeration on growth and morphology are tabulated in Table 3.4. It was observed that the growth was mainly mycelial at low glucose concentrations in shake flasks. On increasing glucose concentrations, transition towards yeast-phase were observed in shake flasks at 28°C. Thus, as glucose concentration is increased in aerobic cultures, the transition of hyphae → yeast is promoted.

Without aeration, the organism grew predominantly in yeast form even in yeast-peptone (YP) medium, although growth was extremely poor. The above results, differed from those on *Mucor* in which, depending on the glucose concentration in an anaerobic environment, hyphal or yeast growth could occur i.e., a low glucose concentration and a high pCO_2 is needed for yeast development and vice-versa, while *M. rouxii* grew in yeast form at high glucose concentration under N_2 atmosphere (usual form is M-phase). The influence of

Table 3.3: Growth and morphogenesis of *B. poitrasii* parent strain grown under air, N_2 and CO_2[a].

Media	Temp.	Air		N_2		CO_2	
		Growth[b]	Morphology	Growth	Morphology	Growth	Morphology
YP	28°C	300	M	100	M	200	Y
	28°C			100	Y		
	37°C	100	M	250	Y	100	Y
YPG	28°C	400	M	1300	Y	1500	Y
	28°C	500	Y				
	37°C	1600	Y	1100	Y	300	Y

a The media (YP and YPG) were boiled to remove air, and sterile CO_2 and N_2 were passed through the media and autoclaved. A final pressure of 1.0 atmosphere was maintained in all the bottles by passing the required gases. 8×10^6 spores/50ml media were injected into the bottles and incubation was carried out at 28°C and 37°C for 48h under shaking conditions.

b Growth is expressed as mg dry weight/litre media.

Table 3.4: Effect of aeration and glucose concentration on the morphology of *B. poitrasii* parent strain at 28°C for 48h.

Media	Shake flasks		Still flasks	
	Morphology	Growth[a]	Morphology	Growth
Yeast-Peptone (YP)	M	60.0	Y	28.2
YP + 0.1% (w/v) glucose	M(Y)	88.6 (20.6)	Y	42.0
YP + 1.0% (w/v) glucose	M = Y	66.3 and 62.7	Y	60.3
YP + 5.0% (w/v) glucose	Y	169.1	Y	98.6
YP + 10.0% (w/v) glucose	Y	143.2	Y	83.4

a Growth is expressed as mg dry weight/100 ml media. Spore inoculum of 8×10^7 spores/ml was used.

M Mycelial-phase.

M(Y) Most of the cells grew as mycelia (M), but a small percent of the cells were found in yeast-phase (Y).

Y Yeast-phase.

kind and concentration of carbon sources used (Sypherd *et al.*, 1987), presence of complex organic nitrogen in the growth medium (San-Blas and San-Blas, 1983) and C:N ratio of the growth medium (Inderlied and Sypherd, 1978) on the morphological transitions in *B. poitrasii* and its mutants are tabulated in Table 3.5. The yeast-phase mutants Y-2 and Y-5 did not respond to these variations (i.e. no mycelia formation occurred and yeast type morphology was maintained throughout) although, all these parameters were found to be responsible for Y $\leftrightarrow$ M transitions in *B. poitrasii* parent strain.

Aerobically, the fungus utilized a wide variety of carbon compounds. Of the pentoses, xylose (1.0%) supported growth of the parent strain (as M-phase) in both defined medium as well as in MYP media. On the other hand, growth of mutants Y-2 and Y-5 was not observed on xylose. Similarly, while the parent strain grew in M-form in MYP (i.e., without glucose), no growth was observed in the same media with respect to Y-2 and Y-5 (Table 3.5). Evidently, the requirements for carbon and energy were not being met in the case of mutants. Among the hexoses, both mannose and glucose supported growth of the parent strain, Y-2 and Y-5 on minimal as well as MYP media; lactose was not utilized either by Y-2 or Y-5 whereas the parent strain (concentration of 1.0%, w/v) gave rise to yeast morphology on complex media. Growth of parent strain on starch (1.0%, w/v) and carboxymethyl cellulose (CMC) was in mycelial form while the mutants were unable to use starch or CMC as carbon source for growth in either defined or complex media.

Failure of the mutants to utilize xylose, lactose, sucrose, starch or CMC can be accounted for by the failure of the above carbon sources to be hydrolyzed, the results of which are discussed below. Our results indicate importance of type of carbon source, presence of complex organic nitrogen and C:N ratio in yeast-mycelial morphogenesis. In general, it was observed that with a high C:N ratio, the fungus developed yeast morphology and vice-versa.

Enzymatic studies: As the mutants were unable to utilize some of the carbon sources it was deemed necessary to check for the levels of extracellular hydrolytic enzymes. Parent strain showed appreciable extracellular induced cellulase activity (0.5-1.0 IU/ml) and amylase activity (0.7-1.2 IU/ml) on defined medium, while growing as M-form. Mutants Y-2 and Y-5 were unable to utilize starch or CMC as sole carbon source as evident from the negligible amounts of extracellular carbohydrases produced. This also explains the lack of growth of Y-2 and Y-5 in the above media. Intracellular alcohol dehydrogenase (ADH) was estimated

Table 3.5: Effect of different carbon sources, complex organic nitrogen and C:N ratio on the morphology of *B.poitrasii* and its morphological mutants[a]

Carbon source		Defined medium				Malt extract - yeast extract peptone medium (MYP)[b]			
Type	% (w/v)	C:N ratio	Parent	Y-2	Y-5	C:N ratio	Parent	Y-2	Y-5
Xylose	1.0	2.2	M	-	-	4.6	M	-	-
Mannose	1.0	2.2	M	Y	Y	4.6	M	Y	Y
Glucose	0.1	0.2	M	Y	Y	1.4	M	Y	Y
	1.0	2.2	M(Y)	Y	Y	4.6	Y(M)	Y	Y
	5.0	11.1	M(Y)	Y	Y	18.5	Y	Y	Y
Sucrose	1.0	2.3	M	Y	-	4.7	M	Y	-
	10.0	23.3	M	Y	-	37.8	Y	Y	-
Lactose	1.0	2.3	M	-	-	4.7	Y	-	-
	5.0	11.7	M	-	-	19.5	Y	-	-
Starch	1.0	ND	M	-	-	ND	M	-	-
CMC	1.0	ND	M	-	-	ND	M	-	-
Control[c]	none	0	M	-	-	0.7	M	-	-

- No growth.

ND Not determined.

M Mycelial-phase.

M(Y) Most of the cells grew as mycelia (M), but a small percent of the cells were found in yeast-phase (Y).

Y Yeast-phase.

Y(M) Most of the cells grew in yeast-phase (Y), but a small percent of the cells were present as mycelia (M).

a As described under Materials and Methods.

b Analysis values of MYP media needed for calculating C:N ratio were taken from Bridson and Brecker (1970).

c In the control samples carbon source was omitted from the media.

in cells grown on 5.0% glucose in MYP medium for 48 h as all the three strains exhibited Y-form at the above concentration. Mutants Y-2 and Y-5 showed higher ADH activity (0.5 U/mg total protein) as compared to parent strain (0.2-0.4 U/mg total protein). Extra- and intra-cellular β-galactosidase activity was also estimated in the above strains grown in MLYP (5.0%, w/v, lactose) medium for 48 h. Parent strain had 0.05 x 10^{-2} IU/ml and 9.1 x 10^{-2} IU/mg total protein while no detectable activity of β-galactosidase could be obtained Y-2 and Y-5. Absence of extra-cellular β-galactosidase activity explains the absence of growth of mutants with lactose as a carbon source.

D. *Effect of inoculum size*

Studies on organisms like *Mucor* have indicated that size of inoculum affects form development. Thus, studies were carried out with four different spore concentrations in order to check if inoculum concentrations affected morphology in *B. poitrasii*. The following spore concentrations were taken into consideration:

(i) 10^4 spores/100 ml media

(ii) 10^5 spores/100 ml media

(iii) 10^6 spores/100 ml media

(iv) 10^7 spores/100 ml media

Growth and morphology were recorded every 24h (Fig. 3.10a,b). It was seen that under aerobic conditions, dissmilarities between growth curves resulting from different inocula concentrations were minor and proportional to the size of inoculum. Inoculum size also did not influence cellular form and the proportion of morphological change remained constant.

E. *Antibiotic studies*

The cell envelope of fungi forms a barrier to many chemicals and drugs. Transition of yeast-to-hyphae in *Mucor rouxii* is inhibited by cycloheximide (Haidlee and Storck, 1966) and thus appears to be dependent on *de novo* protein synthesis. Of the few effective antifungal antibiotics known, studies with cycloheximide (inhibits protein synthesis on 80 s ribosomes of eukaryotic cells), nystatin (a polyene antibiotic acting on the plasma membrane) and with chloramphenicol (inhibits protein synthesis on 70 s ribosomes of mitochondria in eukaryotes) and tetracycline (affects binding of aminoacyl-tRNA to the 70 s ribosome in prokaryotes and mitochondria) gave the following results.

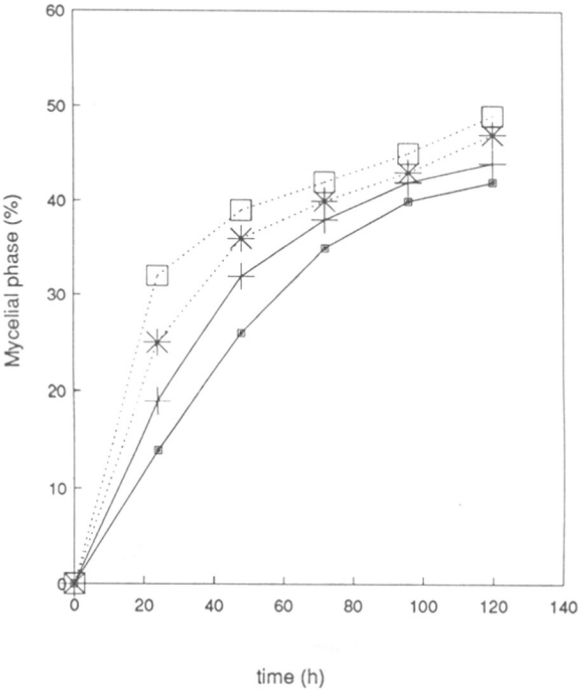
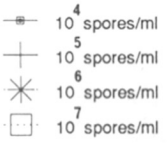


Fig. 3.10a Effect of inoculum size on morphology of *B. poitrasii* under shaking conditions.



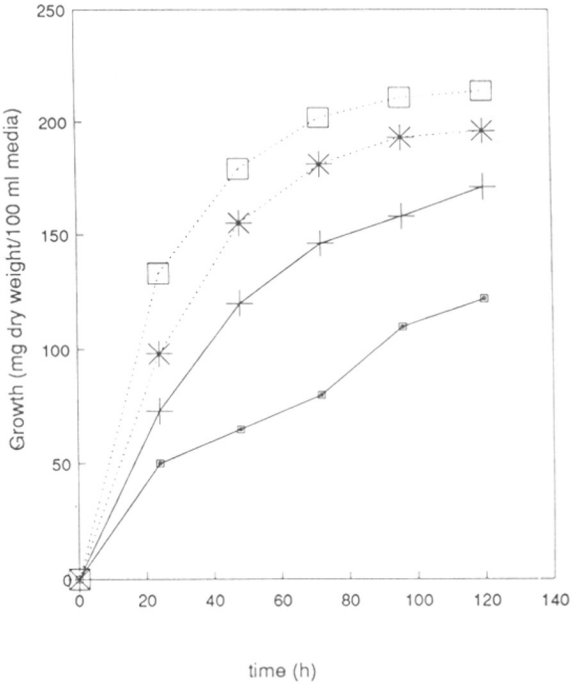


Fig. 3.10b Effect of inoculum size on growth of *B. poitrasii* under shaking conditions.

The mycelial-phase of parent strain grew at concentrations as high as 100 µg/ml of nystatin in MYPG agar media while the yeast-phase did not show any growth under similar conditions. The mutants Y-2 and Y-5 however, showed growth in the concentration range (50-200 µg/ml) studied (Table 3.6 a). Similar trends were obtained with yeast-phase of parent strain, Y-2 and Y-5 mutants with cycloheximide. However, mycelial-phase of parent strain was seen to grow upto 40-50µg/ml of cycloheximide and was inhibited at concentrations above it. In parent strain, it was also observed that while 30µg/ml of cycloheximide could prevent Y→M transition upto 48h, at 40 µg/ml it was upto 72h (Table 3.6b), similar to that reported in *M. rouxii* by Haidlee and Storck (1966). (Table 3.6b). Chloramphenicol and tetracycline which were studied to check the role of mitochondria in the morphological expression of *B. poitrasii*, could not inhibit growth either with mycelial- or yeast-phase in parent strain or with Y-2 and Y-5, thereby showing that mitochondria do not seem to play a significant role in the morphogenesis of *B. poitrasii*, assuming these antibiotics do not appear to have any permeability barriers.

3.3 DISCUSSION

It was anticipated that a full understanding of the phenomena that underlie dimorphism would require the isolation of morphological mutants. To obtain stable morphological mutants of *B. poitrasii* was a pre-requisite which has been fulfilled by the isolation of Y-2 and Y-5 mutants which form yeast-phase cells at ambient temperatures. This state was maintained at both 28°C and 37°C as well as in various liquid and solid media irrespective of carbon and/or nitrogen source. The mutants did not revert to the parental phenotype and the most significant aspect was the correlation of colonial morphology with a permanent alteration of their cellular morphology.

The pattern of growth (i.e., hyphal or yeast) in the parent strain can be induced by (i) temperature and (ii) changes in glucose concentration. Dimorphism in *B. poitrasii* can be directly mediated by temperature, once other conditions (e.g., glucose concentration) are satisfied. It was seen that pure phase yeast cells were obtained at 37°C while mycelial growth occurred at 28°C. The mycelial-to-yeast conversion can occur above 30°C (Table 3.2) and 37°C (Table 3.1), while at 28°C yeast cells transform to mycelia (Table 3.1). The time taken for yeast-to-mycelial transition is independent of the glucose concentration, and the early morphological changes can be seen by 3h. However, the percentage of cells undergoing transition depend on the glucose levels present in the media (Table 3.1). Therefore, transition

Table 3.6a: Effect of antibiotics on growth and morphology of *B. poitrasii* parent strain and morphological mutants Y-2 and Y-5.

Antibiotic[a]	Parent strain		Y-2	Y-5
µg/ml	M-Phase	Y-Phase[b]		
Chloramphenicol				
50	M	M	Y	Y
100	M	M	Y	Y
200	M	M	Y	Y
Tetracycline				
50	M	M	Y	Y
100	M	M	Y	Y
200	M	M	Y	Y
Nystatin				
50	M	-	Y	Y
100	M	-	Y	Y
200	-	-	Y	Y
Cycloheximide				
50	M	-	Y	Y
100	-	-	Y	Y
200	-	-	Y	Y
Control	M	M	Y	Y

\- No growth.

a Mycelial and yeast phase cells (10^6 cells/ml) from parent strain and mutants were inoculated onto petri plates containing MYPG medium supplemented with the above antibiotics and incubated at 28°C for 96h.

b For yeast phase cells, although a yeast inocula was used, the transition of the cells to mycelial phase occured by 96h as seen in the controls.

Table 3.6b: The preventive effect of cycloheximide on Y→M transition in parent strain[a].

Cycloheximide[b] (μg/ml)	Time (h) 24	48	72	96	120
Control	M	M	M	M	M
10	M	M	M	M	M
20	M + Y	M(Y)	M	M	M
30	Y	M + Y	M	M	M
40	Y	Y	Y	Y(M)	M + Y

a Yeast phase cells (10^6 cells/ml) used as inocula.

b Cells were inoculated on YPG agar media (1.0%, w/v glucose) containing the above cycloheximide concentrations, and grown at 28°C.

also depends on the exhaustion or rate of utilization of glucose from the media. The transition from mycelium-to-yeast requires a longer time and the early morphological changes were detected at 8-9h irrespective of glucose concentration. The percentage of yeast phase formed was noted to be higher at glucose concentration of 1.0%. The mutants Y-2 and Y-5 maintained their morphology under the temperatures tested.

A key factor in dimorphism of *B. poitrasii* was found to be the carbon source. Depending on the glucose concentration in aerobic environment, *B. poitrasii* could develop either mycelial or yeast-phase growth at 28°C (Table 3.4). When glucose concentration was decreased below 0.1% (w/v) the fungus predominantly displayed mycelial growth. On the other hand, at higher concentration of glucose (5.0%, w/v and above), *B. poitrasii* grew in yeast form, thus showing that under aerobic conditions yeast growth requires a fermentable carbon source. Under still conditions, yeast phase cells were obtained in YP medium (i.e., in the absence of glucose), although the growth was extremely poor. The mutants were unable to grow on media devoid of a fermentable hexose sugar since the other carbon sources could not be utilized by them (Table 3.5). With the parent strain growth was generally as mycelia on the other carbon sources listed.

Nitrogen source also seems to play an important role in the dimorphism of *B. poitrasii*. Though Y-2 and Y-5 did not grow in MYP and minimal media, the predominant form in parent strain was M-form in the above media. It was observed that while *M. rouxii* yeast-phase requires a complex nitrogen source for growth, it was not so the case in *B. poitrasii*.

Thus, the tendency of *B. poitrasii* to undergo mycelial or yeast morphologies depends upon an interplay of environmental factors like temperature, carbon source, complex organic nitrogen source and C:N ratio in the media. In general, a high C:N ratio led to yeast-phase growth, while a lowered C:N ratio gave rise to mycelial-phase. Similarly, studies with monomorphic mutants Y-2 and Y-5 showed that no growth occurred (since they could not switch to mycelial-phase) on a lowered C:N ratio while they grew in yeast-form at a higher C:N ratio.

Since differences between growth and type of morphology between MYPG and YPG media were negligible, studies henceforth, were carried out with YPG media.

CELL WALL ANALYSIS OF THE YEAST AND MYCELIAL PHASES OF *Benjaminiella poitrasii* AND ITS MORPHOLOGICAL MUTANTS

CHAPTER IV

ISOLATION AND CHEMICAL COMPOSITION OF THE CELL WALLS OF YEAST AND MYCELIAL PHASES AND MUTANTS OF *Benjaminiella poitrasii*

SECTION A

CHAPTER - IV

SECTION - A

4.1 INTRODUCTION

Dimorphism in *B. poitrasii* is influenced by temperature as well as by nutritional conditions (Khale *et al.*, 1990). A number of fungi *viz.*, *Mucor* sp., *Paracoccidiodes brasiliensis*, *Candida albicans* etc., have been studied systematically to search for molecular events leading to dimorphism (Ruiz-Herrera, 1985; San-Blas and San-Blas, 1985; Soll, 1985). The cell walls of fungi play a critical role in their biology, including the determination of form, maintenance of the osmotic pressure, interaction with other organisms, storage of reserve carbohydrates, protection against antifungal compounds as well as being involved with recognition systems associated with cell surface. (Aronson, 1981; Farkas, 1985; Reiss, 1985; Richmond, 1977). Thus, the multifunctional nature of the fungal wall is, undoubtedly, a reflection of its particular composition and of its architecture.

Numerous data exist in literature concerning the chemical composition of cell walls from different fungi (see reviews by Bartnicki-Garcia, 1968; Gander, 1974; Bacon, 1981). The fungal cell walls are composed of (a) polysaccharides, (b) proteins, (c) protein-polysaccharide complexes, and (d) variable amounts of minor constituents (Farkas, 1985). A list of the wall components based on Peberdy (1990) is given in Table 4.1.

The skeletal polysaccharides are water-insoluble, highly crystalline homopolymers and include glucans and chitin. In the cell walls, chitin is usually found to occur in a complex with R-glucan, where the covalent linkage between these two polymers is presumed to occur *via* peptide bridges (Siestma and Wessels, 1979). Their function is to give the wall mechanical rigidity and to maintain its shape. By contrast the polysaccharides of the wall matrix are amorphous, water soluble homo- and heteropolymers, often occurring in chemical complexes with proteins. They fill the space between the skeletal polysaccharides, i.e., serve as cementing substances (Peberdy, 1990).

Table 4.1: Macromolecular components of fungal cell walls

1.	*Skeletal elements*	
	Chitin	β-1,4-linked homopolymer of N-acetyl-D-glucosamine
	β-glucans	β-1,3- glucan homopolymer comprised of D-glucose units with β-1,3- and β-1,6-glucosidic bonds (R-glucans) which are resistant to alkali treatment
2.	*Matrix components*	
	α-glucan	α-1,3-homopolymer of glucose (S-glucans) characterized by its solubility in alkali
		α-1,3- and α-1,4-linked glucans (nigeran)
	Glycoproteins	Polysaccharide-protein complexes
3.	*Miscellaneous components*	
	Chitosan	β-1,4-polymer of D-glucosamine
	D-galactosamine polymers	
	Polyuronides	
	Lipids	
	Melanins	

As pointed out by Bartnicki-Garcia (1968), the chemical composition of the walls is closely correlated with the taxonomic classification of fungi. Most of the reports have confirmed Bartnicki-Garcia's taxonomic conclusions. It is considered that the shape of the cell is determined by its wall. Hence, alterations in morphogenetic changes in dimorphic fungi should accompany either changes in the cell wall constituents or changes in their arrangement to generate different shapes. Therefore, the purpose of this study was to determine the cell wall composition of the yeast and mycelial forms and to correlate it with the morphological yeast phase mutants Y-2 and Y-5.

4.2 RESULTS

Purity of cell wall preparation: After disintegration in Braun's homogenizer, approximately 95-98% cells were broken as seen under light microscope. Cell wall preparations were considered essentially clean and free from cytoplasmic material for the following reasons based on criteria of Taylor and Cameron (1973):

(a) examination under light microscope showed absence of cytoplasmic or membraneous material.

(b) ultra-violet absorbing material was no longer released after the series of washes and ultrasonic treatment as described in Materials and Methods.

Cell wall composition: The various cell wall components in the yeast phase, mycelial phase and mutants Y-2 and Y-5 are presented in Table 4.2.

Carbohydrates: Striking quantitative differences in carbohydrate content were evident between the cell walls of yeast and mycelial phase of *B. poitrasii*. Anthrone analysis for carbohydrate content showed that yeast cell wall contains 2.2 times more neutral sugars than the mycelial phase. Mutants Y-2 and Y-5 had the values for anthrone-positive material lying in between those of yeast and mycelial phases. The glucose content as estimated by glucose oxidase method was found to be a smaller fraction of the total neutral carbohydrates estimated. In the mycelial-phase, the percentage of glucose per total neutral carbohydrate is 21%, while in yeast phase it is 17.7%, in Y-2 20.9% and in Y-5 it is 23.8% . The other neutral

Table 4.2: Chemical composition of yeast and mycelial phases of parent strain and mutants Y-2 and Y-5 of *B. poitrasii*.

Wall constituents[a]	Mycelial phase	Yeast phase	Y-2	Y-5
Total neutral carbohydrate	26.6	48.1	37.4	36.1
Mannose	21.0	39.6	29.6	27.5
Glucose	5.6	8.5	7.8	8.6
Galactose	Tr	Tr	Tr	Tr
Protein	21.3	29.6	14.0	22.9
Total hexosamine	35.6	20.5	23.3	21.2
N-acetylhexosamine	9.0	3.2	3.6	3.9
Uronic acid	ND	ND	ND	ND
Lipids	7.5	6.0	6.6	7.3
Free Lipids	2.0	1.6	1.9	2.1
Bound Lipids	5.5	4.4	4.7	5.2
Phosphate	0.11	0.1	0.05	0.08
Ash	0.2	0.6	0.9	0.8
Recovery	91.3	99.9	82.3	88.4

a Expressed as percentage.

Tr Trace amounts.

ND Not detected.

sugar detected by paper chromatography was galactose, which was present in trace amounts. Therefore, the bulk of neutral sugar is composed of mannose in all the cases, the percentage being the greatest in the yeast phase.

Protein: Yeast phase cells of parent strain contained a higher percentage of protein than mycelial phase. In the case of mutants, the levels in Y-2 and Y-5 were lesser to those of yeast phase, while the amount of protein in Y-2 cell wall was found to be relatively low (Table 4.2). Analysis of cell wall protein to give their component amino acids are tabulated in Table 4.3. Significant amounts of aspartic acid, threonine, serine, glycine and alanine are present in both the forms. The major difference was in the histidine content which was present in trace amounts in yeast phase and undetected in Y-2, but was present in mycelial phase in measurable amounts. Although glucosamine is also detected by amino acid analysis, the values for it are not included in Table 4.3.

Lipids: Appreciable amounts of lipids, predominantly of the bound type were found in all the cell walls. The values for free lipids or readily extractable lipids were comparable for the yeast and mycelial phase as well as for the mutants. The percent of free lipids were similar in all the four cells walls analyzed. The percent of bound lipid was higher in the mycelial phase than in the yeast phase. The total lipid content in all the given cell walls was present in the range of 6.0-8.0%. The fatty acid composition of the cell walls was examined by gas-liquid chromatography (Table 4.4). Lauric acid was the predominant fatty acid though mycelial phase had 1.5 fold its content than yeast phase. The mutants Y-2 and Y-5 had values for lauric acid which were greater than the wild strain levels. Myristic acid, palmitic acid and stearic acid were present in greater amounts in yeast phase than in mycelial phase, while on the other hand palmitoleic acid was absent in mycelial phase. Linoleic acid was absent in all the cell types. Oleic acid levels were comparable in yeast and mycelial phase. On calculating the ratio of unsaturated fatty acids to saturated fatty acids, values of 0.06, 0.06, 0.03 and 0.04 were obtained for mycelial phase, yeast phase, Y-2 and Y-5 respectively.

Phosphate: Very little phosphate could be detected in the cell walls of all the preparations (Table 4.2). Therefore, phosphodiester bonds would be absent in the cell walls

Table 4.3: Amino acid composition of cell walls of yeast and mycelial phase of parent strain and mutants Y-2 and Y-5 of *B. poitrasii*.

Amino acid[a]	Mycelial phase	Yeast phase	Y-2	Y-5
Aspartic acid	4.7	4.2	1.1	2.0
Threonine	4.2	4.4	0.8	4.3
Serine	4.3	4.7	1.0	4.3
Glutamic acid	3.0	3.4	0.9	3.0
Proline	2.1	2.6	ND[b]	3.0
Glycine	4.4	4.9	0.8	4.4
Alanine	4.0	4.5	0.4	4.0
1/2 Cystine	0.3	0.2	ND	0.5
Valine	2.0	0.5	Tr[c]	2.2
Methionine	0.4	0.5	0.2	1.0
Isoleucine	2.0	2.1	0.6	2.3
Leucine	2.9	2.6	1.0	4.3
Tyrosine	2.9	2.0	Tr	2.4
Phenylalanine	2.0	2.0	Tr	2.3
Lysine	0.9	0.3	1.0	1.3
Histidine	0.4	Tr	ND	0.16
Arginine	0.07	0.1	ND	1.0

a Expressed as micromoles /100 mg cell wall.

b Not detected.

c Trace amounts.

Table 4.4: Fatty acid composition of yeast and mycelial phases and mutants Y-2 and Y-5 of *B. poitrasii*.

Fatty acid[a]	Mycelial phase	Yeast phase	Y-2	Y-5
Caprylic acid, C8:0	-	1.1	-	-
Capric acid, C10:0	-	0.9	-	-
Lauric acid, C12:0	82.8	55.5	94.1	90.7
Myristic acid, C14:0	1.1	6.6	0.5	-
Palmitic acid, C16:0	8.3	17.0	2.5	4.2
Palmitoleic acid, C16:1	-	0.4	0.2	0.4
Stearic acid, C18:0	3.7	13.8	1.2	2.4
Oleic acid, C18:1	4.2	4.7	1.8	2.7
Linoleic acid, C18:2	-	-	-	-
Linolenic acid, C18:3	1.4	0.9	1.0	1.1
Recovery	101.5	104.9	101.3	101.5

a Expressed as percentages.

of *B. poitrasii*. Such type of bonds have been shown to cross-link mannan molecules in the cell walls of yeast (Farkas, 1979) while in *Mucor* the nature of phosphate component is not known although it is presumed to be present as polyphosphate (Bartnicki-Garcia, 1962).

Ash: The ash content was found to be 0.2% in the mycelial form while it was 0.6% in yeast form. In Y-2 and Y-5 mutants ash content was 0.9% and 0.8% respectively.

Uronic acids: In our cell wall preparations, uronic acid (e.g., D-glucuronic acid) was not detected, although Bartinicki-Garcia and Reyes (1962) were able to detect appreciable amounts in *Mucor*.

Amino sugar: The total hexosamine content is 1.5 times higher in the mycelial phase than in the yeast phase. Aminosugar values of the mutants Y-2 and Y-5 were comparable to that of the yeast phase. Using aminoacid analysis all the hexosamine was found to be in the form of glucosamine, and neither galactosamine nor mannosamine could be detected. Amino acid analysis was used only for qualitative determinations of aminosugars since refluxing with 6N HCl at 110°C for 16h causes degradation of glucosamine and N-acetylglucosamine. The N-acetylglucosamine (NAG) values were the higher in the mycelial phase and were 3-fold greater than that in the yeast phase, while the values of Y-2 and Y-5 were comparable to those of yeast-phase. The percentage of N-acetylhexosamine per total hexosamine present was 25.2% (mycelial phase), 15.6% (yeast phase). 15.5% (Y-2) and 18.4% (Y-5).

In order to discriminate between chitin (poly N-acetylglucosamine) and chitosan (poly-glucosamine), it was essential to depolymerize chitosan by reacting the free NH_2 groups with HNO_2 (see Materials and Methods), the results of which are shown in Table 4.5. Presence of chitosan can be demonstrated by (i) hydrolysis to give glucosamine (ii) degradation by action of nitrous acid and (iii) resistance to nitrous acid degradation following treatment with acetic anhydride (Briza *et al.*, 1988). Most of the glucosamine was degraded by HNO_2. N-acetylation prior to HNO_2 treatment, largely prevented the glucosamine polymer from degradation (Table 4.5). Similar treatment to N-acetylglucosamine polymer showed that this

Table 4.5: Discrimination between chitin and chitosan in the cell wall fractions.

Treatment[a]	Mycelial phase	Yeast phase	Y-2	Y-5
Glucosamine (Chitosan)				
Untreated	100	100	100	100
Treated with HNO_2	14.2	7.6	8.3	6.0
Acetylated and treated with HNO_2	88.2	83.4	82.1	86.3
N-acetylglucosamine (Chitin)				
Untreated	100	100	100	100
Treated with HNO_2	77.3	80.1	82.2	89.2
Deacetylated with NaOH and treated with HNO_2	10.2	4.7	4.5	8.6

a Glucosamine (GA) and N-acetylglucosamine (NAG) were determined spectrophotometrically as described under Materials and Methods and normalized to their respective contents (GA or NAG) of untreated walls.

polymer was resistant to such degradation. Conversely, deacetylation of NAG polymer with sodium hydroxide prior to HNO_2 treatment led to depolymerization of the polymer (Table 4.5).

4.3 DISCUSSION

A purified cell wall preparation was obtained from the yeast and mycelial forms as well as from mutants Y-2 and Y-5 which were free of cytoplasmic and membrane contamination.

According to the analysis of the hydrolysate, the chemical composition of the cell walls have been tabulated in Table 4.2. The total neutral carbohydrate was found to be two fold greater in the yeast phase than in mycelial phase, while the carbohydrate content in the mutants Y-2 and Y-5 occurred between the yeast and mycelial phase. Analysis of the total neutral carbohydrate showed that the carbohydrates detected were small amounts of glucose, traces of galactose and mainly mannose. The percentage of glucose was 1.5 times greater in the yeast phase, Y-2 and Y-5 than in the mycelial phase.

According to many investigators, the conversion of filamentous phase to yeast phase is accompanied by an increase in the mannose content of the yeast wall (Bartnicki-Garcia, 1963; 1968; Chattaway *et al.*, 1968; Dow and Rubery, 1977). In *M. rouxii*, the percentage of mannose present is greater in yeast form than in mycelium form. In *P. brasiliensis* high amounts of galactose and mannose in the form of galactomannan were found in the mycelial form (Kanetsuna *et al.*, 1969; San-Blas and San-Blas, 1977). Similarly, the mannose content of mycelial cell wall in *H. capsulatum* and *B. dermatitidis* was significantly greater as compared to that in yeast wall (Domer, 1971; Kanetsuna and Carbonell, 1971; San-Blas and Carbonell, 1974). Comparative study on cell wall composition amongst the varied groups of fungi has been given in Table 1.2. As observed by the data on total neutral carbohydrate, the relationship between the former and morphogenesis in *B. poitrasii* does not seem to hold true. The data of the yeast form mutants Y-2 and Y-5 is in good agreement with the above, since the carbohydrate content in this cell wall is found to lie in between those of yeast and mycelial phase.

Protein analysis in the cell walls demonstrated a higher protein content in yeast phase wall, which is similar to that observed in *M. rouxii* (Sypherd *et al.*, 1978), *C. albicans* (Chattaway *et al.*, 1968) and *Yarrowia lipolytica* (Vega and Dominguez, 1986). The protein content in the Y-2 cell wall was found to be much lesser as compared to the other cell wall fractions. Amino acid analysis of the cell wall fractions also exhibit disparities with respect to cell walls of *Mucor* and *Mycotypha*. Based on the cell wall data of *Mucor* (where the yeast wall is aspartic acid rich as compared to the mycelial wall), Bartinicki-Garcia (1963) suggested a hypothesis that incorporation of an aspartic acid rich mannoprotein into the cell wall is responsible for the alteration in the pattern of wall formation, resulting in the morphological change in *Mucor*. In *Mycotypha* the concentration of this amino acid was found to be higher in the mycelial wall. In *B. poitrasii*, however, the percentage of this amino acid was similar in both the fractions i.e., yeast and mycelial. Our results are therefore not in agreement with the above hypothesis suggested by Bartnicki-Garcia (1963). In the mycelial phase, the concentrations of serine, threonine, glutamic acid, glycine, valine and lysine were found to be higher than those in the yeast phase wall. A similar trend has been observed in the case of *Mycotypha* while in *Mucor* vice-versa occurs, i.e., the concentration of these amino acids is higher in the yeast wall than in the mycelial (Cole *et al.*, 1980; Bartnicki-Garcia, 1962). In our studies histidine was the only amino acid present in the mycelial form which could be detected in trace amounts in yeast phase wall.

The total lipid content (free and bound) was slightly higher in mycelial than in the yeast phase walls (Table 4.2), the contents of which are very similar to that reported for *Mucor* (see Table 1.2). The fatty acid composition supports the finding of Wassef (1977) who determined that polyunsaturated fatty acids of chain lengths upto C 18:3 are characteristic of most zygomycetous fungi. However, lauric acid was found to be the most predominant in all the four cell walls investigated, the percentage being 1.5 fold greater in mycelial phase than in yeast cell wall. The lauric acid content in Y-2 and Y-5 cell wall is greater than either of the parent strain cell wall. Of the remaining fatty acids, myristic, palmitic and stearic were greater in the yeast phase than in the mycelial phase. Palmitoleic, caprylic and capric acid though present in yeast phase were found to be absent in the mycelial phase.

Very little phosphate could be detected in the cell wall preparation (Table 4.2) and hardly any quantitative differences between both the forms occurred. This is in contrast to data reported from *Mucor rouxii* (Bartnicki-Garcia, 1962) which contains a high percentage of phosphate (present as polyphosphate) . Similarly, significant differences in the ash content among the four cell walls investigated were found to occur, the content being higher in yeast phase, Y-2 and Y-5.

Hexosamines were also found to be one of the major component of the cell walls. Amino acid and paper chromatography indicated that glucosamine was the sole aminosugar present. Higher amounts of glucosamine (1.5 fold) were present in mycelial phase than in the yeast phase. The percentage of glucosamine in the mutants Y-2 and Y-5, on the other hand, was comparable to the yeast phase. This was the only wall constituent in which the values of mutants Y-2 and Y-5 were comparable to the yeast phase. Cell walls of *M. rouxii*, as well as those from other zygomycetes (Bartnicki-Garcia, 1968; Novaes-Ledieu and Jimnez-Martinez, 1969) are characterized by the presence of deacetylated glucosamine polymer, chitosan. Chitin (acetylglucosamine) is present as a smaller fraction of the total glucosamine. The ratio of chitosan: chitin was found to be in the range of 3.0-3.9 in all the four cell walls. The need of greater amounts of this polymer in the mycelial form of *B. poitrasii* is unknown. Chitin might give additional rigidity to the microfibillar network allowing extension of the wall exclusively at the apex of the hyphae as suggested by Victoria Elorza *et al.*, (1983) for *C. albicans*.

Thus, the major change in chemical composition seems to be with respect to the hexosamine content between the mycelial phase and yeast phase cell wall and the mutants Y-2 and Y-5. Therefore, on comparing the cell wall composition between *B. poitrasii* and *M. rouxii* (a dimorphic zygomycetous fungus), although qualitative differences do not arise, quantitative deviations exist among the wall components. Glucose was found to be absent in *M. rouxii* cell wall while it is present as a minor neutral carbohydrate in *B. poitrasii*. However, in *Mycotypha* (Cole *et al.*, 1980), a zygomycetous fungus, the presence of glucose in the cell wall has also been reported. Secondly, the levels of phosphate in *M. rouxii* cell wall is high, whereas very little phosphate could be detected in *B. poitrasii*. Similarly, although glucuronic acid was detected in *M. rouxii*, this component was not detected in *B. poitrasii*.

It has been suggested that morphogenetic changes in dimorphic fungi must accompany either changes in cell wall structure and biosynthesis or rearrangement of the same compounds to generate different shapes. Thus, while results on *B. poitrasii* do not show any qualitative changes in the chemical composition of cell wall, quantitative changes especially with respect to glucosamine content do occur. Therefore, the next section deals with preliminary investigations which have been performed to see if differences in cross-linking between the major cell wall components occur among the two phases due to morphogenetic change.

CELL WALL FRACTIONATION AND CROSS-LINKING OF WALL POLYMERS

SECTION B

CHAPTER - IV

SECTION B

4.4 INTRODUCTION

The major components of cell walls in most fungi as mentioned in section A were found to be mannans, glucans, chitosan and/or chitin and protein. Although work has been carried out on the arrangement of these components and their architecture throughout the cell walls in various fungi, yet it is still not a well understood subject. The general picture is that the skeletal, microfibrillar wall components such as β-glucans and chitin/or chitosan are embedded in an amorphous polysaccharide and protein-polysaccharide matrix. There is however, rarely observed a sharp boundary between the individual layers; more likely, they merge into one another.

There is little information about the nature of linkages between the different components in the fungal walls. The existence of covalent linkages between chitin and glucan has been described in *Aspergillus*, *Saccharomyces* and *Schizophyllum* (Siestma and Wessels, 1979). Mannan-glucan complexes have been described in yeasts (Kesslar and Nickerson, 1959; Villa *et al.*, 1980). Non-covalent interactions, such as hydrogen bonding and Van der Waals forces, may also play an important role in holding the cell wall structure together (Farkas, 1985).

At first sight fungal walls contain a bewildering number of polymers and constituent monomers (Bartnicki-Garcia, 1968; Wessels and Siestma, 1981). However, when only the alkali-insoluble components are considered, a much more simple picture emerges. The alkali-insoluble wall portions of ascomycetes and basidiomycetes mainly consists of β-D-glucans and chitin (Rosenberger, 1976). In *Candida albicans* also evidence for a covalent linkage between chitin and β-glucan has been provided (Surarit *et al.*, 1988). There is evidence to suggest that this alkali-insoluble portion of the wall provides the wall with increased rigidity which in turn is solely responsible for hyphal morphogenesis (Siestma and Wessels, 1988; Gooday and Trinci, 1980). According to Burnett (1979) and Wessels *et al.*, (1990), the newly synthesized wall is inherently visco-elastic and expandable which gradually develops rigidity

in the hyphal wall. Similarly, in *C. albicans* it has been suggested (Soll *et al.*, 1985; Staebell and Soll, 1985; Shepherd, 1987), that the important difference between the yeast and hyphal modes of growth may be associated with the timing of secondary wall formation, e.g., if glucan-chitin complex were formed immediately behind the apical tip, this would give a rigid structure resulting in a hyphal element. If, however, secondary wall formation with its cross-links were delayed a more plastic wall would result, allowing the formation of a spherical cell. Environmental conditions conducive to hyphal development caused young buds to become transformed into hyphal apices, but after the buds had attained approximately half their final size this was no longer possible; the buds grew to their final size but hyphal arose only from new evaginations (Soll *et al.*, 1985, Wessels *et al.*, 1990).

Since, it has been generally accepted that glucan-glucosaminoglycan linkage is responsible for morphogenesis, it was decided to carry out studies on the chemical extraction of cell walls of mycelial phase, yeast phase, Y-2 and Y-5 mutants of *B. poitrasii*, to see the solubility differences, if any, occur amongst the cell wall components. These studies were carried out in 2 parts:

1. The cell walls were fractionated into their alkali-soluble and alkali-insoluble components which were hydrolyzed for the detection of individual components.

2. Preliminary studies on the solubility characteristics of the major cell wall components (i.e., mannans, glucans and glucosaminoglycans) were examined in order to determine their cross-links. Based on the studies of Siestma and Wessels (1981) cell walls were extracted with the following treatments: 1M KOH extracts all the mannan and also small amounts of glucans (Fleet and Manners, 1976). Most of the glucan can then be extracted with dimethyl-sulfoxide (Bacon *et al.*, 1969). Subsequent treatment with 40% NaOH further reduces this amount of glucan as well leads to the deacetylation of chitin (Horton and Wolform, 1963). The remaining glucan becomes soluble in alkali (KOH), when the deacetylated chitin in the residue is depolymerised with nitrous acid.

4.5 RESULTS

4.5.1 *Cell wall fractionation*

The cell walls of the mycelial phase, yeast phase, Y-2 and Y-5 mutants were fractionated based on their alkali solubility as shown in the Scheme 4.1. The residues were then hydrolyzed and analyzed for their mannan, glucan, protein and glucosaminoglycan contents. Mannose, traces of glucose and protein were detected in the alkali-soluble fraction (Fraction I). Glucose, glucosamine and protein were detected in the alkali-insoluble fraction (Fraction II), although the percentage of protein present in this fraction was much lesser as compared to that in Fraction I (Table 4.6). Fraction III was found to contain glucosamine and small amounts of protein. Hence, the above results indicate that fraction I consists mainly of a mannan-protein complex, fraction II containing a glucan-glucosamine complex while fraction III was composed mainly of glucosamine.

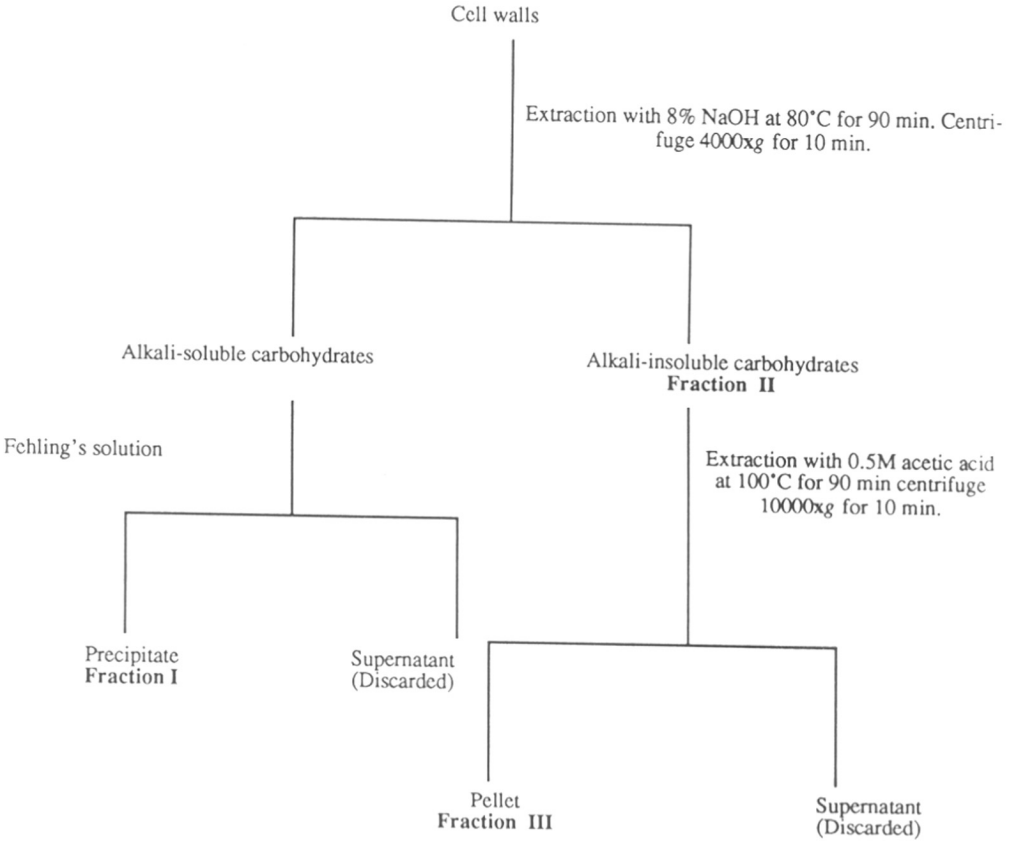
4.5.2 *Cross-linking of wall polymers*

Table 4.7 shows the effect of the various chemical treatments on the removal of anthrone-positive material and glucosaminoglycans from the cell walls of mycelial phase, yeast phase and mutants Y-2 and Y-5.

Most fractionations of cell walls start with a rigorous extraction with alkali to remove the mannan and protein, leaving an insoluble residue containing mainly glucan and glucosaminoglycan (Roberts and Cabib, 1982; Bacon *et al.*, 1966). On similar lines to that of Siestma and Wessels (1981), a mild alkali treatment was used to define our alkali-insoluble wall fraction. This was done in order to minimize alkaline degradation of the cell wall components (Mol and Wessels, 1987). This alkali treatment (KOH, 1M) removed all the mannan as judged from the identity of anthrone and glucose oxidase values in hydrolyzates of the alkali-insoluble fraction, and absence of mannose after paper chromatography of such hydrolyzates.

4.5.2.1 Effect of potassium hydroxide (KOH)

Anthrone: This mild alkali extraction of the cell walls rendered 31% of the anthrone-positive material alkali-soluble in mycelial phase. In the yeast form 60% of the material was alkali soluble while in Y-2 and Y-5 it was 65% and 45%, respectively. This



Scheme 4.1: Fractionation of cell walls

Table 4.6: Cell wall fractionation of yeast and mycelial phase cell walls and of mutants Y-2 and Y-5.

	Sample[a]	Anthrone positive material	Glucose (%)	Glucosamine (%)	Protein (%)
M	Alkali-soluble	19.7	0.7	1.2	16.1
	Alkali-insoluble	7.0	6.8	32.0 (7.8)[b]	7.8
Y	Alkali-soluble	34.9	0.1	1.9	22.8
	Alkali-insoluble	8.6	9.3	19.6 (5.5)[b]	11.0
Y-2	Alkali-soluble	20.3	0.1	1.2	10.8
	Alkali-insoluble	7.4	6.0	17.8 (2.6)[b]	4.6
Y-5	Alkali-soluble	24.5	0.2	1.5	20.1
	Alkali-insoluble	8.1	7.7	16.3 (3.2)[b]	6.9

a Alkali-soluble: Fraction I
Alkali insoluble: Fraction II.

b Percent N-acetylhexosamine.

Table 4.7: Effect of several consecutive treatments on the solubility of various components from the cell walls of yeast and mycelial phase and mutants Y-2 and Y-5.

Treatments	Percentage of anthrone-positive material remaining insoluble[a]				Percentage of glucose remaining insoluble[a]				Percentage of glucosamino-glycan remaining insoluble[a]			
	M	Y	Y-2	Y-5	M	Y	Y-2	Y-5	M	Y	Y-2	Y-5
KOH, 1 M, 20 min, 60°C	69.2	39.6	34.4	54.1	98.5	98.0	100.0	100.0	95.8	94.6	97.3	96.8
DMSO, 16h, room temperature	52.8	35.5	30.7	37.6	60.1	77.9	79.1	65.0	80.3	47.0	42.2	70.1
NaOH, 40%, 1h, 100°C	32.0	30.4	27.6	32.6	19.7	63.2	70.9	60.2	49.5	31.0	34.8	39.7
Nitrous acid, pH 3.0, 1.5h, room temperature	18.5	13.7	14.2	15.3	3.6	26.6	21.8	24.4	13.6	18.5	20.3	18.9
KOH, 1 M, 20 min, 60°C	3.8	1.9	2.3	2.6	4.6	2.5	3.8	3.5	3.0	6.8	6.1	5.7

a The values for all the components were normalized to their contents of untreated wall samples.

material was gathered to be mannan as judged by values of glucose oxidase and anthrone.

Glucose: The amount of glucose extracted in this fraction was almost negligible as judged by the glucose oxidase method. This residue contained most of the glucan (Table 4.7). Because hexosamines do not react with anthrone, the anthrone-positive material in the alkali-resistant fraction refers to glucose.

Glucosaminoglycan: Alkali-treatment removed small fractions of the glucosaminoglycan from all the four cell walls under investigation (Table 4.7).

4.5.2.2 Effect of dimethylsulfoxide (DMSO)

Anthrone: The percentage of the anthrone-positive material remaining insoluble differs in the cell walls analyzed. In the case of the yeast phase and Y-2 mutant, ~5% of the material was found to be soluble in DMSO, whereas in the case of mycelial phase approximately 17% of the material was DMSO soluble.

Glucans: On treatment with DMSO around 40%, 22%, 21% and 35% of total glucans could be extracted from mycelial phase, yeast phase, Y-2 and Y-5, respectively.

Glucosaminoglyan: A large percentage of this component (~ 80%) was insoluble in DMSO in the mycelial phase whereas approximately 50% was soluble in yeast phase and Y-2 mutant. However, in Y-5 around 30% was found to be solubilized on this treatment. Based on the above data, it seems that glucosaminoglycan and possibly a small percent of glucan-glucosaminoglycan fraction (which are DMSO soluble) occur in yeast phase and Y-2 cell wall. From the mycelial cell wall mainly glucans seem to be extracted.

4.5.2.3 Effect of sodium hydroxide (NaOH)

Anthrone: Subsequent treatment with 40% NaOH at 100°C further reduces this material in the case of mycelial phase, whereas little anthrone reacting material could be extracted for yeast phase, Y-2 and Y-5.

Glucans: A large percentage of glucans of mycelial phase were solubilized on NaOH treatment, but the residue from yeast phase and mutants Y-2 and Y-5 was found to be resistant to this treatment.

Glucosaminoglycan: About 30-40% of this fraction was found to be NaOH soluble in the mycelial phase wall. However, the fraction soluble in NaOH in yeast phase

was much lesser as compared to the mycelial phases. Treatment with NaOH not only extracts glucans but also leads to the deacetylation of chitin (N-acetylglucosaminoglycan) converting it to glucosamine. The above data indicates that a glucan-glucosaminoglycan fraction may be solubilized by this treatment in mycelial phase.

4.5.2.4 Effect of nitrous acid (HNO_2)

Treatment with nitrous acid caused depolymerization of glucosamine but has no effect on N-acetylglucosamine (Shivley and Conrad, 1970; Horton and Philips, 1973; Datema *et al.*, 1977). In the studies carried out, the deacetylation of chitin occurs during the previous NaOH treatment step. Therefore, most of the glucosaminoglycan would now be present as glucosamine.

Anthrone: The anthrone-positive material in all the four cell walls investigated was found to be solubilized on HNO_2 treatment.

Glucans: A large faction of glucans was solubilized in all the cell wall type studied the extraction being 16%, 36%, 48%, 36%, for mycelial phase, yeast phase, Y-2 and Y-5, respectively. However, around 20-25% of this fraction did remain insoluble in the case of yeast phase Y-2 and Y-5 mutant.

Glucosaminoglycan: This treatment was effective in solubilizing a large fraction of the glucosaminoglycan in mycelial phase (36%) while in the yeast phase (13%), Y-5 (21%) and Y-2 (15%) of this fraction was found to be solubilized.

4.5.2.5 Effect of KOH treatment

Anthrone, Glucans: This subsequent alkali treatment rendered most of the anthrone-positive material as well as glucans soluble. However, a small fraction remained in the residue after all the above treatments.

Glucosaminoglycans: Similarly, while most of the fraction was rendered soluble in alkali, a small fraction remained insoluble. It is possible, that the insolubility in the above two fractions could be due to their cross-links with one another.

4.6 DISCUSSION

Studies on cell wall fractionation showed that alkali treatment solubilized most of the mannans, protein and a small fraction of glucosaminoglycan. The small amounts of

glucosaminoglycan removed by alkali treatment is probably contained in the di-N-acetylchitobiose bridges that are known to link mannans to cell wall proteins (Sentandreu and Northcote, 1968; Nakajima and Ballou, 1974). Results on cell wall fractionation (Table 4.6) have shown that fraction I contained mainly mannose and protein with traces of glucose and glucosamine. Glucose, glucosamine and protein were detected in fraction II, while fraction III was composed of glucosamine and traces of protein.

Most of the studies characterizing glucan-chitin linkages in the alkali-insoluble fractions have been carried out on ascomycetes and basidiomycetes. (Burnett, 1979; Gooday and Trinci, 1980; Wessels and Siestma, 1981). Studies with *C. albicans* have also shown the presence of a covalently linked glucan-chitin complex in its cell wall (Surarit *et al.*, 1988).

Data on solubilization of anthrone positive material by KOH treatment (Table 4.7) has shown that approximately 30% is extracted from mycelial phase and 60% from yeast and Y-2 cell walls. This anthrone positive material is most likely mannose, since the amount of glucans solubilized by KOH treatment was relatively less. DMSO treatment solubilized glucans to varying degrees in all the cell wall fractions under investigation. DMSO extracted less glucan from the walls of yeast phase and Y-2 mutant, while a reasonable fraction could be extracted from mycelial walls. The quantities of glucosaminoglycans solubilized from yeast phase and Y-2 mutant were large. Thus, it seems likely that a small amount of glucan-glucosaminoglycan or a free glucosaminoglycan fraction soluble in DMSO exists in yeast phase and Y-2 mutant, while in mycelial phase such a fraction does not seem to be extracted. Glucans, however, were solubilized by DMSO treatment in the mycelial phase. Sodium hydroxide solubilized a greater fraction of glucan/glucosaminoglycan in mycelial phase, while no such effect was observed with either yeast phase or Y-2 and Y-5 cell walls. Since such data was obtained only for mycelial phase it could be possible that this fraction may be responsible for the maintenance of mycelial morphology, although, further characterization of this above fraction is necessary. The most important evidence for postulating linkages between glucan-glucosaminoglycan chains is that glucan chains become soluble after specific depolymerization of (acetyl) glucosamine containing polymers (Wessels, 1988). In all the cell wall fractions studied in *B. poitrasii*, depolymerization of glucosaminoglycan by HNO_2 treatment rendered the solubilization in alkali of a large percentage of glucan in

alkali. Thus, the evidence for a glucan-glucosaminoglycan link seems to exist in *B. poitrasii*. In this organism, however, a large number of residues in glucosaminoglycan occur in a deacetylated form, i.e., as glucosamine. This polymer is extensively degraded by nitrous acid releasing glucans into solution in the mycelial-phase, while little effect is observed in yeast phase. In *M. mucedo* also a large fraction of glucosaminoglycan is deacetylated resulting in polycation (Datema *et al.*, 1977). According to them, linkages in the cell wall of *M. mucedo* occur possibly through amino groups of glucosamine residues. Such a situation could also be possible in case of *B. poitrasii*.

According to Mol *et al.*, (1988) even though a portion of the alkali-insoluble glucan of *Aspergillus nidulans* and *Neurospora crassa* can be extracted by DMSO, they strongly suggest that all glucan is linked to glucosaminoglycan. Their studies on the alkali-insoluble complex of *Agaricus bisporus* (which contains a large fraction as glucosamine) suggests that most of the glucan must be linked to glucosamine residues. Mol and Wessels (1987) have also suggested linkages between glucosamine and glucan in *Schizophyllum commune*.

As discussed earlier, in *B. poitrasii* results indicate the presence of a glucan-glucosaminoglycan fraction soluble in NaOH in the mycelial phase. It is necessary to further characterize and obtain the chemical structure of this fraction, since it may be responsible, in some manner, for the maintenance of cell shape. A large percentage of glucans were solubilized in mycelial phase on nitrous acid treatment. This difference may arise due to its cross links with the glucosamine polymer. Wessels (1988) has suggested that in *Mucor*, that since the glucosamine can be extracted by alkali, the linkage between these two polymers is probably ionic and not covalent. This may also be true for *B. poitrasii*. Although, generally the linkage between glucan and glucosaminoglycan is covalent, the exact nature of this linkage remains a matter of conjecture. Similarly, the nature of the presumed inter-polymer linkages in chitin-protein complexes have not been resolved (Poulicek *et al.*, 1985). Linkage of protein through glucosamine are surmised to occur in chitin-protein complex in *M. Mucedo* (Datema *et al.*, 1977). Such a possibility may also occur in *B. poitrasii* as fraction III of cell wall

fractionation contained small quantities of protein along with glucosamine. With respect to the inter-polymer linkage in chitin-glucan complexes, direct links between free amino groups in glucosamine and the reducing end of the glucan chain is a possibility (Wessels *et al.*, 1990).

To summarise, in *Benjaminiella poitrasii*, results indicate that cross-links occur between mannan and protein, while glucans seem to be linked to glucosaminoglycan. The kind and nature of this glucan-glucosaminoglycan linkage may probably determine the morphology of the fungus. The estimation of the cell wall components and the effect of treatments listed in Table 4.7 have shown that changes in morphology are associated with changes in the levels of one or more of these structural polymers. The levels of glucosamine in the yeast phase mutants (Y-2 and Y-5) clearly support the above fact, as lower levels of this component were present in both yeast phase and yeast form morphological mutants than in the mycelial phase. These results lead to the conclusion that cell-wall composition is closely associated with morphology in *B. poitrasii* and that the shift from mycelial to yeast phase may be due to changes in the relative levels of glucosamine polymer as well as due to the nature of its cross-links with glucan.

DIMORPHISM IN *Benjaminiella poitrasii*: SIGNIFICANCE OF NADP:NAD - GLUTAMATE DEHYDROGENASE RATIO IN TRANSITION OF PARENT STRAIN AND MORPHOLOGICAL MUTANTS

CHAPTER V

CHAPTER V

5.1 INTRODUCTION

Dimorphism, i.e., yeast-to-mycelial transition, is a freely reversible process that can be provoked by temperature or factors controlling environmental conditions (Bartinicki-Garcia, 1963). *Benjaminella poitrasii* is a dimorphic phycomycete and can serve as an useful system for studying cell differentiation. In *B. poitrasii* under aerobic conditions, in complex media, development is mycelial-like at 28°C while it is yeast-like at 37°C. The organism is more fastidious in yeast phase than in mycelial phase with respect to both carbon and nitrogen sources. Cells in the yeast phase require a fermentable hexose whereas the mycelial phase can use a variety of carbon sources under aerobic conditions (Khale *et al.*, 1990). A complex medium containing yeast extract and peptone is required for optimal growth of yeast cells, while mycelial cells can grow with ammonium salts as nitrogen source. Thus, the more stringent growth requirements of yeast cells as compared to mycelial cells suggests differential expression of some key enzymes of carbon and nitrogen metabolism in both the morphological forms.

The carbon and nitrogen metabolism in fungi is connected *via* ammonium assimilation by two pathways. In the first, the reductive amination of α-ketoglutarate to form glutamate and the dissimilation of glutamate releasing ammonia are the reactions catalyzed by glutamate dehydrogenase (GDH) :

$$\text{Ammonium} + \text{2-oxoglutarate} + \text{NAD(P)H} \leftrightarrow \text{glutamate} + \text{NAD(P)}^{+}$$

Fungi possess either or both, an NAD-dependent (EC 1.4.1.2) and an NADP-dependent (EC 1.4.1.4) glutamate dehydrogenase; the former catalyzing the catabolic reaction while the latter the anabolic reaction. The second pathway involves the action of both glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 2.6.1.53).

$$\text{Ammonium} + \text{glutamate} + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + \text{Pi}$$

$$\text{Glutamine} + \text{2-oxoglutarate} + \text{NAD(P)H} \rightarrow \text{2-glutamate} + \text{NAD(P)}^{+}$$

Biochemical analysis of morphological mutants is an useful approach in distinguishing alterations associated with dimorphic transitions, from those, which have no correlation with morphogenesis but which are induced by environmental conditions common to both (Ruiz-Herrera *et al.*, 1983). Recently, we have reported the isolation of two yeast phase mutants growing in yeast form both at 28°C and 37°C (Khale *et al.*, 1990).

The present investigation aims to study the dimorphic transition in *B. poitrasii* (regulated by temperature and carbon - nitrogen balance) in terms of biochemical correlates, i.e., with respect to the involvement of the above enzymes. Comparative studies of the parent strain with the morphological mutants have also been undertaken in order to elucidate the phenomenon of dimorphism in *B. poitrasii*.

5.2 RESULTS

Since NAD-dependent and NADP-dependent GDH seem to function differently by catalyzing opposing reactions, they may be subjected to some form of concurrent regulation. Therefore, the changes in NADP:NAD-GDH ratio (as NADP-GDH is the biosynthetic enzyme) along with changes in levels of GOGAT and GS were studied to ascertain their role in the morphogenesis of *B. poitrasii*.

5.2.1 *Influence of glucose concentration*

Our previous studies have shown that temperature, or glucose concentration, or both act as agents which trigger a dimorphic change (Khale *et al.*, 1990). In low glucose concentrations (less than 0.5% glucose in YPG medium) mycelial form (M) develops while yeast form (Y) development occurs at high glucose concentration (5.0% glucose in YPG medium). However, no change in the morphological mutants Y-2 and Y-5 was observed with respect to either glucose concentration or temperature. Therefore, in the studies carried out, it was essential to monitor the specific activities of GDH, GS and GOGAT as a function of temperature as well as glucose concentration.

The GDH activity detected in extracts of *B. poitrasii* was both NAD- as well as NADP-dependent. In 48h, for both the morphological types, i.e., Y-phase and M-phase (Table 5.1), an increase in glucose concentration in YP media led to an increase in NADP:NAD-GDH ratio at 28°C and 37°C. This increase in NADP:NAD-GDH ratio occurs because a higher NADP-GDH activity was obtained while NAD-GDH was repressed for increasing glucose concentration. This suggests, as reported in other fungal systems that NAD-GDH in *B. poitrasii* also seems to be controlled by carbon catabolite repression (Magasanik, 1961). Thus, the NADP:NAD-GDH ratio (henceforth termed as GDH ratio) seems to be regulated at two levels: a) change in glucose concentration, as increasing glucose concentrations led to an increase in GDH ratio and b) change in temperature, as a higher GDH ratio was needed to maintain the parent strain in yeast form at 28°C than at 37°C.

Table 5.1: Effect of glucose concentration on the morphology and specific activities of GS, GOGAT and GDH ratio in *B. poitrasii*.

Glucose concentration[a] (%)	Morphology[b]	GS (U/mg protein)	GOGAT (U/mg protein)		GDH ratio
			NAD-linked	NADP-linked	
28°C					
YP	M	3.3	ND	33.7	0.002
0.1	M	6.9	ND	16.6	0.005
0.5	M	7.6	2.2	7.3	0.028
	Y	8.5	25.5	6.2	0.23
1.0	M	8.0	3.0	ND	0.16
	Y	14.5	29.0	6.6	0.76
5.0	Y	24.3	33.4	8.3	12.8
37°C					
YP	M	9.0	16.8	35.9	0.004
0.1	M	19.2	12.8	27.8	0.013
	Y	11.6	11.9	4.7	0.2
0.5	M	20.6	13.0	8.5	0.011
	Y	12.8	7.3	4.5	0.32
1.0	Y	16.4	4.5	5.1	0.37
5.0	Y	26.9	ND	1.2	0.5

a Cells were grown for 48h at 28°C and 37°C in YP media with varying glucose concentrations as indicated.

b Separation of mycelial and yeast phase cells was carried out as described earlier, (Khale *et al.*, 1990).

ND Not detected.

M Mycelial phase.

Y Yeast phase.

The GOGAT activity detected in cell-free extracts of cells was found to be both NADP- and NAD-linked. Levels of maximal GOGAT activity were approximately ten fold lower than maximal NAD-GDH activity both at 28°C and 37°C, while the levels were comparable with those of NADP-GDH activity at the same temperature. On comparing the trends between GOGAT and GDH ratio at 28°C, in mycelial phase increase in glucose concentration led to an increase in GDH ratio and GOGAT (NAD) activity, while GOGAT (NADP) decreased. In the case of yeast phase increase in GDH ratio corresponded to steady GOGAT (NAD and NADP) levels. At 37°C on employing higher glucose concentrations in mycelial phase, the GDH ratio was seen to increase with a simultaneous decrease in GOGAT (NAD and NADP) activity levels. Similar trends were also noticed with yeast phase cells.

GS levels were found to be low in the complex media (YP) used and the specific activity was found to be stable for at least 3-4 days after extraction when stored at 4 °C. This enzyme is of importance in morphogenesis because of its function to produce glutamine, an important component leading to the synthesis of chitin, a cell wall constituent (Bartinicki-Garcia, 1963). With increasing glucose concentration in mycelial and yeast phase, irrespective of temperature, a higher GS activity was observed. In *Neurospora crassa*, it has been suggested that the low levels of GS in glucose limited cultures possibly reflects modification, degradation or decreased synthesis of GS to conserve the carbon skeleton (Cooper *et al.*, 1978; Espin *et al.*, 1980; Marzluf, 1981).

5.2.2 *Spore germination*

Spores of parent strain germinate on YP media (containing 0.1% glucose) to give mycelia at 28°C while at 37°C yeast phase is obtained. Microscopic studies show that irrespective of the temperature, the initial morphological stages were common upto 12h. Spores began to swell after 3h and attained maximum size (4-6 times the original size) by about 8h. Thereafter, the spore put forth bud-like structures similar to those of yeast phase cells and the process continued upto 12h. Depending on the temperature of incubation (i.e., 28°C or 37°C), the morphological changes occurred. At 28°C , these bud-like structures gave rise to germ tubes which led to mycelium formation, while at 37°C, the budding process continued until a yeast type colony was obtained. It was therefore important to check the correlation, if any, between the morphological form during spore germination and the levels of GS, GOGAT and GDH activities.

During spore germination upto 8-10h, the GDH ratio decreased and followed one another closely and proportionately, in cultures incubated both at 28°C and 37°C (Fig. 5.1a). This is due to the fact that although NADP-GDH levels changed, a steady increase in NAD-GDH was observed (the levels of the NAD-dependent enzyme in the spores are very low). By 12h the cells had probably been committed to its ultimate morphology since the ratio dropped further in case of cultures at 28°C (leading to M-phase formation), while at 37°C (leading to Y-phase) a steady ratio was maintained. These results coupled with our observations on the effect of glucose concentration confirms the fact that a high GDH ratio is required for the maintenance of yeast morphology.

GOGAT (NAD) activity was absent in the spores and its levels were detected only after 24h, by which time the morphological phases were already formed. Therefore, the involvement of this enzyme in *B. poitrasii* morphogenesis seems to be remote. GOGAT (NADP) activity was high in spores and increased nearly 4-fold upto 8h (Fig. 5.1b), after which it decreased for both yeast and mycelial phase cells. In *N. crassa* it has been shown that GOGAT (NAD) is essential for the germination of conidia (Hummelt and Mora, 1980). This may also be the case with GOGAT (NADP) in *B. poitrasii* during spore germination, since, after 8h the enzyme levels were found to decrease. Thus, although the correlation of GOGAT (NAD and NADP) activity with morphogenesis seems remote, this enzyme may be involved in the assimilation of ammonia in both forms. Similarly, a spurt in GS activity at 4-8h could be due to the need for glutamate or glutamine during spore germination (Fig. 5.1c). Since GS activity trends follow that of GOGAT(NADP), it is suggested that GS/GOGAT pathway may be acting in conjunction for glutamate production during germination of spores.

In general, glucose is more rapidly metabolized than other carbon compounds. Cells grown on glucose can degrade it *via* triose phosphate, as well as *via* gluconic acid. With the aid of these two independent pathways, glucose produces very rapidly pyruvate and α-ketoglutarate which in turn are the precursors for a number of amino acids, including glutamate (Magasanik, 1961). It was therefore important to check the effect of these compounds on the transition of *B. poitrasii*.

5.2.3 *Transition studies*

For these studies yeast cells were grown at 37°C for 48h in YPG medium containing 0.1% (w/v) glucose. A high glucose concentration was not used since relatively pure

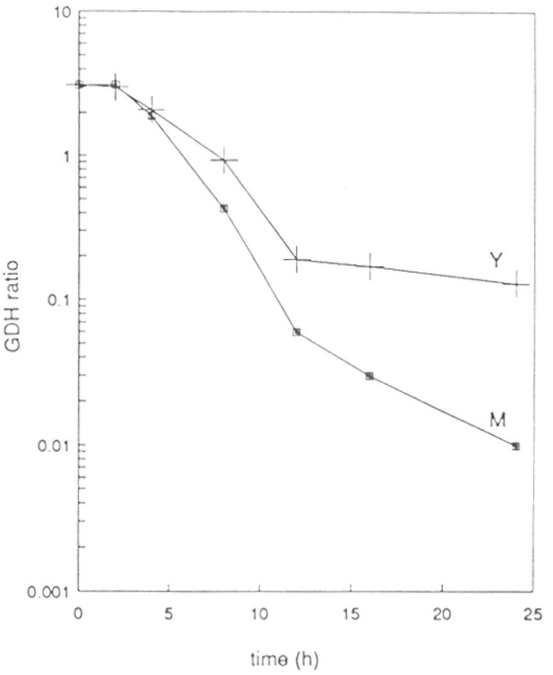


Fig. 5.1a: GDH ratio during spore germination in *B. poitrasii* parent strain.
Y - yeast phase
M - mycelial phase

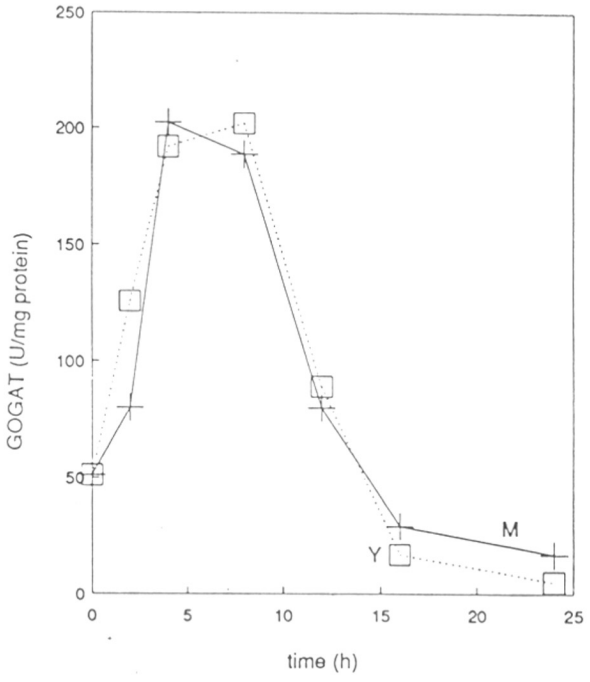


Fig. 5.1b: Glutamate synthase (NADP) activity during spore germination in *B. poitrasii* parent strain.
Y - yeast phase
M - mycelial phase

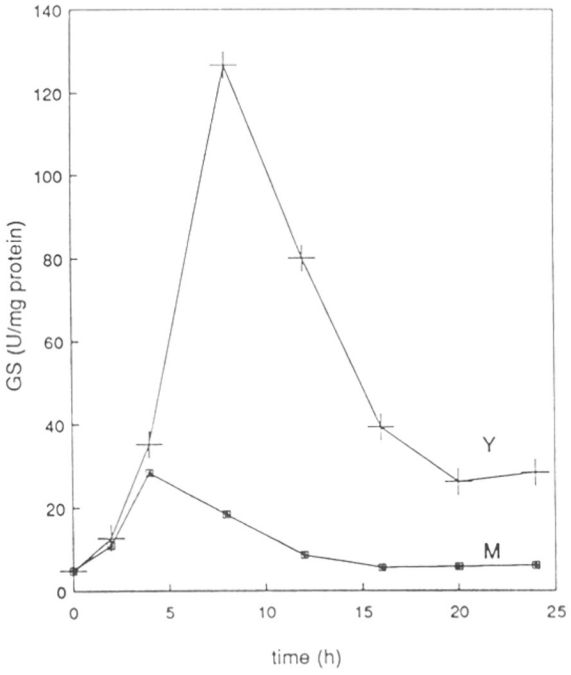


Fig. 5.1c: Glutamine synthetase activity during spore germination in *B. poitrasii* parent strain.
Y - yeast phase
M - mycelial phase

phase cells were obtained with 0.1% glucose and secondly, repression was observed in the case of NAD-GDH at higher glucose concentrations (Table 5.1). For yeast-to-mycelium transition studies, the yeast cells (8 x 10^6 cells/50 ml medium) were used to inoculate YPG (0.1% glucose) medium and incubated at 28°C under shaking conditions. The morphological differentiation as seen microscopically by germ tube formation was monitored along with GDH, GOGAT and GS activities. The cells initially grew for 2-3h in yeast form after which some of the cells put forth germ tubes. The percentage of cells forming germ tubes increased rapidly thereafter.

The criteria employed to determine which of the three enzymes (GDH, GOGAT or GS) in yeast and mycelial phase of *B. poitrasii* was a biochemical correlate of the morphological transition were similar to those used by Peters and Sypherd (1979) :

(1) The first criterion used was that the biochemical correlate should precede the morphological outcome. In the present studies, it was observed that the decrease in GDH ratio preceded the morphological outcome i.e., germ tube formation. The drop in GDH ratio began by 1h, with the maximal drop between 1-2h, i.e., before the visible germ tube formation which occurred at 3h (Fig. 5.2a). However, during mycelium-to-yeast (M → Y) transition studies carried out at 37°C (using mycelial phase cells as an inoculum), reverse trends were observed (Fig. 5.3a). While the percentage of hyphal cells decreased, there was a concomitant increase in GDH ratio (5h), which once again preceded the morphological outcome (6-8h). During yeast-to-mycelium transition studies GOGAT (NAD) was found to decrease by 2h and was undetectable on further incubations, while GOGAT (NADP) levels increased throughout the transition (Fig. 5.2b). In mycelium-to-yeast transition, 15-20% of the cells were found in yeast form by 6-8h, though GOGAT (NAD) activity could only be detected at 24h. Thus, GOGAT (NAD) activity does not seem to have a significant role in *B.poitrasii* morphogenesis. The levels of GOGAT(NADP) on the other hand were found to decrease (Fig. 5.3b). During yeast-to-mycelium transition in the parent strain, the levels of GS dropped and followed a trend similar to that of GDH ratio (Fig. 5.2b). In the case of mycelium-to-yeast transition however, the trend of this enzyme was not opposed to that of yeast-to-mycelium transition, which would have been expected had it been involved in the dimorphic transition. In fact, GS trend during mycelium-to-yeast transition initally increased upto 5h after which the levels dropped (Fig. 5.3b).

(2) The second criterion used was that the biochemical correlate should be sensitive to signals that affect the morphological outcome. These studies were carried out in 2 parts,

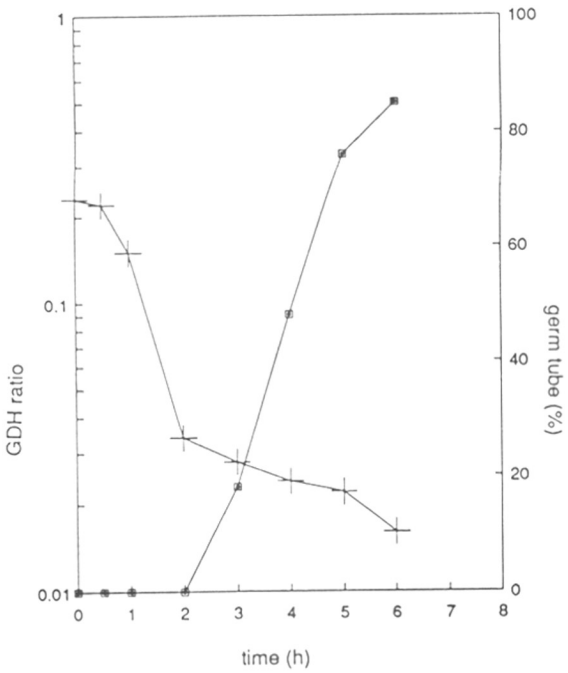


Fig. 5.2a: Change in GDH ratio during yeast-to-mycelial (Y→M) transition in *B. poitrasii* parent strain.

germ tube
GDH ratio

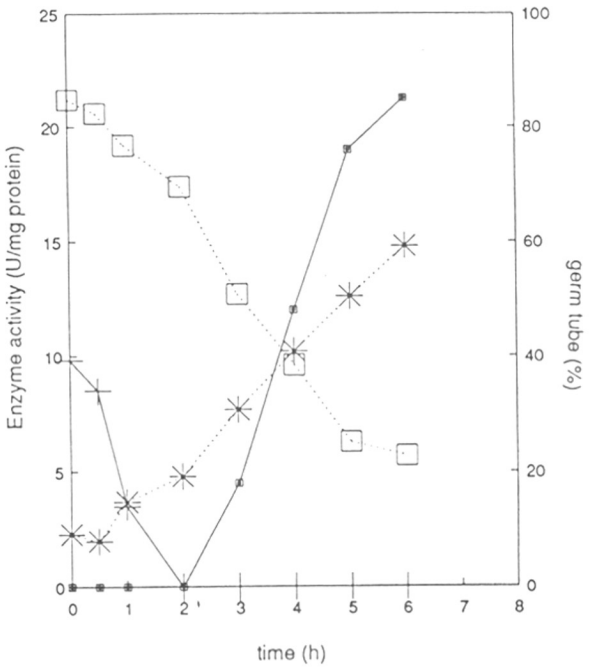


Fig. 5.2b: Changes in enzyme trends of GS and GOGAT during yeast-to-mycelial (Y→M) transition in *B. poitrasii* parent strain.

germ tube
GOGAT(NAD) activity
GOGAT(NADP) activity
GS activity

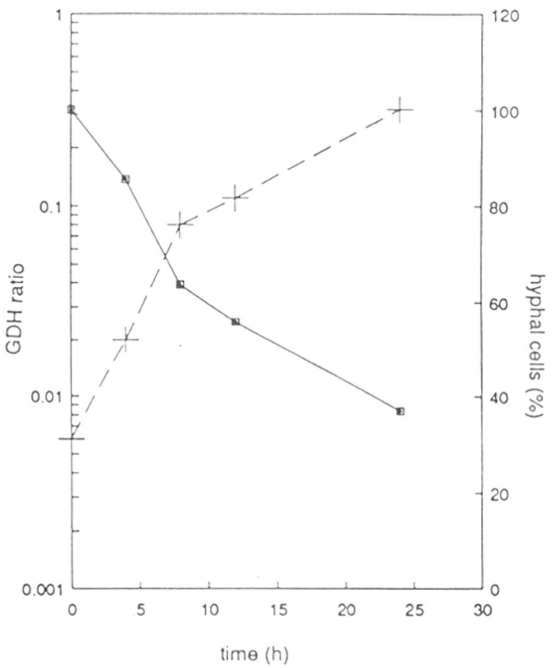


Fig. 5.3a: Change in GDH ratio during mycelium-to-yeast (M→Y) transition in *B. poitrasii* parent strain.

hyphal cells
GDH ratio

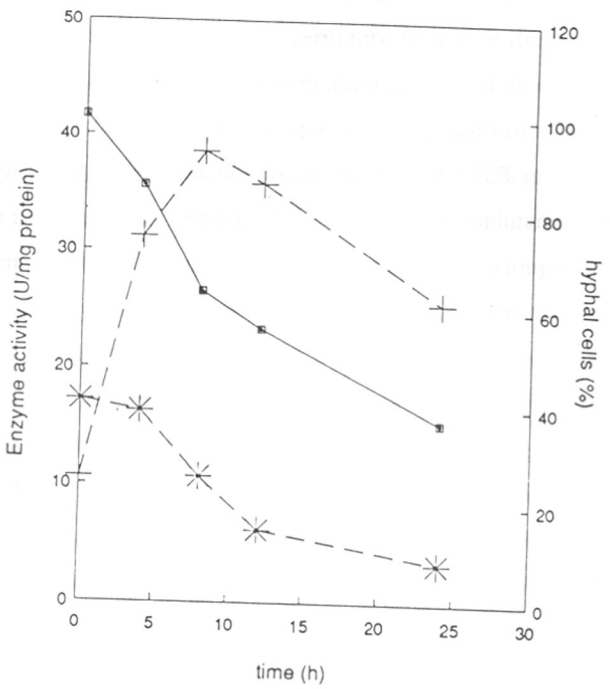


Fig. 5.3b: Changes in enzyme trends of GS and GOGAT during mycelium-to-yeast (M→Y) transition in *B. poitrasii* parent strain.

namely, (a) exogenous addition of α-ketoglutarate and glutamate to YPG medium (0.1%, glucose) to see the effect on transitional events, and (b) Exogenous addition of inhibitors to determine the role of enzyme(s) in transitional events.

5.2.4 *Effect of α-ketoglutarate on yeast-to-mycelium transition*

Varying concentrations of α-ketoglutarate (1.0 mM - 10.0 mM) in YPG medium were tested to observe their effect on germ tube formation during transition. While 1.0 mM α-ketoglutarate inhibited germ tube formation upto 3h, at 4h only 10-12% of the cells were able to form germ tubes (Fig. 5.4). Higher concentrations of α-ketoglutarate (2.5 mM-10.0 mM) delayed germ tube formation to varying degrees (22% in 5h, 18% in 5h and 10% in 6h at 2.5, 5.0 and 10.0 mM, respectively). In short, α-ketoglutarate when added externally to the medium was able to maintain the cells in yeast form for a longer period of time as compared to controls. Enzymatic studies carried out during transition in presence of 1.0 mM α-ketoglutarate showed an increase in GDH ratio upto 2h, after which it decreased (Fig. 5.5a). This may be either due to an increase in NADP-GDH levels (observed upto 2h as α-ketoglutarate acts as substrate for this enzyme) or due to the end product inhibition of NAD-GDH activity. As shown in Fig. 5.5a, the decrease in GDH ratio which preceded germ tube formation probably occurs once the α-ketoglutarate levels decreased. The trends of GOGAT (NAD and NADP) and GS activities during transition in presence of α-ketoglutarate were found to be the same (Fig. 5.5b). Increased levels of GOGAT (NAD) and GS also point towards their role in ammonia assimilation.

5.2.5 *Effect of glutamate on yeast-to-mycelium transition*

It was seen that at 1.0 mM glutamate, the percentage of cells forming germ tubes was higher at 3h than the control (Fig. 5.4). As shown in Fig. 5.6a, the GDH ratio dropped initially upto 3h, after which a slight increase in the ratio was observed. The initial lowering of GDH ratio may be due to the greater specific activity of NAD-GDH observed during transition. Glutamate is the substrate for the oxidative deamination reaction while it also acts as a product inhibitor of the NADP-GDH catalyzed reaction (as substantiated by the low levels of NADP-GDH activity). GOGAT (NAD and NADP) seemed to be inhibited by glutamate, which can be attributed to end product inhibition. Higher GS levels (Fig. 5.6b) can be correlated to the increased availability of the substrate, glutamate.

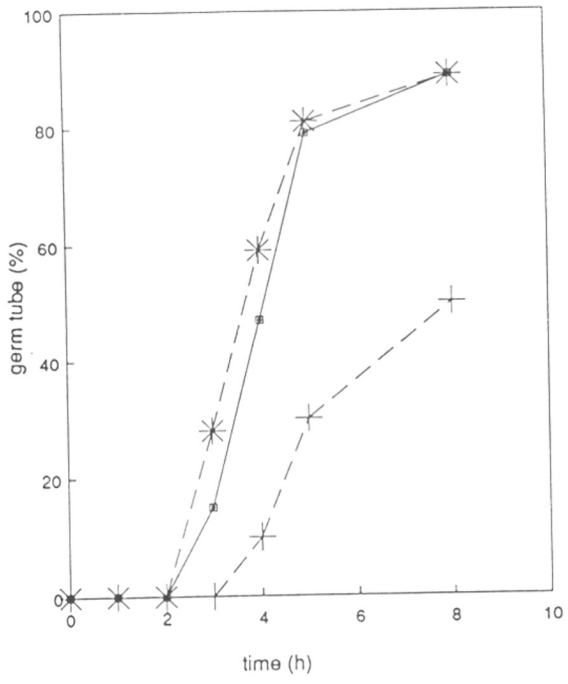


Fig. 5.4: Effect of α-ketoglutarate (1.0 mM) and glutamate (1.0mM) on germ tube formation during yeast-to-mycelial (Y→M) transition in *B. poitrasii* parent strain.

control
. -ketoglutarate
glutamate

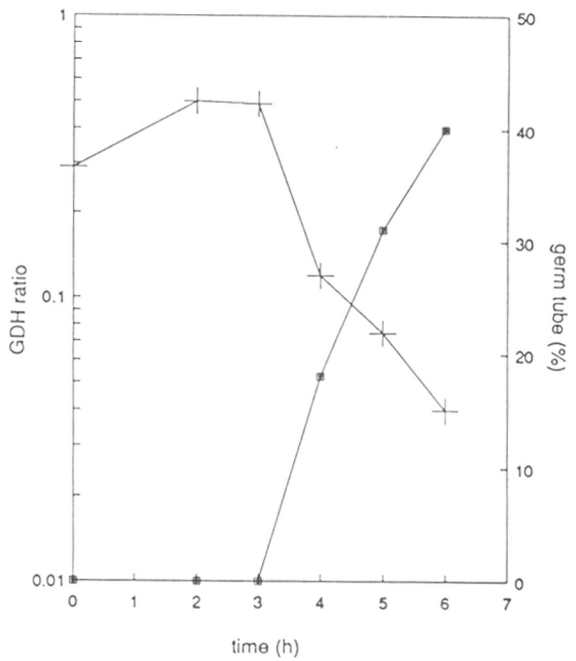


Fig. 5.5a: Effect of α-ketoglutarate (1.0mM) on GDH ratio during yeast-to-mycelial (Y→M) transition in *B. poitrasii* parent strain.

germ tube
GDH ratio

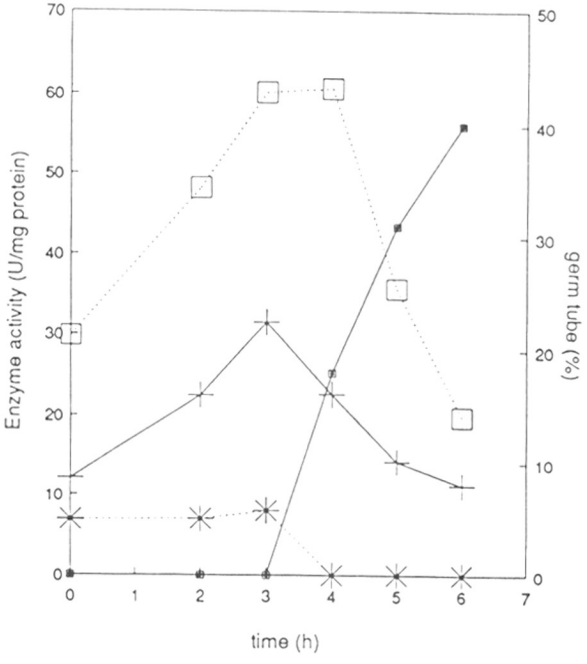


Fig. 5.5b: Effect of α-ketoglutarate (1.0mM) on GS and GOGAT activities during yeast-to-mycelial (Y→M) transition in *B. poitrasii* parent strain.

germ tube
GS activity
GOGAT(NAD) activity
GOGAT(NADP) activity

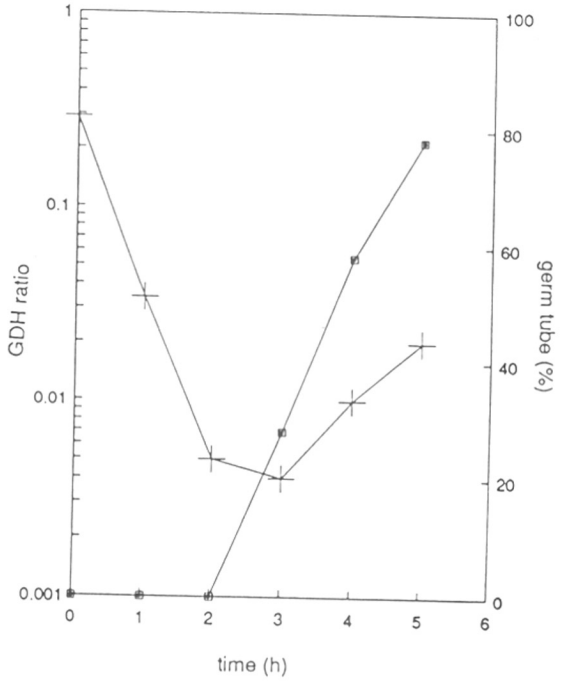


Fig. 5.6a: Effect of glutamate (1.0 mM) on GDH ratio during yeast-to-mycelial (Y→M) transition in *B. poitrasii* parent strain.

germ tube

GDH ratio

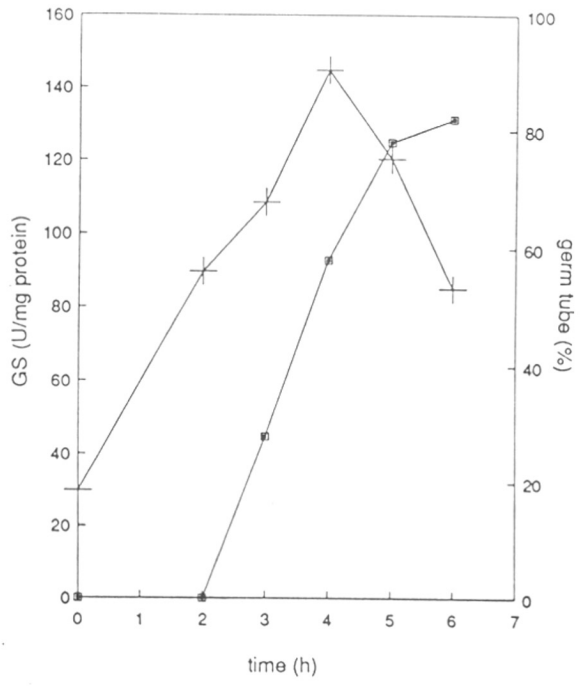


Fig. 5.6b: Effect of glutamate (1.0 mM) on GS activity during yeast-to-mycelial (Y→M) transition in *B. poitrasii* parent strain.

germ tube
GS activity

5.2.6 *Studies using inhibitors during yeast-to-mycelium transition*

Studies were carried out using cycloheximide (a protein synthesis inhibitor) at a concentration (5 μg/ml), where although inhibition of transition occurred, growth was not inhibited. Similar studies were also carried out using specific inhibitors at varying concentrations for GDH, GOGAT and GS. Isophthalic acid, is known to be a strong competitive inhibitor of NAD-GDH (Veronese *et al.*, 1974). L-Methionine-D-sulfoximine (MSX) is phosphorylated *in vivo* and then binds tightly to the active site of GS, irreversibly inhibiting the enzyme (Ronzio and Meister, 1968). Azaserine, a glutamine analog is a well known GOGAT inhibitor (Holmes *et al.*, 1989).

Effect of cycloheximide: In *Mucor racemosus* it has been shown that cycloheximide at a concentration of 200 μg/ml completely inhibited protein synthesis, NAD-GDH activity, and germ tube formation (Peters and Sypherd, 1979). Since the concentration of cycloheximide used in the above study was high, experiments were initially carried out using concentrations in the range of 2.5 - 50 μg/ml. This enabled selection of those concentration values which would inhibit yeast-to-mycelium transition without affecting growth (monitored by percent budding). The results indicated that cycloheximide inhibited growth at concentrations above 20-30 μg/ml. It was also observed that at 5 μg/ml, germ tube formation was delayed and occurred at 5h as compared to 3h in control (at 10μg/ml germ tube formation occurred at 7h), while no effect on growth was seen.

Using 5μg/ml, the effect of cycloheximide on GDH activities was studied (Fig. 5.7a). An initial increase in GDH ratio of almost 10-fold was observed upto 2h, after which it declined rapidly. It was seen that while NAD-GDH inhibition occurred by cycloheximide, no such effect was observed on NADP-GDH (Fig. 5.7a, inset). It was also noted that a decrease in GDH ratio preceded the onset of germ tube formation. From the above observation it seems that the synthesis of NAD- and NADP-dependent GDH activity occurs independent of one another. Additionally, it was also seen that cycloheximide inhibited GOGAT (NAD and NADP) activities, since the levels of this enzyme could not be detected upto 5h, i.e., before germ tube appearance. No inhibition of GS activity was observed and the enzyme levels began to drop after 3h (Fig. 5.7b), and the trend was similar to that of yeast-to-mycelium transition in the controls.

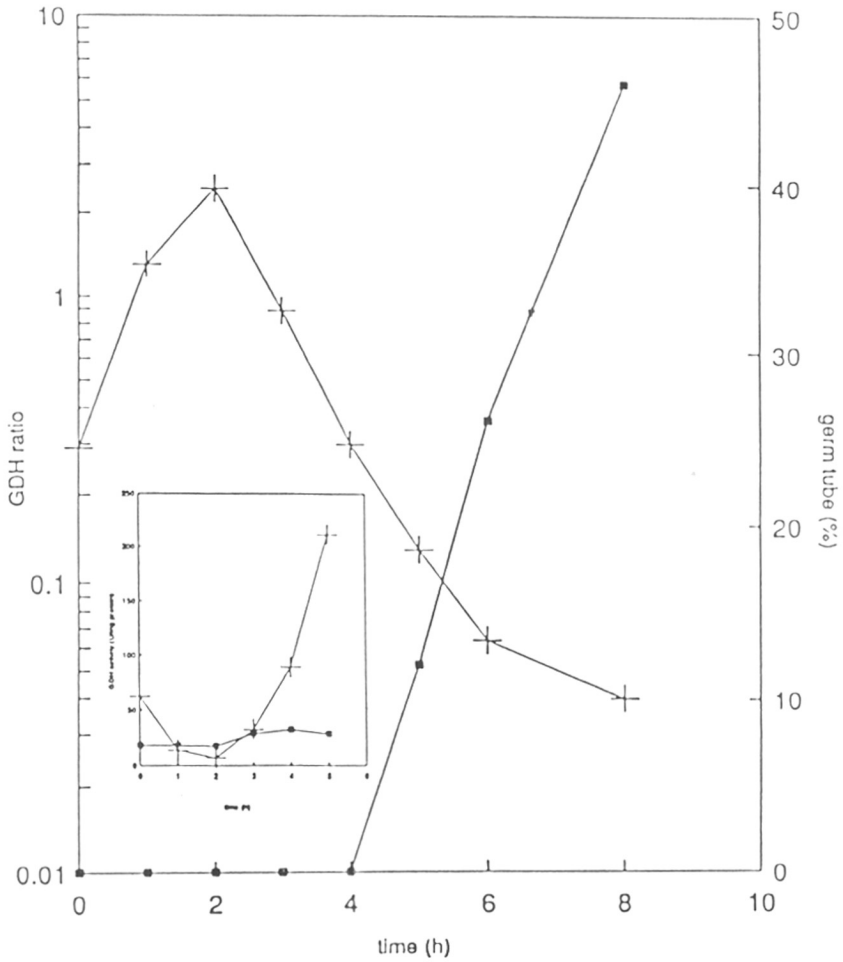


Fig. 5.7a: Effect of cycloheximide (5.0 µg/ml) on germ tube formation and GDH ratio during yeast-to-mycelial (Y→M) transition in *B. poitrasii* parent strain.

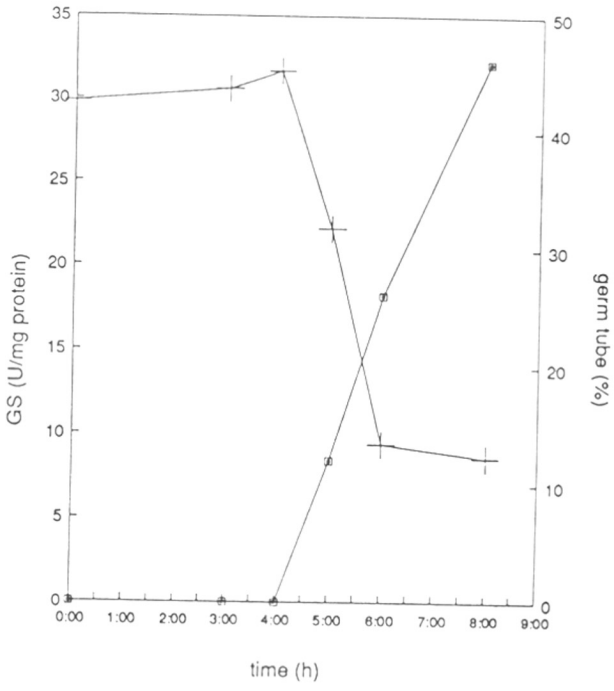


Fig. 5.7b: Effect of cycloheximide (5.0 µg/ml) on germ tube formation and GS activity during yeast-to-mycelial (Y→M) transition in *B. poitrasii* parent strain.

germ tube
GS activity

Enzyme inhibition studies: In the concentration range studied (1.0 mM - 10.0 mM) isophthalic acid (GDH inhibitor) at 2.5 mM inhibited germ tube formation upto 5h (6.0% germ tubes formed). Higher concentrations delayed germ tube formation to varying degrees (20% germ tube in 6h at 5.0 mM and 16% germ tube in 7h at 10.0 mM). An increase in GDH ratio occurred (Fig. 5.8) which was due to inhibition of NAD-GDH activity while no effect on NADP-GDH activity was observed (Fig. 5.8, inset). GS inhibitor, methionine sulfoximine (MSX), could also inhibit germ tube formation although to a lesser extent than isophthalic acid. MSX (2.5 mM) was able to inhibit upto 3h, but the percentage of cells forming germ tubes was lesser (12%) than that compared to controls (20%). However, higher MSX concentrations inhibited the germ tube formation for longer time periods (13% germ tubes for 4h at 5.0 mM and 17% germ tubes for 5h at 10.0 mM). On the contrary, azaserine (GOGAT inhibitor) was unable to inhibit germ tube formation at the concentration range tested (0.1 mM - 2.5 mM).

5.2.7 *Studies with cyclic AMP*

The possible involvement of cyclic AMP(cAMP) in the development and morphogenesis of fungi has attracted the attention of several laboratories (Pall, 1981). Therefore, studies were undertaken to check for the possible involvement of cAMP and other adenine nucleotides in the yeast-to-mycelium transition.

Lower concentrations (2.0 mM and below) of the compounds tested had no effect on the transition. The results of exogenous addition of higher concentrations (5.0 mM) of the various adenine nucleotides are tabulated in Table 5.2. The adenine nucleotides inhibited germ tube formation to varying extents, the maximum inhibition obtained by cAMP, adenine and adenosine. Cyclic AMP was able to inhibit germ tube formation upto 7h, although the cAMP analog dibutyryl cAMP (dbcAMP) was found to be less effective than cAMP. Our observations are in contrast to that of *M. racemosus* (Peters and Sypherd, 1979) where dbcAMP at concentration of 3.0 mM and 6.0 mM could effectively maintain the culture in the yeast form.

5.2.8 *Phosphorylation-dephosphorylation studies*

Protein phosphorylation and degradation are recognized as the most important mechanisms for controlling the activity of regulatory enzymes in eukaryotic cells (Goldberg and St.John, 1976; Krebs and Beavo, 1979; Mazon and Hemmings, 1979). It has been clearly shown that NAD-dependent GDH from *Candida utilis* is regulated by phosphorylation

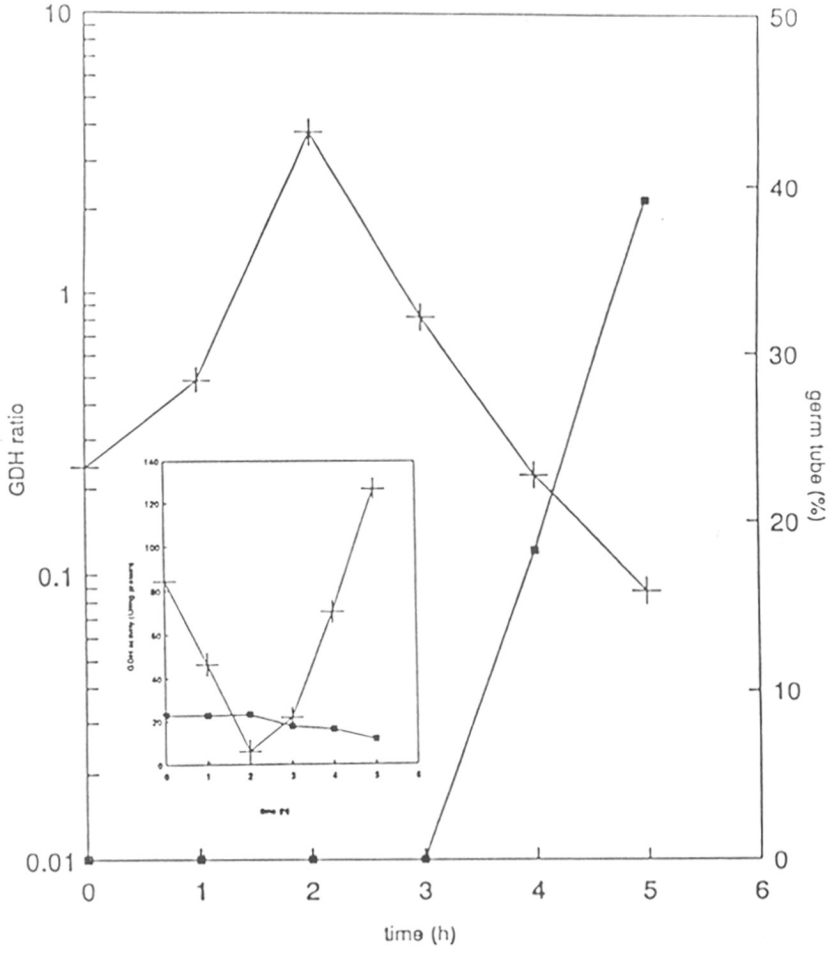


Fig. 5.8: Effect of isophthalic acid (2.5 mM) on germ tube formation and GDH ratio during yeast-to-mycelial (Y→M) transition in *B. poitrasii* parent strain.

(Hemmings, 1978). Similarly, inactivation of NAD-GDH activity in *Saccharomyces cerevisiae* also occurs by phosphorylation followed by proteolysis (Hemmings, 1980; Uno *et al.* 1984). On the other hand, NAD-dependent GDH from *Trignopsis variablis* and *Rhodotorula glutinis* were not regulated in a similar manner (Hemmings, 1978).

Cell-free extracts from yeast and mycelial phase cells grown for 48h on YPG (0.1% glucose) medium were incubated with the various components (Table 5.3), for 10 min prior to GDH assay. In phosphorylation studies, addition of 1.0 mM ATP or 1.0 mM ATP with 10μM cAMP did not lead to significant activation of NAD-GDH of either mycelial or yeast phase cells. Similarly, NADP-GDH of yeast phase also did not show appreciable activation, although activation of NADP-GDH from mycelial phase enzyme did occur on addition of ATP and cAMP (Table 5.3).

Loss of NAD-GDH activity from yeast and mycelial phase cells and NADP-GDH from yeast cells was observed on incubation with 10 mM $MgSO_4$ and alkaline phosphatase. Addition of 40 mM sodium fluoride (NaF) to incubation mixtures containing $MgSO_4$, largely prevented this inactivation (Table 5.3). Thus, this inhibition seems to be mediated by a specific protein requiring Mg^{+2} and was inhibited by NaF. The above data suggests that NAD-GDH is present in an active (phosphorylated) state in both the forms which can be inactivated on incubation with $MgSO_4$ and alkaline phosphatase. Similarly, NADP-GDH of yeast phase is also present in an active (phosphorylated) state, although in mycelial phase it seemed to be present in an inactive (dephosphorylated) state.

5.2.9 *Effect of proteolysis*

Proteolytic enzymes have been shown to have differential effects on interconvertible enzymes (Huang and Cabib, 1974). Incubation of crude cell extracts with trypsin (20 μg/ml) inactivated both NAD and NADP-GDH in *B. poitrasii* (Table 5.3). In *S. cerevisiae* similar observation also have been reported (Hemmings, 1980). However, in *C. utilis* it has been reported (Hemmings, 1978) that incubation of inactive preparations of NAD-GDH with trypsin resulted in rapid reactivation of the enzyme.

5.2.10 *Effect of adenine nucleotides*

In these experiments, the influence of 1.0 mM ATP and 10μM of the various adenylates on GDH activities were studied (Table 5.4). In the mycelial phase, maximum activation was observed with cAMP. Dibutyryl cAMP was found to be relatively less active than cAMP, while 5′AMP inhibited the NAD-GDH enzyme. No change in activity was seen

Table 5.2: Exogenous addition of adenine nucleotides on yeast-to-mycelium transition in *B. poitrasii*

Adenine nucleotides[a] (5.0 mM)	Time[b] 2h	3h	4h	5h	6h	7h
Control	-	22	43	78	82	87
Adenine	-	-	-	-	21	46
Adenosine	-	-	-	-	18	32
3′ AMP	-	14	40	67	82	84
5′ AMP	-	18	38	51	80	88
ADP	-	8	16	40	58	61
ATP	-	13	21	35	46	69
cAMP	-	-	-	-	-	10
dbcAMP	-	-	-	15	26	49

a The inoculum was grown for 48h at 37°C in YPG medium and $8x10^6$ cells/50 ml media were inoculated to the same medium containing the above nucleotides at a concentration of 5.0 mM and incubated at 28°C.

b Measured as percent germ tube formation.

\- No germ tube formation.

Table 5.3: Studies on phosphorylation-dephosphorylation and proteolysis on GDH activity in mycelial and yeast phase of parent strain and mutants Y-2 and Y-5.

Additions[a]	Percentage activity							
	M-phase		Y-phase		Y-2		Y-5	
	NAD	NADP	NAD	NADP	NAD	NADP	NAD	NADP
Control	100	100	100	100	100	100	100	100
Phosphorylation								
ATP (1.0 mM)	120	240	106	103	110	104	114	102
ATP + cAMP (10μM)	160	342	118	130	131	108	124	103
Dephosphorylation								
$MgSO_4$ (10 mM)	19	103	45	0	81	0	33	0
$MgSO_4$ + NaF (40 mM)	87	106	80	62	ND	ND	ND	ND
Alkaline Phosphatase (1000 U)	24	98	52	13	72	0	61	0
Proteolysis								
Trypsin (20 μg/ml)	14	0	68	59	ND	ND	ND	ND

a For enzyme activites, cell extracts prepared in extraction buffer containing 1.0 mM PMSF (except for trypsin experiment, in which case PMSF was omitted) were incubated for 10 min at 30°C. To all the samples NaF (40 mM) was added (except for $MgSO_4$ in which case NaF was added after termination of reaction). GDH activity was measured as described under Materials and Methods.

ND Not determined

Table 5.4: Effect of adenine nucleotides on GDH activity in yeast and mycelial phases of *B. poitrasii*.[a]

Addition[b] (10 μM)	Mycelial phase		Yeast phase	
	NAD-GDH (U/mg protein)	NADP-GDH (U/mg protein)	NAD-GDH (U/mg protein)	NADP-GDH (U/mg protein)
Control	1123.3	4.0	219.4	22.9
cAMP	2176.2	18.6	247.0	29.7
dbcAMP	1413.8	7.8	198.6	29.7
3′AMP	1153.6	10.6	228.6	18.9
5′AMP	554.2	5.6	198.2	18.0
Adenine	1109.1	3.9	237.8	29.7
Adenosine	1067.8	3.9	246.9	29.7

a Cells were grown in YPG (0.1%, glucose) at 37°C and 28°C, respectively.

b As described in Table 5.3. Except control, all the additions contain ATP (1.0mM) and the adenine nucleotide (10 μM) as indicated.

with adenine, adenosine or 3′AMP. With the yeast phase enzyme similar results to that of mycelial phase were observed. Studies with NADP-GDH showed activation with cAMP, dbcAMP and 3′AMP in the mycelial phase, though no significant change was observed in the case of yeast phase NADP-enzyme.

5.2.11 *Studies with morphological mutants*

Morphological mutants Y-2 and Y-5 exhibited yeast form irrespective of growth temperature (28°C or 37°C) or of glucose concentration in the medium (Khale *et al.*, 1990). In both the mutants, the GDH ratio was found to increase with increase in glucose concentration (Table 5.5). The elevated GDH ratio occurred due to an increase in NADP-GDH as a function of glucose concentration (the higher the glucose concentration, the greater NADP-GDH activity).

In YPG medium containing 1.0% glucose, the parent strain at 28°C exhibited both mycelial and yeast phases. The data in Table 5.5 indicates that at 1.0% glucose concentration, a 3-fold increase in GDH ratio was observed in the case of mutants Y-2 and Y-5 when compared to yeast phase cells of the parent strain. It may be concluded that yeast morphology in the mutants is being maintained due to their high GDH ratio. As reported earlier (Khale *et al.*, 1990), no growth of Y-2 and Y-5 was observed on YP media in the absence of a fermentable hexose. No changes in the levels of GOGAT (NAD and NADP) or GS were observed in the mutants Y-2 and Y-5 as a function of either glucose concentration or temperature. The levels of these enzymes were comparable to those of yeast phase of parent strain.

Similarly, 37°C-to-28°C transition studies showed that GDH ratio increased, especially after 2h, while maintaining yeast morphology. This may be due to the fact that both Y-2 and Y-5 require a higher GDH ratio for maintaining their yeast morphologies at 28°C than at 37°C (Table 5.6). These results are in agreement with the data on the effect of glucose concentrations on yeast phase of parent strain. Levels of GOGAT and GS did not vary as a result of shift in temperature in both the mutants.

In studies carried out with exogenous glutamate addition (1.0 mM), no germ tube formation was observed with either Y-2 or Y-5. This could also be correlated to the maintenance of a high GDH ratio (Table 5.7) since an increase in NAD-GDH levels did not occur in the mutants. Similar data with respect to NAD-GDH has been reported in *Mucor*

Table 5.5 Effect of glucose concentration on GDH ratio in the mutants Y-2 and Y-5.

Glucose concentration (%)	Y-phase[a]		Y-2		Y-5	
	28°C	37°C	28°C	37°C	28°C	37°C
YP	-	-	-	-	-	-
0.1	-	0.2	1.7	0.16	0.6	0.2
1.0	0.76	0.37	4.3	0.27	2.7	0.9
5.0	12.8	0.48	11.1	0.39	13.3	1.6

a Yeast phase of parent strain.

\- No growth of mutants observed in YP media, whereas growth occurs as mycelia in parent strain.

Table 5.6: Transition (37°C-to-28°C) studies on the enzyme activities in mutants Y-2 and Y-5.

Mutant[a] (%)	Time (h)	GS (U/mg protein)	GOGAT (U/mg protein)		GDH ratio
			NAD-linked	NADP-linked	
	0	36.8	65.9	3.9	0.11
Y-2	2	35.9	75.1	3.8	0.22
	3	33.7	84.7	4.0	0.81
	5	34.2	73.6	3.8	1.2
	0	35.7	40.2	8.4	0.2
Y-5	2	41.6	31.3	7.6	0.6
	3	43.2	24.0	8.1	0.62
	5	44.6	18.8	7.7	0.68

a No germ tube formation was observed during transition in the case of mutants, i.e., morphology (Y form) is maintained.

Table 5.7: Effect of glutamate (1.0 mM) on enzymes of Y-5 and Y-2 during transition studies.

Mutant (%)	Time (h)	GS (U/mg protein)	GOGAT (U/mg protein)		GDH ratio
			NAD-linked	NADP-linked	
	0	36.2	46.9	8.0	0.3
Y-2	2	144.6	-	-	0.68
	3	63.1	-	-	0.77
	5	39.2	-	-	1.6
	0	31.4	36.8	4.3	0.24
Y-5	2	103.8	-	-	0.9
	3	186.3	-	-	1.7
	5	157.0	-	-	2.2

- Not detected.

bacilliformis mutants (Ruiz-Herrera *et al.*, 1983). The rapid increase in levels of GS (2h) in the presence of glutamate indicates that the enzyme has some role in the catabolism of this amino acid (Table 5.7).

5.3 DISCUSSION

The enzymes, namely, GDH, GS and GOGAT occupy a strategic position in nitrogen metabolism. GDH is an important branch point between carbon and nitrogen metabolism since it catalyzes the reductive amination of α-ketoglutarate to yield glutamate and also the oxidative deamination of glutamate, which provides ammonia. Most micro-organisms possess two GDHs, *viz.*, NADP-GDH which appears to have a role in the bio-synthesis of glutamate, while NAD-GDH is responsible for the reverse reaction in which ammonia and α-ketoglutarate are formed (Pateman and Kinghorm, 1975). *Benjaminiella poitrasii* was found to have both the NAD- and NADP-dependent GDH. In both, mycelial and yeast phases, the NADP-GDH constitutes a lesser fraction of the total GDH activity, although, in yeast phase the NADP-GDH activity is almost 6-7 fold higher as compared to mycelial phase. In higher fungi, the two GDH activities are reciprocally regulated by glucose (Peters and Sypherd, 1979), with the NADP dependent enzyme being glucose inducible and the NAD-dependent enzyme being glucose repressible. A similar type of regulation seems to occur with GDH in *B. poitrasii*. In this system it was observed that while the NADP:NAD-GDH ratio increased with increasing glucose concentration, a concomitant change in morphology from mycelium to yeast form occurred (Table 5.1). The control exerted by hexoses over morphogenesis has been well documented by Bartnicki-Garcia (1968). In *M. rouxii*, a high glucose concentration (8-10%) inhibits hyphal morphogenesis and almost all the cells remain in yeast form (Bartnicki-Garcia, 1968). However, in *B. poitrasii* a higher GDH ratio is required to maintain the cells in yeast morphology at 28°C as compared to 37°C. This finding is supported by the data on yeast phase mutants Y-2 and Y-5, where, a higher GDH ratio (almost 3-4 times more than Y-phase cells of parent strain) enables the mutants to maintain yeast morphology at 28°C.

The parent strain grew on YPG medium (0.1%, glucose) in mycelial form at 28°C and mycelial and yeast form at 37°C with the growth of Y-2 and Y-5 occurring in yeast form at both these temperatures. Under these conditions it was expected that NADP-dependent GDH would be higher in mycelial phase than in yeast phase cells. However, the experiments showed that a higher activity was found in yeast phase cells. Our results are

similar to those reported by Peters and Sypherd on *M. racemosus* (Peters and Sypherd, 1979). It therefore appears that GDH does not seem to be the only enzyme involved in ammonia assimilation in *B. poitrasii*. On calculation of the GOGAT : GDH (NADP) ratio it was seen that this ratio was 5 - 10 fold greater in mycelial cells as compared to the yeast cells. This is in agreement with reports on *Candida albicans* and *Candida tropicalis* (Holmes *et al.*, 1989) where a significant role for GOGAT has been assigned in ammonia assimilation. However, in *S. cerevisiae* GS/GOGAT pathway was found to have limited importance in ammonia assimilation, since, the levels of NADP- dependent GDH were high (Holmes *et al.*, 1989). Ruiz-Herrera *et al.* (1983), have also suggested the involvement of NAD-dependent GDH or GS/GOGAT pathway in ammonia assimilation in *M. bacilliformis*.

Peters and Sypherd (1979) studied the *in vivo* regulation of GDH during *M. racemosus* morphogenesis and found that yeast-to-hyphal transition was accompanied by an increase in NAD-GDH activity which in turn preceded germ tube appearance. Similarly, in *B. poitrasii* a decrease in NADP:NAD-GDH ratio precedes germ tube formation (by 1-2h) during yeast-to-mycelial transition and the vice-versa (i.e., an increase in ratio) during mycelium-to-yeast transition. In yeast-to-mycelium transition (Fig. 5.2), the decrease in ratio is due to an increase in NAD-GDH activity with a simultaneous decrease in NADP-GDH activity.

Factors affecting the GDH ratio were found to affect germ tube appearance. Studies with α-ketoglutarate showed an increase in GDH ratio (as a result of increased NADP-GDH activity) with a concomitant inhibition of germ tube formation (Fig. 5.5a). On the other hand, in the presence of glutamate, the percentage of cells forming germ tubes was greater at 3h as compared to controls (Fig. 5.4). The analysis of GDH ratio showed that a steeper decrease occurs in presence of glutamate when compared to control values (Fig. 5.6a). In mutants Y-2 and Y-5 no change in GDH ratio was found as compared to control samples. Increase in NAD-dependent enzyme levels did not occur although glutamate should have induced higher levels of NAD-dependent activity (as observed in parent strain), taking into account the reported catabolic function of this enzyme. A similar observation has been reported for *M. bacilliformis* and its monomorphic mutants (Ruiz-Herrera *et al.*, 1983) where the mutants had a lowered NAD-GDH activity as compared to the parent strain. Levels of

GS were unaffected in *M. bacilliformis* parent strain and its mutants (Ruiz-Herrera *et al.*, 1983). In contrast, *B. poitrasii* mutants had a higher GS activity compared than the controls. Thus, it seems likely that catabolism of glutamate could be occurring *via* GS.

Cycloheximide (5 µg/ml) inhibited NAD-dependent GDH (no effect was observed on NADP-GDH), while inhibiting the germ tube appearance (Fig. 5.7). This led to a higher GDH ratio while maintaining a yeast morphology. Therefore, it may be concluded that protein synthesis, especially of NAD-GDH synthesis is necessary for yeast-to-mycelial transition to occur in *B. poitrasii*. Enzyme inhibitor studies on *B. poitrasii* gave similar results. Isophthalic acid (a specific NAD-GDH inhibitor) was found to inhibit NAD-GDH with no effect on NADP-GDH, and also delayed germ tube formation due to increase in GDH ratio (Fig. 5.8). The degree to which methionine-sulfoximine (a GS inhibitor) inhibited the germ tube appearance was lesser when compared to isophthalic acid. Therefore, GS may not be directly involved in yeast-to-mycelial morphogenesis, but may have an indirect role. Azaserine (a GOGAT inhibitor) could not inhibit germ tube formation, which clearly rules out the involvement of this enzyme in the morphogenesis of *B. poitrasii*.

The involvement of cAMP in the differentiation of higher fungi has been described (Pall, 1981). In several species of *Mucor*, cAMP is known to produce yeast like cells as opposed to mycelial morphology (Cantore *et al.* 1980; Larsen and Sypherd, 1974; Paveto *et al.*, 1975). The main evidence supporting this proposal is : (i) there is correlation between measured endogenous cAMP levels and yeast like growth versus mycelial growth in *M. racemosus* (Larsen and Sypherd, 1974) and *M. rouxii* (Cantore *et al.*, 1980; Paveto *et al.*, 1975) and (ii) exogenous dbcAMP induces a transformation from mycelial to yeast like growth in several species of *Mucor* (Larsen and Sypherd, 1974; Paveto *et al.*, 1975). The lowering of NAD-dependent GDH which is characteristic of normal yeast like growth could be obtained with dbcAMP (Peters and Sypherd, 1979), while high intracellular cAMP levels showed low NAD-GDH activity in Lev 9 and Lev 11 yeast mutants of *M. bacilliformis* (Ruiz-Herrera *et al.*, 1983). However, cAMP could not produce yeast-like morphology in other *Mucor* species (Jones and Bulock, 1977).

The relationship between cAMP and morphology in *Histoplasma capsulatum* are opposite to that proposed for *Mucor* sps. (Maresca *et al.* 1977). In *H. capsulatum*, cAMP levels are found to be higher in mycelia than in yeast cells and addition of exogenous dbcAMP converted yeast cells to mycelia. The pattern in *Candida* is very similar to that observed in

Histoplasma (Chattaway *et al.*, 1981; Niimi *et al.*, 1980). In *B. poitrasii*, during yeast-to-mycelium transition, lower concentrations of cAMP (2.0 mM and below) when added to YPG media failed to inhibit germ tube formation whereas higher cAMP concentrations (5.0 mM), led to germ tube inhibition, resulting in maintenance of yeast morphology (Table 5.2). Exogenous addition of adenine and adenosine to YPG media also inhibited germ tube formation, while dbcAMP was not as active as cAMP. In *Aureobasidium pullulans* adenine and adenosine have been shown to be taken up by the cells and metabolized to cAMP (Cooper *et al.*, 1985).

Studies with phosphorylation and dephosphorylation showed that in both mycelial and yeast phases a slight activation of NAD-GDH occurs on treatment with ATP and cAMP (Table 5.3). This is in contrast to the observation of Hemmings in *Candida* and *Saccharomyces* (Hemmings, 1978,1980) wherein inhibition of NAD-dependent activity occurred on phosphorylation. In yeast and mycelial phase cells of *B. poitrasii*, NAD-GDH seems to be present in an active (phosphorylated) form since incubation of the crude extracts with $MgSO_4$ and alkaline phosphatase (Table 5.3) resulted in loss of its activity. On the other hand, NADP-GDH seems to be present in a dephosphorylated (inactive) state in M-phase, since incubation with ATP and cAMP led to an increase in enzyme activity, while no effect was observed with either $MgSO_4$ or alkaline phosphatase. In yeast phase, the NADP-dependent enzyme appears to be in a phosphorylated (active) state as no further activation could be observed with ATP and cAMP, although a loss in activity was observed on treatment with $MgSO_4$ and alkaline phosphatase.

The effect of exogenous addition of cAMP on transition (i.e., delayed germ tube formation) can be correlated to the existence of NADP-GDH in an active form (phosphorylated) in yeast phase cells, since, in mycelial phase extracts, cAMP brought about an activation of this enzyme. It is therefore possible that cAMP, *via* a cAMP dependent protein kinase, activates the NADP-GDH to levels where yeast morphology can be maintained. It is clear from the data (Table 5.3) on mutants Y-2 and Y-5 that NADP-GDH is present in an active phosphorylated form, since the addition of ATP and cAMP did not have any effect on its activity. The enzyme was however inactivated on incubation with $MgSO_4$ and alkaline phosphatase.

In conclusion our results indicate that morphogenesis in *B. poitrasii* may be regulated by NADP:NAD-GDH ratio, as a higher GDH ratio is required for the maintenance of yeast morphology. Regulation of this GDH ratio seems to occur two levels, namely: (i) at the translational level, since synthesis of NAD-GDH protein (i.e., lowering of the ratio) is essential for the yeast-to-mycelial transition, and (ii) at the protein level, as phosphorylation or dephosphorylation of NADP-dependent GDH could lead to a shift in GDH ratio, thereby affecting morphogenesis. It is also possible that both these mechanisms could be operating simultaneously during the morphological transitional events.

Hence, characterizing the above phosphorylation-dephosphorylation mechanism(s) (cAMP dependent protein kinase and phosphoprotein phosphatase) of NADP-dependent GDH and genetic approaches to elucidate the concurrent regulation of NAD-dependent and NADP-dependent enzymes may be useful in throwing some more light on the morphogenetic phenomenon in *Benjaminella poitrasii*.

DIMORPHISM IN *Benjaminiella poitrasii*: COMPARISON OF CHITIN SYNTHETASE AND CHITINASE IN YEAST, MYCELIAL FORMS AND MORPHOLOGICAL MUTANTS

CHAPTER VI

CHAPTER - VI

6.1 INTRODUCTION

Differentiation from yeast to hyphae in dimorphic fungi is accompanied by significant changes in the chemical composition of the cell wall (see Table 4.2). The levels of chitin, one of the cell wall constituents, was 3 times more in mycelial phase than in the yeast phase cells. Moreover, the levels of chitin in the mutants Y-2 and Y-5 correspond with those of the yeast phase cells in the parent strain. According to Stewart and Rogers (1978), dimorphism maybe regarded as cell wall morphogenesis and hence the control of the developmental process should be reflected in the regulation of enzyme (s) required for the synthesis of cell wall polymer. The differences in chitin levels in both the forms (yeast and mycelial) and the mutants suggests that chitin synthase (EC 2.4.1.16) may have a regulatory role in the yeast-mycelial dimorphism in *B. poitrasii*. In *S. cerevisiae*, the regulation of the enzyme has been described by Cabib *et al.* (1971, 1973, 1975, 1990) and Duran *et al.* (1975). In the above organism, the enzyme is attached to plasma membrane and is activated by a protease contained in a vacuolar fraction (see Section 1.8.3.3). This compartmentalization accounts for the localized activation of the zymogen at the sites on cell wall synthesis. A third component of the system, a heat stable protein found in the cytosol, acts as an inhibitor of the protease and may function as a regulatory factor. Studies on the regulation of these events in dimorphic fungi have started only in recent times. In *C. albicans*, chitin synthetase preparation from both yeast and mycelial forms was in a zymogenic form, activatable by trypsin (Hardy and Gooday, 1983). Chiew *et al.*, (1980) studied the enzyme with respect to germ tube formation in *C. albicans*. During germ tube formation the chitin synthetase activity increased five fold which accounted for the reported five fold increase in chitin content observed during yeast-to-mycelial transformation.

Studies from *M. rouxii* showed that crude chitin synthetase preparations from mycelial and yeast forms behaved differently (Ruiz-Herrera and Bartnicki-Garcia, 1976). It was deduced that chitin synthetase in yeast preparations was present mainly in a latent, zymogenic form, which could be activated by protease. In mycelial preparations, the chitin synthetase was present in an active state and was rapidly degraded by proteolysis. The activation of chitin synthetase was inhibited by a soluble protein in the cell free extract.

The other enzyme responsible for the levels of chitin in cell wall is chitinase (EC 3.2.1.14). A morphogenetic role in growth and differentiation in all chitin containing fungi has been implicated, wherein a delicate balance between wall synthesis and wall lysis could control apical or spherical growth (Gooday *et al.*, 1986). In *M. mucedo*, chitinase has been found to be associated with chitin synthetase (Humphreys and Gooday, 1984).

In the present investigation, comparisons between chitin synthetase/chitinase were carried out only on one of the mutants (i.e., Y-2) as biochemical characterization studies in Chapters 3-5 have shown similarities between Y-2 and Y-5 mutants. The results of the investigations employing mycelial, yeast phase and Y-2 mutant of *B. poitrasii* are presented in the next Section.

6.2 RESULTS

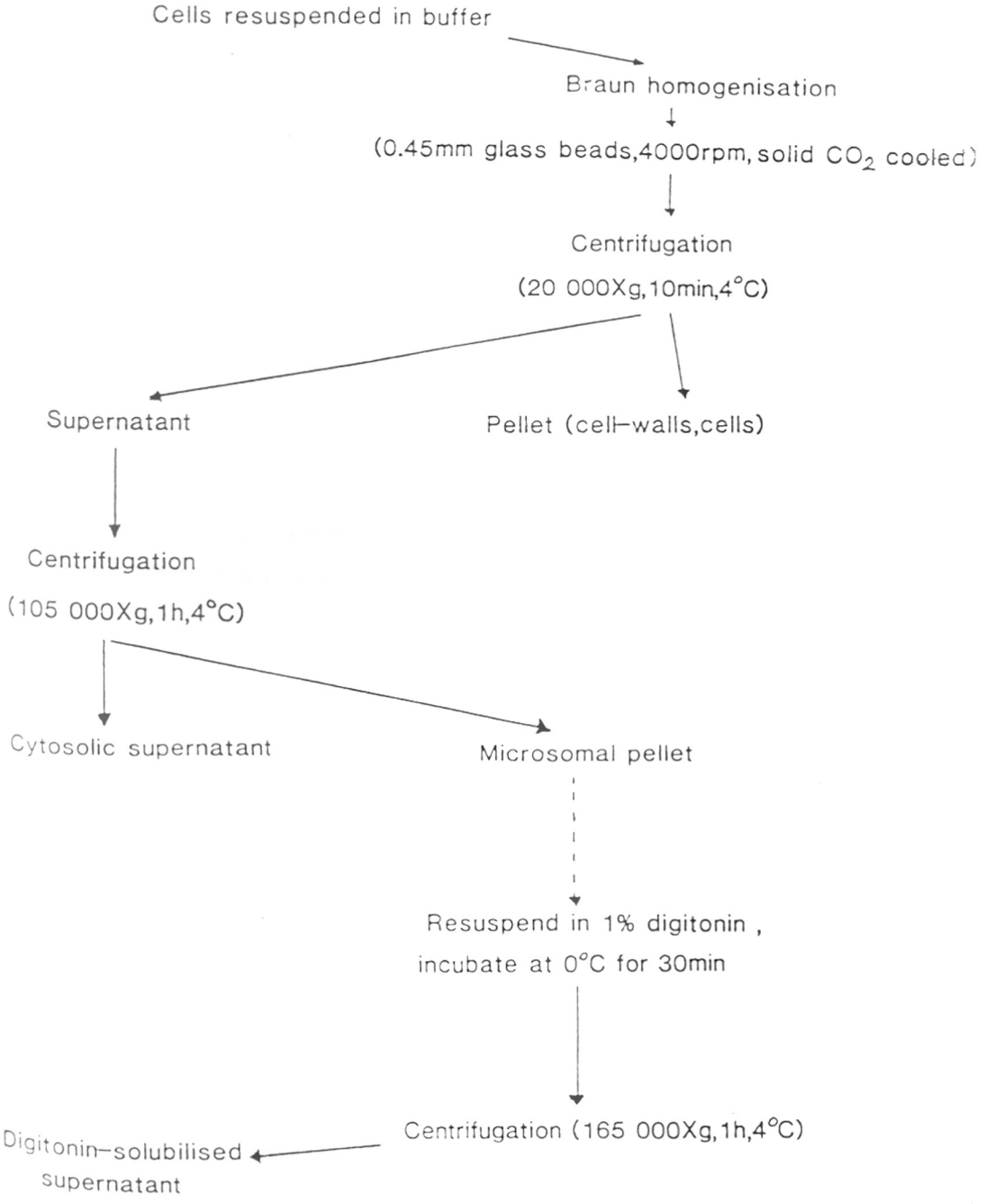
6.2.1 *Cell fractionation*

The cells were fractionated into the various fractions as described in Scheme 6.1 and the enzyme assayed and the specific activity calculated as mentioned under Materials and Methods. Proteolytic activation of the chitin synthetase enzyme using trypsin (200µg/ml) and incubation at 25°C was carried out. The reaction was stopped by the addition of soyabean trypsin inhibitor. Protein was measured according to Bradford (1976).

6.2.2 *Subcellular localization of chitin synthetase*

Cells were grown, homogenized and fractionated according to Scheme 6.1. The activity of each fraction was determined in the presence and absence of trypsin.

In mycelial phase cells grown for 36h, 73% of the native activity was associated with the membrane fraction while 27.5% was found in the cell wall fraction. No activity was detected in the cytosol fraction. In contrast, in the yeast phase cells, 53.8% of the activity was detected in cytoplasm, while 46.2% could be detected in the cell wall. No activity could be detected in the membrane fraction. In Y-2 mutant 84% activity was detected in the cell wall fraction while the activity was absent in the membrane fraction as in the case of the yeast phase cells (Table 6.1).



Scheme 6.1: Preparation of cell free extracts.

In cells grown for 48h, the trends of chitin synthetase activity varied. Cell wall fraction contained 87.8% of the native activity in mycelial cells, while 75% of the activity in yeast cells was present in the cell wall fraction. In Y-2 mutant, most of the activity (56.7%) was found in the cytoplasm (Table 6.1).

With trypsinization, the total activity increased four fold, ten fold and five fold in mycelial, yeast phase cells and Y-2 mutant respectively when grown for 36h. In 48h grown samples, ten fold and seven fold increase was observed in yeast phase cells and Y-2 mutant respectively, while no change in the activity was observed in case of mycelial cells (Table 6.1). Thus, the activation ratios showed that preparations from both yeast and mycelial cells, were present as zymogen (except for M-phase grown for 48h).

Data on M-phase cells grown for 36h in MYP and MYPG media showed that cells grown in the former had higher activity as compared to cells grown in the latter. The only difference in the two media was the presence of glucose (1.0%) im MYPG. Therefore, it is possible that glucose may have a role in the regulation of chitin synthetase activity. Additionally, in glucose grown media, the tendency to occur in a zymogen form has been observed (Table 6.2).

6.2.3 *Effect of digitonin on chitin synthetase activity*

As most of the chitin synthetase activity was found to be associated with the cell wall and membrane, solubilization by digitonin was carried out to check its effect, if any. Digitonin solubilized the enzyme of yeast phase cell wall, albeit in a zymogenic form as observed by the increase in activity on trypsinization of the supernatant fraction. In mycelial phase, digitonin was unable to solubilize the activity although an increase in activation ratio was observed in the digitonin cell wall pellet (Table 6.3a). In the membrane fractions, the activation ratio of the total digitonin activity showed a four and ten fold increase for both mycelial and yeast cells respectively. No native activity could be detected in the digitonin membrane supernatant fraction for the mycelial phase, while in the yeast phase on trypsinization activity could be detected (Table 6.3b).

Table 6.1: Subcellular localization of chitin synthetase activity in yeast, mycelial phases and Y-2 mutant of *B. poitrasii*[a].

Time[b]	Fraction	Source	Native activity	Trypsinized activity	Activation ratio[c]
36 h	Cell wall	M	4.4	14.2	3.3
		Y	5.4	75.9	14.1
		Y-2	18.9	88.5	4.7
	Cytoplasm	M	0.0	0.1	-
		Y	6.3	4.4	0.7
		Y-2	3.6	1.8	0.5
	Membrane	M	11.6	51.1	4.4
		Y	0.0	40.0	-
		Y-2	0.0	14.2	-
	Total activity	M	16.0	65.4	4.1
		Y	11.7	120.3	10.3
		Y-2	22.5	104.5	4.6
48 h	Cell wall	M	7.2	4.7	0.7
		Y	2.1	20.6	9.8
		Y-2	0.6	21.1	35.2
	Cytoplasm	M	0.0	0.0	0.0
		Y	0.5	0.04	0.08
		Y-2	1.7	0.0	0.0
	Membrane	M	1.0	1.6	1.6
		Y	0.2	7.8	39.0
		Y-2	0.7	0.0	0.0
	Total activity	M	8.2	6.3	0.77
		Y	2.8	28.44	10.2
		Y-2	3.0	21.1	7.0

a Activity expressed as pkatals (see Section 2.2.11 under Materials and Methods).

b Cells were grown for 36h and 48h in MYPG media and the treatment for trypsin was 7 min and 10 min, respectively, at room temperature.

c Activation ratio = Trypsinized activity:Native activity.

Table 6.2: Difference in chitin synthetase activity in mycelial phase cells[a] grown in MYP and MYPG media.

Fraction	Media	Native activity	Trypsinized activity	Activation ratio[b]
Cell wall	MYP	31.1	60.3	1.9
	MYPG	4.4	14.2	3.2
Cytoplasm	MYP	0.24	0.0	0.0
	MYPG	0.0	0.1	-
Membrane	MYP	14.0	10.0	0.7
	MYPG	11.6	51.1	4.4
Total activity	MYP	15.1	23.4	1.6
	MYPG	5.3	21.8	4.1

a Cells were grown for 36 h.

b See the foot note of Table 6.1.

Table 6.3a: Effect of digitonin on the chitin synthetase activity in cell wall fraction.

	Fraction	Source[a]	Native	Trypsinized	Activation ratio[b]
1.	Cell wall	M	7.2	4.7	0.7
		Y	2.1	20.6	9.8
		Y-2	0.6	21.1	35.2
2.	Digitonin cell wall supernatant	M	0.0	0.0	0.0
		Y	0.0	10.4	-
		Y-2	5.8	0.0	0.0
3.	Digitonin cell wall pellet	M	4.7	9.8	2.1
		Y	-	21.5	-
		Y-2	1.4	12.2	8.7
4.	Total digitonin activity	M	4.7	9.8	2.1
		Y	0.0	31.9	-
		Y-2	7.2	12.2	1.7

a Cells were grown in MYPG media for 48h.

b See the foot note of Table 6.1.

Table 6.3b: Effect of digitonin on the chitin synthetase activity in the membrane fraction.

	Fraction	Source[a]	Native	Trypsinized activity	Activation ratio[b]
1.	Membrane	M	1.0	1.6	1.6
		Y	0.2	7.8	39.0
		[illegible]	[illegible]	[illegible]	0.0
2.	Digitonin membrane fraction	M	0.0	0.0	0.0
		Y	0.0	6.6	-
		Y-2	[illegible]	[illegible]	).0
3.	Digitonin membrane pellet	M	[illegible]	[illegible]	5.0
		Y	0.05	6.8	136.0
		Y-2	0.0	0.0	0.0
4.	Total digitonin activity	M	1.6	8.0	5.0
		Y	0.05	13.4	268.0
		Y-2	12.8	0.0	0.0

a Cells were grown in MYPG media for 48h.

b See the foot note of Table 6.1.

6.2.4 *Activation by trypsin*

Since most of the chitin synthetase activity in the yeast phase was present in a zymogenic form, the kinetics of trypsin activation in yeast membrane fraction (as the activation ratio was maximal) was studied with respect to time. As shown in Fig. 6.1, the activation was rapid and maximal activity was obtained at 10 min. and was followed by a progressive loss of activity, the loss in activity being maximal between 10-20 min.

6.2.5 *Chitinase levels present in yeast and mycelial phases*

Two assays were carried out as mentioned in Section 3.2 of Materials and Methods. Using the phorogenic assay four times more activity (8.9 U/mg dry wt.) was detected in yeast phase cell as compared to mycelial cells (0.65 U/mg dry wt.)and Y-2 mutant (0.5 U/mg dry wt.) grown on MYPG media. Mycelial phase cells grown on YP media showed 1.2 U/mg dry weight of chitinase activity. Similar trends were obtained using the reducing sugar assay in 48h samples with 4.2, 0.8 and 0.44 U/mg protein being the observed chitinase activity in yeast, mycelial phases and Y-2 mutant respectively.

6.2.6 *Nikkomycin effect*

Nikkomycin, a nucleoside-peptide antibiotic is a highly specific competitive inhibitor of chitin synthetase, mimicking its substrate UDP-NAG (Gooday, 1990). Cells were grown on YP, MYPG and synthetic media (aspargine, 0.2%; KH_2PO_4, 0.175%; glucose, 1.0; $MgSO_4$. 0.0175% and agar, 1.2%). The plates were incubated with spores (for parent strain) and yeast cells (for Y-2 mutant). All the plates were incubated at 28°C for 64h. Growth of mycelial phase on MYPG and synthetic medium was approximately the same (1.5 divisions/2min.) on the basis of hyphal tip elongation. Nikkomycin at a concentration of 0.05-5.0 mM in 2.0% sorbitol was added to the plates. A concentration of 0.05 mM caused the growing hyphal tips to burst in 6 min. while it occurred in 1.5 min. at concentration of 0.5 mM and above (Fig. 6.2 a,b) in MYPG medium. In synthetic medium hyphal bursting was observed in 10 min, 4 min, and 3 min for 0.05 mM, 0.5 mM and 5 mM, respectively. The yeast phase cells remained unaffected for 4h at 0.5 mM nikkomycin concentration (Fig. 6.3). However, cells of Y-2 mutant in control as well as in nikkomycin (0.5 mM) treated samples showed weakening of cell wall and bursting of the cells in 4h (Fig. 6.4 a,b). In YP media although

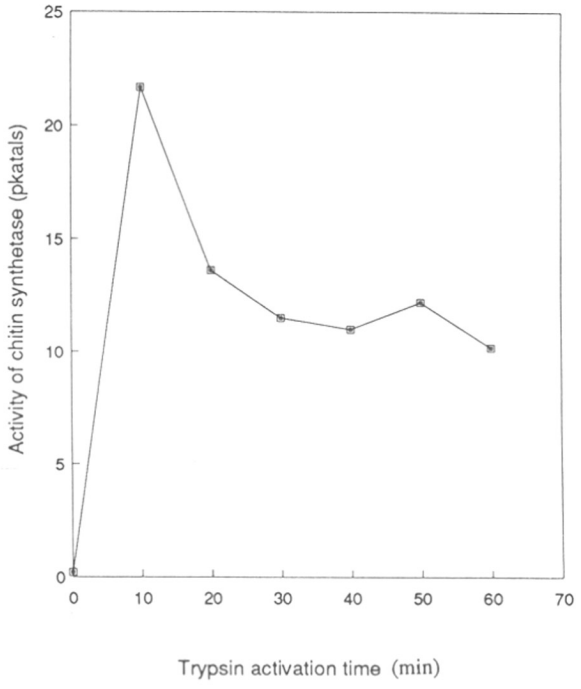


Fig. 6.1: Time behaviour for the activation of chitin synthetase by trypsin in yeast microsomal fraction.

Fig. 6.2: Effect of nikkomycin (5 mM) on hyphal cells of *Benajaminiella poitrasii* grown on agar under polypropylene membrane.
a,b: Bursting of hyphal tips

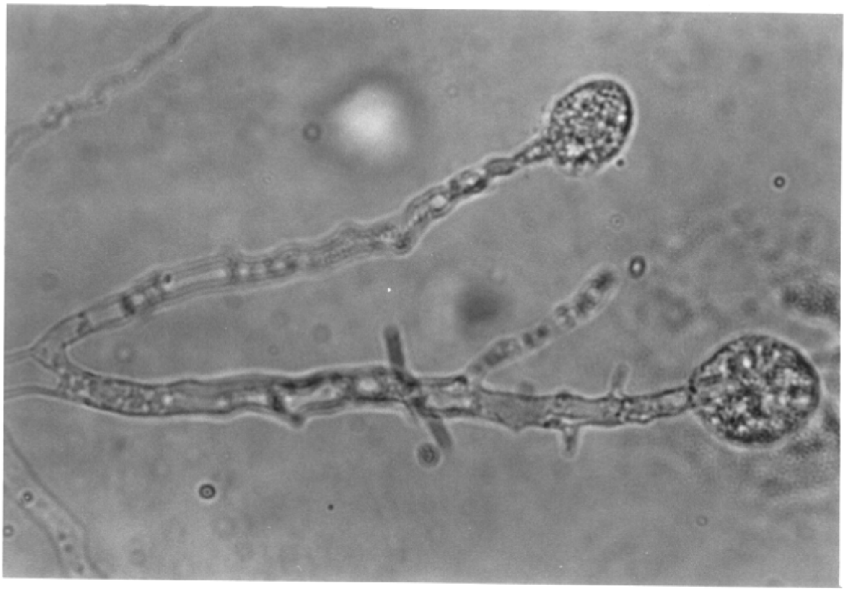


Fig.6.2a

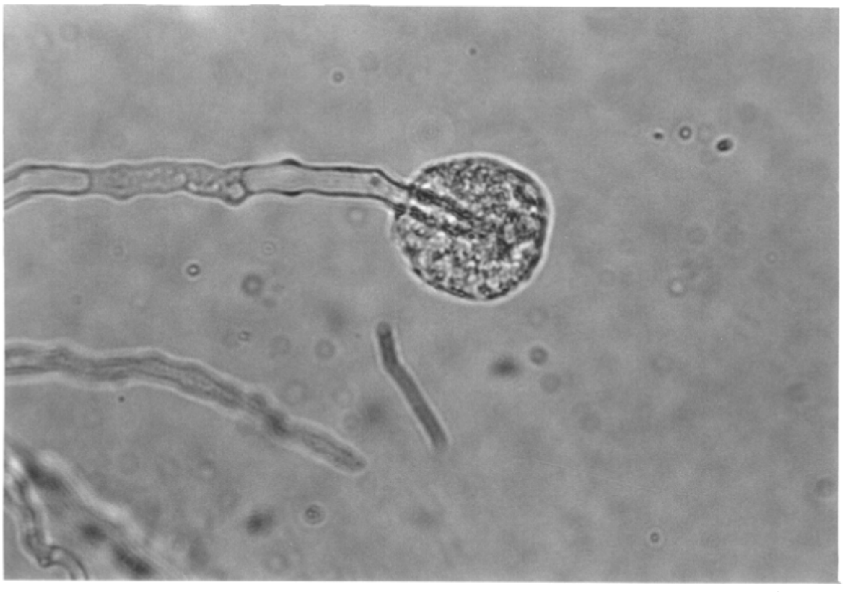


Fig.6.2b

Fig. 6.3: Effect of nikkomycin (0.5 mM) on yeast phase cells of *Benjaminiella poitrasii* grown on synthetic medium. No bursting occurs upto 4h.

Fig.6.3

Fig.6.3

Fig. 6.4: Effect of nikkomycin (0.5 mM) on Y-2 mutant of *Benjaminiella poitrasii* grown on synthetic medium. Bursting of some cells observed in both control and nikkomycin grown samples.

a: Control

b: Nikkomycin grown cells at 4h

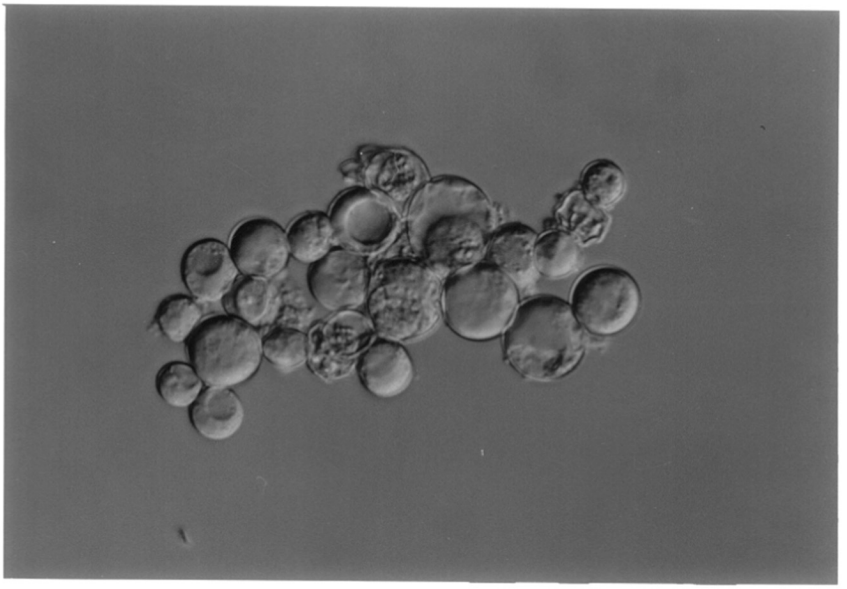


Fig.6.4a

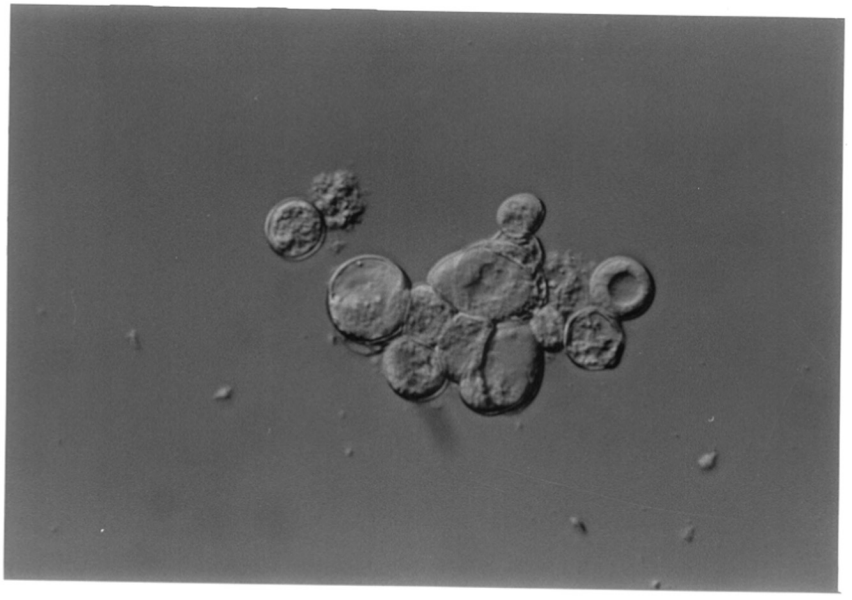


Fig.6.4b

no bursting of mycelial cells was observed in 10-15 min with 5 mM nikkomycin concentration, a large number of intermediate forms without a well defined yeast or hyphal morphoilogy were seen, indicating an alteration in the pattern of development.

6.3 DISCUSSION

The chitin synthetase activity was found to be located in the cell wall fraction in both the mycelial and yeast phase cells. The amount of native activity was greater in the mycelial phase than the yeast phase and mutant Y-2 in 48h grown samples. On trypsinization an increase in activity was observed for both yeast phase and Y-2 mutant (Table 6.1) while no change in the activity for mycelial phase was observed. Therefore, it seems likely that the enzyme is present in a zymogen form in the yeast phase and Y-2 mutant, which gets activated on proteolysis. In the mycelial phase the enzyme already seems to occur in an activated form as proteolysis did not lead to any appreciable change in activity.

It is assumed that digitonin acts partly as a detergent and partly by complexing with membrane sterols. The zymogen from yeast-phase was solubilized on digitonin treatment in both cell wall and membrane fractions. Activation of the enzyme was observed in all the three strains in contrast to its inhibitory action on *C. albicans* chitin synthetase (Hardy and Gooday, 1983). Gooday and de Rousset-Hall (1975) noted that digitonin increased the stability of chitin synthetase from *Coprinus cinerus*. The 3-5 fold greater native activity of mycelial phase cells as compared to yeast phase cells and mutants is in agreement with the data on chemical analysis of cell walls wherein the chitin content was 3-fold higher in mycelia.

A higher chitinase activity in yeast phase cells as compared to mycelial phase cells points to a greater turn over of chitin in yeast cells which in turn would also account for the lowered chitin levels in its cell wall. According to Gooday *et al.* (1986), in *M. mucedo* chitinase has been implicated in morphogenesis in association with chitin synthetase.

Nikkomycin, a chitin synthetase inhibitor caused the bursting of growing hyphal tips in *B. poitrasii* while no effect was observed upto 4h with either yeast phase cells or Y-2 mutant. Similar observation was noticed for hyphal cells of *M. rouxii* (Bartnicki-Garcia and Lippman, 1972) using polyoxin D (an analog of nikkomycin), another chitin synthetase inhibitor. In *C. albicans* however no lysis of germ tubes was observed with polyoxin D (Chiew

et al., 1980). They concluded that polyoxin D is unable to permeate the cell envelope. A similar effect maybe possible with respect to the yeast phase cells of *B. poitrasii.* Also, as the enzyme is largely present in zymogenic form in the yeast-phase cells, the drastic bursting effect is not observed. Since nikkomycin did bring about a change in the pattern of morphogenesis in YP medium, it can be concluded that the overall rate of chitin synthesis seems to be a decisive factor in the dimorphic development in *B. poitrasii.* The levels of latency of the chitin synthetase and chitinase activity, therefore, can account for the amount of chitin present in the cell wall and thus determine the cell morphology in the organism.

CONCLUSION

CONCLUSION

Based on the studies carried out, it can be concluded that in *Benjaminiella poitrasii*, a dimorphic fungus, spore germination can lead to the formation of either mycelial or yeast like growth depending on the temperature of incubation. In the mycelial phase, formation of sporophores and zygospores occurred while the yeast phase multiplied by budding. The isolation of morphological mutants Y-2 and Y-5 (mutants able to grow in yeast form only, at both 28°C and 37°C) was a pre-requisite in order to understand the phenomena of dimorphism and was successfully carried out.

The factors affecting or triggering dimorphism in *B. poitrasii* were mainly, (1) temperature and (2) nutritional conditions. Yeast form developed only at 37°C while mycelial growth occured only at 28°C. The mycelial-to-yeast (M→Y) transition or *vice versa* could be accomplished by shifting the temperature, provided the nutritional conditions were satisfied. Thus, M→Y transition occurred on a shift from 28°C→37°C while Y→M transition could be induced by a 37°C→28°C temperature shift. The M→Y transition required a longer time (8-9h) as compared to the Y→M transition which was observed at 3h. The mutants, however, maintained their morphology (i.e., Y-form) at both these temperatures. The results show that carbon and nitrogen sources are key factors in *B. poitrasii* dimorphism. At low glucose concentrations (< 0.1%), the fungus developed as mycelia, while at higher concentrations (>5.0%), yeast form was observed, irrespective of the temperature of growth. In media containing non-fermentable hexose sugars, the fungus developed in M-form, while the mutants Y-2 and Y-5 were unable to grow. Similarly, on minimal media, growth of Y-2 and Y-5 was arrested while the parent strain grew in M-form. Therefore, C:N ratio of the media seems to play an important role in the dimorphic phenomena. In general, a high C:N ratio led to yeast phase growth, while a lowered C:N ratio gave rise to mycelial phase. Mutants Y-2 and Y-5 could not grow on a lower C:N ratio (due to their inability to switch to M-phase) while they grew in yeast form at a higher C:N ratio.

In view of the fact, that cell wall is considered to play an important role in the maintainence of cell shape, analysis of cell wall studies showed that the major difference between the yeast and mycelial phase cell walls is in the hexosamine content. The amount

of chitin was 2.5-3 fold greater in mycelial phase than in the yeast phase cells of parent strain. The percentages of glucosamine in the cell walls of mutants Y-2 and Y-5 were comparable to yeast phase of parent strain. This result seems to indicate that glucosamine plays a role in the dimorphic change. Cross-linking studies among the various cell wall components in *B. poitrasii*, suggests that mannans seem to be linked to proteins, while glucans and glucosaminoglycans are linked together. Therefore, characterization and chemical structure of the glucan-glucosaminoglycan fraction insoluble in DMSO in yeast phase and Y-2 mutants, and that of a similar fraction susceptible to NaOH treatment in mycelial-phase may prove to be helpful in understanding the dimorphic phenomena.

The more stringent growth requirements of yeast cells as compared to the mycelial cells, suggests that some key enzymes of carbon and nitrogen metabolism might be differentially expressed in the two morphological forms. Therefore, studies were carried on glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamate synthase (GOGAT) which are the enzymes linking the carbon and nitrogen metabolism together. Studies on these enzymes were carried out as a function of nutritional conditions and morphological states in *B. poitrasii*. Results showed that while GS/GOGAT system maybe involved in ammonia assimilation, NADP:NAD-GDH ratio appears to play an important role in morphological transition of the organism. Exogenous addition of α-KG (1.0 mM) to YPG medium at 28°C, during yeast-to-mycelium transition led to an increase in GDH ratio, resulting in the maintenance of yeast form. In contrast, glutamate (1.0 mM) lowered the GDH ratio, leading to induction of germ tube. Mutants Y-2 and Y-5 (which do not undergo transition) showed a higher GDH ratio, thereby, substantiating the above observations. Exogenous addition of cycloheximide and specific enzyme inhibitors also conclusively proved the role of a high GDH ratio for the maintainence of yeast form. Addition of adenine, adenosine and cAMP (5.0 mM) to YPG medium, delayed the appearance of germ tubes. Phosphorylation/dephosphorylation studies on NAD- and NADP-GDH led to alterations in their levels, thus affecting the GDH ratio, which seems to be another factor playing a role in the observed dimorphic behaviour. Thus, regulation of NAD- and NADP-GDH maybe one of the early steps in the cascade of events leading to dimorphism in *B. poitrasii*.

Since the total glucosamine (chitin and chitosan) levels were found to be 1.5 fold higher in mycelial than yeast, Y-2 and Y-5 cell walls, it was necessary to check the levels of enzymes involved in synthesis and degradation of chitin. The amount of native activity was greater in mycelial cells than in yeast phase cells and Y-2 mutant. However, trypsinized activity was greater in Y-phase and Y-2 mutant than in M-phase. Trypsinization did not lead to any change in M-phase chitin synthetase activity. Hence, it seems that the enzyme exists in an active form in M-phase while occurring as a zymogen in Y-phase and Y-2 mutant. A higher chitinase activity was present in yeast-phase cells as compared to mycelial, pointing to a higher turnover of chitin in the former. This resulted in lowering of chitin levels in Y-phase and Y-2 cell wall. Nikkomycin, a chitin synthetase inhibitor, caused bursting of hyphal tips in 1.5 min while no effect on Y-phase cells or Y-2 mutant was observed upto 4h. This could either be due to the inability of nikkomycin to permeate the cell wall of Y-phase and Y-2 cells, or due to the existence of the enzyme in zymogenic (latent) form in the latter. These results seem to suggest a correlation between wall composition and dimorphism in *B. poitrasii*. Levels of chitin and the bursting of hyphal walls also convey that chitin is an essential component of the cell wall in *B. poitrasii*. Also, since nikkomycin brought about a change in the pattern of morphogenesis, a decisive factor in dimorphic development of the organism could be the overall rate or extent of chitin synthesis in the cell wall.

A recent study (Sburlati and Cabib, 1986), reports the presence of a second chitin synthetase (Chs 2) in *S. cerevisiae* which catalyzes chitin synthesis. The role of chitin synthetase 1 (Chs 1), which is present in the cell at a much higher level, has also been questioned. According to the authors, the answer may perhaps be found in evolution by the following hypothesis. In filamentous fungi that contain chitin, the polysaccharide is found both in cell walls and in septa. Since growth of wall and septa must be independently controlled, two different chitin synthetases may be laying down chitin at each of these two locations. Yeasts may have evolved from filamentous fungi by a series of changes in mechanisms of cell wall growth and deposition. In fact, intermediate organisms (the dimorphic fungi) that can grow either in filamentous or in yeast form exist. In a putative filamentous precursor of *Saccharomyces*, Chs 1 may have been endowed with the task of synthesizing lateral cell wall chitin. Among the evolutionary changes involved during the

transition to the yeast form, there may have been a loss in the *in vivo* ability to activate the Chs 1 zymogen, thus, decreasing the ability to form cell wall chitin. It is remarkable that a similar behaviour has been observed in *B. poitrasii,* where, in the yeast phase cells and Y-2 mutant chitin synthetase exists in an inactive (zymogenic) form with the consequent lowering of the chitin content in the cell walls. The dimorphic phenomena observed in *B. poitrasii* may, therefore, provide an ideal system to verify the above hypothesis. It would be interesting to study the presence or absence of a second chitin synthetase in this simple morphological system.

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LIST OF PUBLICATIONS

Dimorphism in *Benjaminiella poitrasii* : Isolation and biochemical studies of morphological mutants, A. Khale, S.S. Deshmukh, M.C. Srinivasan, and M.V. Deshpande, *Antonie van Leeuwenhoek*, **57**, 37 (1990).

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(4) Cell wall composition of dimorphic fungus *B. poitrasii* and its morphological mutants, A. Khale, M.C. Srinivasan, and M.V. Deshpande, Presented at the 56th Annual General Body Meeting of SBC(I) at Tirupati, 1987.

(5) Dimorphism in *Benjaminiella poitrasii*: Inosi ige caused by ethanol and iso-propanol, A. Khale, M.C. Srinivasan, and M.V. Deshpande, Presented at the 57th Annual General Body Meeting of SBC(I) at New Delhi, 1988.

(6) Expression of GDH in the dimorphic fungus *Benjaminiella poitrasii*, A. Khale, M.C. Srinivasan, and M.V. Deshpande, Presented at the 58th Annual General Body Meeting of SBC(I) at Izaatnagar, 1989.