Role of Cell Wall in the Dimorphism of *Benjaminiella poitrasii*

A thesis submitted to the
University of Pune
for the
Degree of Doctor of Philosophy (in Biotechnology)

By
Manisha Vasant Chitnis

Division of Biochemical Sciences
National Chemical Laboratory
Pune- 411 008, India

June 2001

Dedicated with love and respect to

my parents,

Suman and Vasant Chitnis

Table of Contents

List of Tables 7
List of Figures 9
Acknowledgement 10
Declaration 11
List of Abbreviations 12
Abstract 14

1. Introduction 23

1.1 Dimorphism in fungi 24
1.1.1 Events correlating dimorphic transition in fungi 24
1.1.1.1 Phenotypic events 27
1.1.1.2 Biochemical events 28
1.1.1.3 Genetic events 30
1.1.2 Factors influencing dimorphic transition 32
1.2 Fungal cell wall: Components, organisation and chemistry 34
1.2.1 Cell wall composition, structure and organisation 34
1.2.1.1 Chitin and chitosan 37
1.2.1.2 Glucans 38
1.2.1.3 Glycoproteins and proteins 38
1.2.2 Chemistry of wall synthesis and degradation 39
1.2.3 Cell wall and morphogenesis 41
1.3 Protoplasts as a tool to study cell wall biosynthesis 41
1.3.1 Methods for protoplastation 41
1.3.2 Protoplastation using lytic enzyme systems 42
1.3.3 Factors influencing protoplast isolation using lytic enzyme systems 44
1.3.3.1 Culture conditions 44
1.3.3.2 Age of culture 44
1.3.3.3 Pretreatment of organisms 45
1.3.3.4 Lytic enzymes 45
1.3.3.5 Effect of osmotic stabilizers 46
1.3.3.6 Treatment conditions 46
1.3.4 Regeneration of protoplasts 47
1.4 Chitin synthases and their role in cell wall metabolism 49
1.4.1 Properties and regulation of chitin synthases from fungi 49
1.4.2 Structure of chitin synthases 55
1.4.3 Chitin synthase genes and their classification 57
1.5 Cell wall as a target for antifungal drugs 59
1.6 Present investigation and thesis organisation 62
1.6.1 *Benjaminiella poitrasii* as a model system 62
1.6.2 Objectives of this study 64
1.6.3 Thesis organisation 65

2. Materials and Methods 66

2.1 Chemicals 67
2.2 Organism and cultivation 67
2.2.1 Organisms and culture conditions 67
2.2.2 Yeast-mycelium transition in *B. poitrasii* 69
2.2.3 Determination of the growth curve and glucose utilization pattern under different conditions in *B. poitrasii* 70
2.2.4 Production of chitisanase-rich cell wall lytic enzyme system 71
2.2.4.1 Screening of microorganisms for chitosanase production 71
2.2.4.2 Production of cell wall lytic enzymes 71
2.2.5 Production of chitin synthase inhibitors 72
2.2.6 Growth conditions and harvesting of cells for isolation of total RNA from *B. poitrasii* 72
2.2.7 Hyphal tip bursting test 73
2.2.8 Isolation of protoplasts 73
2.2.9 Regeneration of protoplasts 74
2.3 Biochemical methods 74
2.3.1 Isolation of cell wall 74
2.3.2 Isolation and solublisation of cell membrane 75
2.3.3 Isolation of genomic DNA from *B. poitrasii* 75
2.3.4 Plasmid DNA isolation from *E.coli* 76
2.3.4.1 Small scale plasmid DNA isolation 76
2.3.4.2 Medium scale plasmid DNA isolation 77
2.3.5 Isolation of total RNA from *B. poitrasii* 78
2.3.6 Polymerase chain reaction (PCR) and RT-PCR 78
2.3.7 Cloning of DNA 80
2.3.8 Digestion of DNA with restriction enzymes 80
2.3.9 Southern blot analysis 81
2.3.10 Southern hybridization 82
2.3.11 DNA sequencing reaction 82
2.3.12 Determination of protein 83
2.3.12.1 Lowry method 83
2.3.12.2 Optic al method 83
2.3.13 Determination of nucleic acid 83
2.3.13.1 Optical method 83
2.3.13.2 Ethidium bromide method 83
2.3.14 Determination of glucose 84
2.3.15 Enzyme assays 84
2.3.15.1 Chitinase 84
2.3.15.2 Chitosanase 84
2.3.15.3 β-1,3-Glucanase 85
2.3.15.4 Mannanase 85
2.3.15.5 Protease 85
2.3.15.6 Chitin synthase 86
2.4 Analytical methods 86
2.4.1 Identification of *Streptomyces* sp. 86

2.4.2 Fluorescence microscopy 87
2.4.3 Vital staining 87
2.4.4 Preparative isoelectric focusing 87
2.4.5 Agarose gel electrophoresis 87
2.4.6 Formaldehyde gel electrophoresis 88
2.4.7 Analysis of DNA sequence data 89
2.4.8 Phylogenetic analysis 89

3. Isolation and regeneration of protoplasts from *Benjaminiella poitrasii* 90

3.1 Chitosanase-rich enzyme lytic preparation for protoplastation 91
3.1.1 Introduction 91
3.1.2 Results 92
3.1.2.1 Screening for chitosanase producers and production of lysing enzyme mixture 92
3.1.2.2 Properties of chitosanase-rich lysing enzyme mixture 93
3.1.2.3 Use in protoplastation of zygomycetous fungi 94
3.1.3 Discussion 95
3.2 Isolation and regeneration of protoplasts in *Benjaminiella poitrasii* 97
3.2.1 Introduction 97
3.2.2 Results 98
3.2.2.1 Isolation of protoplasts from mycelium and yeast-form cells of *B. poitrasii* 98
3.2.2.2 Regeneration of protoplasts under mycelium and yeast favouring conditions 100
3.2.2.3 Effect of different inhibitors on the regeneration of mycelial protoplasts of *B. poitrasii* 102
3.2.3 Discussion 105

4. Biochemical studies on chitin synthases of *Benjaminiella poitrasii* 110

4.1 Biochemical characteristics of chitin synthases from *Benjaminiella poitrasii* 111
4.1.1 Introduction 111
4.1.2 Results 112
4.1.2.1 Chitin synthase activity during yeast-mycelium transition 112
4.1.2.2 Biochemical characterization of membrane-bound chitin synthases from yeast- and mycelium-form cells 112
4.1.2.3 Isoelectric focusing for chitin synthases from yeast and mycelium-form cells 114
4.1.3 Discussion 116
4.2 *Benjaminiella poitrasii*: A model system to screen chitin synthase inhibitors 120
4.2.1 Introduction 120
4.2.2 Results 121

4.2.2.1 Hyphal tip bursting test 122
4.2.2.2 Germ tube formation in the presence of cell wall metabolism inhibitors 125
4.2.2.3 Chitin synthase activities in the presence of cell wall metabolism inhibitors 127
4.2.2.4 Effect of culture filtrates on chitin synthase activity from pathogenic fungi 128
4.2.3 Discussion 130

5. Molecular studies on chitin synthases from *Benjaminiella poitrasii* 134

5.1 Introduction 135
5.2 Results 136
5.2.1 PCR amplification of chitin synthase genes from *B. poitrasii* genome 136
5.2.2 Cloning of PCR-amplified products, sequence determination and analysis 137
5.2.3 Southern analysis and the organisation of *CHS* genes in the genome 141
5.2.4 RT-PCR for studying expression of chitin synthase genes 141
5.3 Discussion 145

6. Conclusion 149

7. References 155

List of publications, patents and presentations 173

List of Tables

Table No.	Title	Page No.
1.1	Some known biochemical events relevant to dimorphism in selected fungi	26
1.2	Different genes involved in morphogenesis for selected fungi	31
1.3	Factors influencing dimorphic transition in selected fungi	33
1.4	Main components of the fungal cell wall	35
1.5	Major polymers occurring in the fungal cell walls of various fungal classes	36
1.6	Different method used for protoplast isolation from fungi	42
1.7	Biochemical properties of chitin synthases from fungi	50
2.1	Sources of different chemicals used	68
3.1	Hydrolytic enzyme activities of commercial lysing enzyme preparations	92
3.2	Screening of microrganisms for extra-cellular chitosanase production	93
3.3	Properties of chitosanase-rich lytic enzyme mixture prepared using *Streptomyces* sp. MC1	94
3.4	Effect of osmotic stabilizer on the isolation of protoplasts for different organisms	95
3.5	Effect of the osmotic stabiliser on the isolation of protoplast of *B. poitrasii*	99
3.6	Optimisation of the incubation time for the protoplastation	100
3.7	Effect of different cell wall metabolism inhibitors on the regeneration of mycelial protoplast of *B. poitrasii*	104
4.1	Effect of meta1 ions on chitin synthase activity	113
4.2	Effect of different additives on chitin synthase activity of *B. poitrasii.*	114
4.3	Screening for inhibitors of cell wall metabolism	124
4.4	The effect of chitin synthase inhibitor producers on yeast-mycelium ransition in *B. poitrasii*	126
4.5	Screening for chitin synthase inhibitors	128

4.6	Chitin synthase activity from pathogenic fungi in the presence of potential chitin synthase inhibitors	129
5.1	Comparison of different chitin synthase gene fragments	138
5.2	*CHS* gene expression during yeast-mycelium transition in the absence of glucose at 28°C	148

List of Figures

Figure No.	Title	Page No.
1.1	Cell wall: Organisation of different components	36
1.2	Pathway for chitin metabolism	40
1.3	Generic protocol for protoplast isolation using lytic enzyme systems	43
1.4	Conserved regions in chitin synthase sequences	56
3.1	Regeneration of protoplasts of *Benjaminiella poitrasii*	101
3.2	Staining of the intial irregular mass formed (12h growth) during protoplast regeneration using FITC-WGA	102
3.3	Regeneration of mycelial protoplasts of *Benjaminiella poitrasii* in the presence of inhibitors	103
4.1	Isoelectric focussing of yeast-cell and mycelium mixed membrane fraction.	115
5.1	Pile-ups of the amino acid sequences	139
5.2	Phylogenetic tree to show the relatedness between *CHS* genes of *B. poitrasii* and other organisms (figure generated using Clustal W program)	140
5.3	Southern analysis of *CHS* gene fragments	142
5.4	Expression of chitin synthase genes	144

Acknowledgement

I would like to express my sincere gratitude to Dr MV Deshpande for agreeing to serve as my research advisor and offering me an interesting research topic.

I thank the Head, Biochemical Sciences Division, National Chemical Laboratory (NCL) and the Director, NCL for allowing me to pursue my PhD research work at NCL and permitting the full use of institutional facilities.

I also thank the Council of Scientific and Industrial Research, New Delhi for the award of Junior and Senior Research Fellowships between 1996-2001.

The work on chitin synthase genes from *B. poitrasii* was done in Molecular and Cell Biology Department at the University of Aberdeen. I am thankful to Prof. A.J.P. Brown, Prof. G.W. Gooday, Prof. N.A.R. Gow and Dr. Carol A. Munro for the discussions, suggestions and the help that I always received from them during the course of this work. I am also grateful to the British Council's Higher Education Link Programme that enabled me to carry out this work.

The scintillation counter and ultracentrifuge required for the estimation of chitin synthase activity were at National Centre for Cell Sciences (NCCS), Pune. I am thankful to the Director, NCCS and Dr. Padma Shastry, NCCS for permitting to use these facilities.

I wish to thank all the divisional members for the time-to-time help I received from them. In particular, I am grateful to all my lab-mates - present and past - for their support, help and company. I am also thankful to Dr V Shankar and Dr (Mrs) S Barnabas for the useful discussions with them.

I am indeed grateful to my parents and parents-in-law for the constant encouragement I received from them during the course of this PhD. Finally, I would like to thank my husband, Premnath, for his help, support and encouragement.

Manisha V. Chitnis

Declaration

Certified that the work incorporated in this thesis entitled **"Role of Cell Wall in the Dimorphism of *Benjaminiella poitrasii*"** submitted by **Manisha Vasant Chitnis** was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Mukund V Deshpande

Research Guide

List of Abbreviations

Abbreviation	Full form
AA	Amino acid
ADP	Adenosine diphosphate
AIDS	Aquired immuno-deficiency syndrome
ATP	Adenosine triphosphate
BME	β-Mercaptoethanol
BSA	Bovine serum albumin
cAMP	Cyclic-3',5'-adenosine monophosphate
CHS	Chitin synthase gene
CoA	Coenzyme A
dCTP	Deoxycytidine triphosphate
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
FASTA3	Software to compare a protein or nucleotide sequence against a sequence database. Software available at http://helix.nih.gov/science/seq.html
FITC-WGA	Fluroscein isothiocyanate labelled wheat germ agglutinin
GCG	Genetics Computer Group software to access, manipulate and analyze nucleotide and protein sequences.
GlcNAc	*N*- acetyl glucosamine
IEF	Isoelectric focusing
MGYP	Malt extract, glucose, yeast extract, peptone medium
MOPS	3-*N*-Morpholinopropanesulphonic acid
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
Na-EDTA	Sodium EDTA
PB	Phosphate buffer
PCR	Polymerase chain reaction

PDA	Potato dextrose agar
PHYLIP	Philogeny Interference Package. A package of programs for inferring phylogenies (evolutionary trees).
PMSF	Phenyl methyl sulphonyl fluoride
POPOP	1,4 – bis [5-Phenyl-2-oxazolyl]-benzene
PPi	Inorganic diphosphate
PPO	2,5-Diphenyloxazole
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulpate
SSC	Sodium citrate, sodium chloride solution
TAE	Tris-acetate, EDTA buffer
TCA	Trichloroacetic acid
TE	Tris-EDTA buffer
TFP	Trifluoroperazine
UDP	Uridine diphosphate
UMP	Uridine monophosphate
UTP	Uridine triphosphate
UV	Ultra violet light
YP	Yeast extract peptone medium
YPG	Yeast extract peptone medium containing glucose

Abstract

Abstract

Dimorphism is a phenomenon where fungi exist in two different morphological forms – spherical (yeast-like) and filamentous (mycelium-like) - depending upon the environmental conditions and reversibly transit between the two forms. Fungi from different taxonomic groups display yeast-mycelium transition, which include saprophytes such as *Mucor*, *Mycotypha*, human pathogens *Candida*, *Histoplasma* and plant pathogens *Taphrina*, *Ceratocystis*, to name a few.

The aim of the work was to understand the phenomenon of dimorphism in a zygomycetous fungus, *Benjaminiella poitrasii* with a special emphasis on the role of the cell wall metabolism in morphological transition. More specifically, the objectives were as follows:

1. Isolation of yeast and mycelial protoplasts and their regeneration under different dimorphism triggering conditions as well as in the presence of inhibitors like nikkomycin (chitin synthase inhibitor), tunicamycin (inhibitor of *N*-glycosylation of proteins) etc.
2. Biochemical and molecular studies on chitin synthase(s), the enzyme involved in the synthesis of chitin, a main structural component of the cell wall, with respect to their regulation and correlation with yeast-mycelium transition.
3. Evaluation of chitin synthase as a possible target for the treatment of fungal infections.

Isolation and regeneration of protoplasts from *Benjaminiella poitrasii*

The isolation and the regeneration of protoplasts of *B. poitrasii* under different dimorphism triggering conditions was studied to ascertain the role of cell wall components in the determination of morphological outcome.

a) Chitosanase-rich enzyme lytic preparation for protoplastation

For the isolation of protoplasts from *B. poitrasii*, different commercial lytic enzyme preparations and a chitinase-rich lytic enzyme preparation from *Myrothecium verrucaria* were used. (A patent entitled, "A process for the preparation of mycolytic enzyme complex used for the isolation of fungal protoplasts." submitted to CSIR for filing of Indian patent). Though most of these preparations were useful for protoplastation of higher fungi, appreciable yields of protoplasts from *B. poitrasii* were not observed using any of these lytic enzyme preparations. A stable chitosanase – rich lytic enzyme preparation was developed from *Streptomyces* sp. MC1 that produced protoplasts in *B. poitrasii* and other zygomycetous fungi such as *Rhizopus* sp. and *Conidiobolus* sp. The optimum pH, pI and optimum temperature for chitosanase activity in the preparation were found to be 5.0, 8.5 and 50°C, respectively. This lytic enzyme preparation was suitable for obtaining stable protoplasts from *B. poitrasii*. The lytic enzyme system used for protoplaststion of yeast cells included Sigma lysing enzyme, Lyticase and Zymolyase in addition to the chitosanase-rich preparation from *Streptomyces* sp MC1 that was sufficient for the protoplastation of mycelium form cells. (A patent entitled "A process for the preparation of mycolytic enzymes containing mainly chitosanase" submitted to CSIR for filing of Indian patent).

b) Isolation and regeneration of protoplasts in *Benjaminiella poitrasii*

Studies on the isolation and regeneration of protoplasts from the mycelium- and yeast- form cells of the dimorphic fungus *B. poitrasii* have been reported in this section. Though isolation of protoplasts from yeast-form cells of *B. poitrasii* required

the pre-treatment with protease, BME (β-mercaptoethanol) and DTT (dithiothreitol), the mycelial protoplasts were obtained in good yields without pretreatment. A chitosanase-rich preparation from *Streptomyces* sp. MC1 was used to obtain maximum yields of protoplasts $2 \pm 0.3 \times 10^{6}$/ml and $3 \pm 0.4 \times 10^{7}$/ml for the mycelium and yeast- form cells respectively within 5h at 28°C. Optimisation of the protoplast isolation with respect to the osmotic stabiliser, incubation conditions and age of the cells was also carried out. The inorganic stabiliser mixture (0.48M KCl plus, 0.12M $MgSO_4$), 16h old cells, 28°C and 5h incubation were found to be suitable for maximum yield.

Regeneration of protoplasts from yeast and mycelium-form cells of *B. poitrasii* was carried out on yeast extract peptone (YP) medium containing 0.6M KCl as the osmotic stabiliser under specific dimorphism triggering conditions of temperature (28°C and 37°C) and YP medium with and without glucose (5%). The protoplasts from both yeast and mycelial cells formed irregular masses during the initial 10-12 h possibly due to the non-regulated cell wall synthesis. The morphological distinction (yeast or mycelium) was seen after 36h.

Regeneration of protoplasts was similar for protoplasts from yeast as well as mycelium-form cells. Therefore, the regeneration of the protoplasts from mycelium-form cells was studied in the presence of different cell wall metabolism inhibitors. In the presence of cycloheximide (protein synthesis inhibitor, 3-17 μM), no differentiation either in the yeast-or mycelial- form up to 36 h was noted. The presence of nikkomycin (chitin synthase inhibitor, 5-20 μM) led to the formation of deformed cells under mycelium favoring conditions while that of phenyl methyl sulfonyl fluoride (serine protease inhibitor, 0.5-5.5 mM) led to the formation of deformed cells under yeast- favoring conditions. In the presence of glucono-δ- lactone

(glycosidase inhibitor, 2-30 mM) only yeast- form cells were observed under all regeneration conditions. The glucan and mannan synthase, Ca-calmodulin, cAMP-dependent protein kinase inhibitors studied did not affect the morphological outcome. Thus, among all the tested cell wall synthesis inhibitors, chitin metabolism inhibitors showed distinctive effect on the regeneration of protoplasts suggesting that the respective enzymes significantly contribute in determining the morphological outcome of a dimorphic fungus *B. poitrasii* (Manuscript communicated to *Microbiological Research*).

Biochemical studies on chitin synthases from *Benjaminiella poitrasii*

The protoplast regeneration studies discussed earlier indicated that chitin metabolism contributed significantly in the morphological outcome. Therefore, further studies on the biochemical properties of membrane bound chitin synthases from *B. poitrasii* were undertaken. Various producers for inhibitors of chitin metabolism were also screened.

a) Biochemical characteristics of chitin synthases from *Benjaminiella poitrasii*

Chitin synthase (UDP-GlcNAc: chitin 4-β-*N*-acetylglucosaminyltransferase; EC 2.4.1.16) is an important enzyme which accepts the nucleotide sugar uridine diphospho-*N*-acetyl glucosamine (UDP-GlcNAc) as a substrate from cytosol and donates the GlcNAc moiety to the growing chitin chain, thereby synthesizing polymerised chains into the cell wall. Chitin synthases from fungi are either cytosolic or membrane bound. Membrane bound chitin synthases are thought to be involved in the dimorphic transition. The mixed membrane fractions (MMF) isolated from yeast and mycelium-form cells of *B. poitrasii* were used to study the properties of

membrane bound chitin synthases. The MMF was obtained by centrifuging the cell wall free lysate of yeast and mycelium-form cells at 100000 x g. Chitin synthase assay was performed using C^{14} labelled UDP-GlcNAc as the substrate. The reaction was carried out at 37°C for 1h. Chitin synthase activity is five times higher for mycelium-form cells as compared to that for yeast-form cells. Different mechanisms are suggested to be regulatory for chitin synthesis in fungi. These include: activation of zymogenic chitin synthases by trypsinisation, phosphorylation- dephosphorylation, membrane stress, involvement of chitinases and chitin synthase inhibitors in controlling chitin synthesis. Earlier studies have shown that chitin synthase from yeast-form cells of *B. poitrasii* was more zymogenic as compared to that from mycelium-form cells. In this study, metal ions, like Mg^{2+}, Co^{2+} and Mn^{2+}were found to be effective as activators for chitin synthases from both yeast and mycelium-form cells. The activity without metal ions was used as a control. Chitin synthases of yeast- and mycelium-form cells of *B. poitrasii* were activated in the presence of 10 mM metal ions (Mg^{2+}, Mn^{2+} and Co^{2+}). The addition of H-7, an inhibitor of cAMP-dependant protein kinases, did not have any significant effect on the chitin synthase activity of mycelium-form cells but reduced chitin synthase activity of yeast-form cells slightly. On the other hand, TFP (a calcium calmodulin inhibitor) completely inhibited chitin synthase activity in both yeast- and mycelium-form cells thus indicating that primary signal transduction mechanism associated with chitin synthase activity involves calcium calmodulin dependant kinases. However, further experimentation with the purified enzymes is necessary to ascertain the role of phosphorylation in the activation of chitin synthases in *B. poitrasii.*

In this study, membrane solubilisation of mixed membrane fractions of yeast- and mycelium- form cells using digitonin, Triton X-100 and Tween 80 resulted in

very high levels of activation of the chitin synthases. The isoelectric focussing experiment with digitonin solubilized mixed membrane fractions (MMF) of yeast- and mycelium-form cells in the pH range 3-10 resolved chitin synthase activities into 3 peaks for yeast MMF and 4 peaks for mycelium MMF.

b) *Benjaminiella poitrasii* : A model system to screen chitin synthase inhibitors

Three different tests were used for the initial screening for the chitin metabolism (mainly chitin synthase) inhibitor producers. These were hyphal tip-bursting, observations of decreased germ tube formation during yeast-mycelium transition in *B. poitrasii* and chitin synthase activity from mycelium-form cells of *B. poitrasii* in the presence of cell free extracellular broth of various microbial cultures grown in chitin synthase inhibitor-containing production medium for 96h. Hyphal tip bursting (HTB) was the test used for the screening of the cell wall metabolism inhibitors. In this the cell free extracellular broth (10 µl) of each culture in the presence of sorbitol (0.6M) was added to the plates having 16-18 h old growth of *B. poitrasii.* The hyphal tips that burst in a stipulated time were counted. For determining decrease in the germ tube formation in liquid culture, *B. poitrasii* cells were observed during yeast to mycelium transition after 6h incubation and percentage of cells forming germ tubes were determined. Reduction in germ tube formation was determined by comparing with the control. The addition of these culture filtrates inhibited growth and/or cell wall metabolism. Cell free extracellular broth of *Chaetomium* sp., *Volutella* sp. and *Sclerotium* sp. inhibited chitin synthase activity from mycelium-form cells of *B. poitrasii*. Cell free extracellular broth of *Chaetomium* sp., *Volutella* sp inhibited chitin synthase activities from mycelium-form cells of pathogenic fungi *C. albicans* and *A. niger*, respectively. In the presence of cell free

extracellular broth of *Chaetomium* sp., *Volutella* sp. and *Sclerotium* sp reduction in the germ tube formation during yeast to mycelium transition in *B. poitrasii* , as compared to the control was observed. Hyphal tip bursting in *B.poitrasii* was observed only in the presence of the cell free extracellular broth of *Chaetomium* sp. (In press, *Journal of Biocontrol*)

Molecular studies on chitin synthases from *Benjaminiella poitrasii*

Chitin synthase genes from different fungi have been studied in the literature and multiplicity of these genes has been commonly observed. Chitin synthase genes from *Saccharomyces cerevisiae* have been well studied and individual genes have been assigned different functions like lateral growth, bud separation, repair functions, septum formation etc. Chitin synthase genes have been shown to be differentially regulated. For instance, these genes have been shown to be differentially regulated during dimorphic transition in *C. albicans*.This chapter describes the studies on the isolation and molecular analysis of chitin synthase genes and their expression during dimorphic transition in *B. poitrasii*.

PCR amplification of *B. poitrasii* genomic DNA using degenerate PCR primers towards conserved regions in known chitin synthase genes from other fungi, identified eight different chitin synthase gene fragments. This indicated the presence of at least eight distinct chitin synthase genes in the genome of *B. poitrasii*. These genes were labeled as *BpCHS1-8*. Southern analysis for these genes further confirmed the presence of eight chitin synthase genes. Sequence determination and analysis of these genes showed that these genes belonged to chitin synthase classes I, II, IV and V. The phylogenetic tree constructed (using the CHS gene sequences) to study the evolutionary relationship between *B. poitrasii* and other fungi revealed that *B.*

poitrasii is closely related to *Rhizopus oligosporus* and *Phycomyces blakesleeanus*. Expression of these genes was studied through RT-PCR. Different chitin synthase genes were found to be differently expressed during various stages of dimorphic transition and growth. *BpCHS3* and *BpCHS4* expressed exclusively in the mycelium-from cells. *BpCHS1* and *BpCHS5* expressed prominently in the young yeast cells. *BpCHS8* was the only gene that was found to be expressed constitutively. (In preparation for *Fungal Genetics and Biology*)

Conclusion

Thus, during the course of this work, chitin metabolism was shown to be important in determining the morphological outcome during protoplast regeneration in *B. poitrasii*. Further biochemical and molecular studies of chitin synthases showed their multiplicity and different biochemical characteristics that significantly contributed in the dimorphic behavior of *B. poitrasii*. The use of chitin synthesis as a potential target for the antifungal drugs was also evaluated.

Chapter 1: Introduction

1.1 Dimorphism in fungi

Dimorphism is a phenomenon exhibited by a variety of fungal species where fungi exist in two different morphological forms – yeast and mycelium - depending upon the nutritional and environmental conditions. The transition between the two forms is reversible. Dimorphism in fungi has been extensively reviewed in the literature (Gow, 1995). Several pathogenic fungi are known to be dimorphic with one of the morphological forms showing virulence. While the mycelial form of *Candida albicans* and *Ustilago maydis* is considered to be more pathogenic, the yeast form of *Histoplasma capsulatum*, *Paracoccidiodes brasiliensis* and *Blastomyces dematiditis* is pathogenic. So while there is confusion and controversy in the literature ascribing any functional relationship between the vegetative growth form and pathogenicity (Gow, 1995), there is consensus that understanding the phenomenon will be significant in the control of diseases caused by fungi in humans and animals. The dimorphism has also been viewed as a useful model eukaryotic system to study the basis of morphogenesis. Fungal dimorphism is also relevant to industrial production of single-cell protein, since the capacity to form pellicles or mats by yeast used in biomass production may alleviate difficulties inherent in harvesting individual cells from large quantities of culture fluid (Stewart and Roger, 1978).

1.1.1 Events correlating dimorphic transition in fungi

In general, the mycelium to yeast transition (M→ Y) can occur by lateral budding, terminal budding or by arthrospore formation resulting from fragmentation. On the other hand, yeast to mycelium (Y → M) transition occurs by germ tube formation and elongation (Stewart and Rogers, 1978). One of the models describing

these transitions is based on the studies on *P. brasiliensis* and has been reviewed by San-Blas and San-Blas (1983).

Irrespective of the details of the process, dimorphic transition involves spatial differentiation of cell wall synthesis and degradation since the cell wall ultimately determines the cell shape of the fungus (Gow, 1995). The patterns of cell wall synthesis and degradation are in turn related to the activities of the cytoskeleton which directs the vesicles that carry the enzymes responsible for cell wall metabolism to the cell surface. While transition to the yeast cell represents simultaneous synthesis of the whole cell wall without polarization, transition to mycelium represents increasing polarization in cell growth. In different organisms, the details of the process of dimorphic transition including the various morphological forms seen during transition, the cellular biochemical/genetic events and the influencing factors differ.

Gow (1995) has reviewed the cellular events and the factors influencing dimorphic transition for several important pathogenic dimorphic fungal systems. Some of the key biochemical events that have been correlated with dimorphic transition in the case of selected dimorphic fungi are summarized in Table 1.1. These include events relating to a) cell wall composition changes and metabolism, b) signal transduction pathways and c) carbon, nitrogen and sulphur metabolism.

Table1.1: Some known biochemical events relevant to dimorphism in selected fungi

Fungal system	Cellular events identified as relevant to dimorphism	References
Benjaminiella poitrasii	(Y→M) Decrease in NADP/ NAD-GDH ratio. Decrease in endochitinase activity Increase in native chitin synthase activity. Increase in *N*-acetyl glucosaminadase activity. Increase in hexosamine and decrease in mannose content in the cell wall. (M→Y) Reverse trend in comparison to Y→M transition	Khale *et al.* (1992), Khale-Kumar and Deshpande (1993), Deshpande *et al.* (1997), Ghormade *et al.* (2000)
Candida albicans	(Y→M) Changes in second messenger system including cAMP, calcium ions, calmodulin or inositol phosphate. Increase in cAMP level. Increase in native chitin synthase activity. (M→Y) Decrease in cAMP level. Decrease in native chitin synthase activity.	Soll (1985), Gow (1995), Brown and Gow (1999)
Histoplasma capsulatum	(Y→M) Expression of sulphide reductase. Increase in cAMP level. (M→Y) Initial uncoupling of oxidative phosphorylation followed by a drop in ATP levels and a decline in the rate of respiration. Expression of heat shock proteins. Expression of cystein oxidase. Expression of cystine reductase.	Maresca and Kobayashi (1989,2000), Maresca *et al.* (1994).
Mucor species	(Y→M) Increase in NADP-GDH activity. Decline in cAMP level. Increase in ornithine decarboxylase activity. Demethylation of DNA. Decrease in methylation of CUP gene. Oxidative phosphorylation. (M→Y) Increase in cAMP level. Increase in mannan content. Preference for fermentative carbon metabolism pathway.	Stewart and Rogers (1978), Orlowski (1991)
Paracoccidiodis brasiliensis	(Y→M) Change from α−glucan to β−glucan. (M→Y) Change from β−glucan to α−glucan. Increase in levels of polyamines.	San-Blas and San-Blas (1983)
Saccharomyces cerevisiae	(Y→M) Increase in cAMP. Decreased aminoacid uptake. (M→Y) Decrease in cAMP. Increased aminoacid uptake.	Niimi *et al.* (1980); Egidy *et al.* (1989), Sabie and Gadd (1992)

1.1.1.1 Phenotypic events

The morphological and ultrastructural changes during yeast-to-mycelium (Y→M) transition have been studied using electron microscopy of thin sections of transiting cells (Garrison, 1978). The transition process in *P. brasiliensis, B. dermatidis, H. capsulatum and S. schenckii* is initiated by the formation of a bud-like structure termed transition cell. The transition cell often contains abundant reserve material, ribosomes and mitochondria. Occasionally, the complete septum can be seen separating the parent yeast from the elongation. In *W. dermatitidis*, the thin-walled yeast form becomes thick-walled before transition occurs. This is also accompanied by accumulation of endogenous reserves (Lane and Garrison, 1970). In *C. albicans*, an electron transparent layer emerges through the blastospore wall to form the germ-tube initial followed by degenerative changes of the overlying wall structures and re-establishment as germ-tube elongation proceeds (Cassone *et al.*, 1973).

Most detailed analysis of mycelium-to-yeast (M→Y) transition has been done in *H. capsulatum* (Pine and Webster, 1962). Here, the events relating to morphological change are: a) formation of yeast cells by budding of mycelial cells, b) conversion of mycelial cells by monilial chain formation, c) formation of stock yeast cells, d) conversion of microconidia to yeast cells. In *B. dematitidis*, the transition occurs *via* oidial cell formation or by budding of intercalary or terminal chlamydospores (Howard and Herndon, 1961; Miyaji and Nishimura, 1977). In *P. brasiliensis*, under conditions favouring yeast-form cells, the diameter of the hyphae increase at the interseptal spaces (Carbonell, 1969). The outer wall appears to rupture in places. As the transition progresses, the thickness of the cell wall and the cracking of its outer layer increases. Then separation occurs at the interseptal spaces forming cells that become rounded in shape. In *S. schenckii*, the M→Y transition involves the

formation of budding, club-shaped structures at hyphal tips or on lateral branches and the formation of oidia and subsequent fragmentation of the chains into their constituent yeast-phase elements (Garrison *et al.*, 1975).

1.1.1.2 Biochemical events

Since it is the cell wall that determines cell shape, differences in cell wall composition and metabolism between the two forms and during transition are expected. Significant differences in cell wall composition between yeast and mycelium have been shown for *M. rouxii, C. albicans, S. schenckii, B. dermatitidis, H. capsulatum* and *P. brasiliensis* (San-Blas and San-Blas, 1983). Differences in cell wall composition have also been demonstrated for *B. poitrasii* (Khale and Deshpande, 1992). Detailed cell wall compositions and differences in cell wall composition between yeast and mycelium-form cells are described later in Sections 1.2.1 and 1.2.3. The evidence in the literature also indicates that the enzymes involved in cell wall metabolism are differentially regulated in yeast and mycelium-form cells. For example, in *C. albicans*, chitin synthase was ten times more active in mycelium-form cells than that from yeast-form cells (San-Blas and San-Blas, 1983). Similar trends were observed in *Mucor* (San-Blas and San-Blas, 1983) and *B. poitrasii* (Deshpande, 1998). In *P. brasiliensis*, glucan synthetase activities were correlated with the morphological form. Yeast preparations synthesized glucan from UDP-glucose more efficiently at 37°C that 23°C while the mycelial preparations did the same in the reversed fashion (San-Blas, 1979). In *B. poitrasii*, endochitinase activity was reported to be higher in the yeast-form cells while *N*-acetylglucosaminadase activity was higher in mycelial form cells (Ghormade *et al.*, 2000).

Differences in signal transduction systems in yeast and mycelium-form cells have also been recorded in the literature. For example, differences in the cAMP levels were been reported for the case of *C.albicans*, *H. capsulatum*, *M. rouxii* and *S. cerevisiae* (Table 1.1). In *C. albicans*, several second messenger systems were implicated in dimorphic regulation including those based on cAMP, Ca^{2+} and calmodulin or inositol phosphates or intracellular pH (Gow, 1995).

Differential regulation of the carbon, nitrogen and sulphur metabolism pathways have also been observed in various organisms. Carbon metabolism was studied extensively in *M. racemosus.* Here, yeast growth was associated with fermentative metabolism. Most of the glucose in the medium was catabolised to ethanol, CO_2 and glycerol through EMP (Embden-Meyerhoff-Parnas) pathway. Only 14-20% of the glucose was channelled through the hexose monophosphate (HMP) shunt (Inderlied *et al.*, 1978). Chattaway *et al.* (1973) have found differences in the enzyme activities associated with carbohydrate metabolism (in particular, activity of phosphoglucose isomerase, phosphofructokinase and the first enzyme of the hexose monophosphate pathway) in the two morphological forms of *C. albicans*. The difference in the activity of glutamate dehydrogenase (GDH) between the yeast and mycelium-form cells of *B. poitrasii* (Khale-Kumar and Deshpande, 1993) and *M. rouxii* (Orlowski, 1991) is an example of differential regulation of nitrogen metabolism. The differential regulation of cysteine oxidase, cystine reductase and sulfite reductase in yeast and mycelium form cells of *H. cap*sulatum is an example of the involvement of sulphur metabolism in dimorphic transition (Maresca and Kobayashi, 1989, 2000).

1.1.1.3 Genetic events

Genes related to various functions in the cell have been extensively studied in different dimorphic systems (Table 1.2). Genes encoding for cellular regulators like protein kinases (*STE12, STE20, PKC1*), transcriptional activators (*TUP1, EFG1*), heat shock proteins (*HSP70*) are thought to be playing an important role in the yeast-mycelium transition of *S. cerevisiae*, *C. albicans* and *H. capsulatum*, respectively. In *C. albicans*, northern analyses indicated that the expression of some genes coding for cellular building blocks vary during yeast-mycelium transition (Gow, 1995). For instance, the genes *ECE1* (gene that is expressed in relation to the extent of cell elongation) and *CHS2* (chitin synthase gene) were preferentially expressed in the hyphal form. The genes that express in the cells of only one form, such as *HYR1*, a hypha-specific gene in *C. albicans* (Bailey *et al*, 1996) and *YPS3*, a yeast- specific gene in *H. capsulatum* (Maresca and Kobayashi, 1989) are known but the information about their products is not clear. However, there has been no gene reported to be linked constitutively to the dimorphic transition.

Table 1.2. Different genes involved in morphogenesis for selected fungi[1]

Genes coding for	*C. albicans*	*H. capsulatum*	*M. racemosus*	*P. brasiliensis*	*S. cerevisiae*	*U. Maydis*	*Y. lipolytica*
Cellular regulators	*ACPR, CPH1, CST20, EFG1, HST7, RAS2, RBF1, SAP1-7, TUP1*	*HSP70, HSP83*	*CUP*	*HSP70*	*BEM1, CDC24, CUP, ELM1, ELM2, ELM3, PKC1, STE7, STE11, STE12, STE20, SHR3*		*XPR2*
House-keeping enzymes					*CDC3, CDC10-12, CDC30*		
Cellular building blocks	*CHS1, CHS2, ECE1*	*TUB1, TUB2*			*CAP2, CHS2, CHS3, CHS5, MYO2, TPM1, PFY2*		
Unknown products	*HYR1*	*YPS3*				*EG11*	

Function of genes:

S. cerevisiae – *BEM*, bud emergence (Cabib *et al.*, 1998); *CAP2*, capping protein; *CHS2, CHS3, CHS5*, chitin synthase; *ELM1, ELM2, ELM3*, protein kinase (Blacketer *et al.*, 1993), *MYO2*, myosin; *PFY2*, profiling (Harold, 1995); *STE7, STE11, STE12, STE20*, protein kinases (Liu *et al.*, 1993); *TPM*, tropomyosin (Harold, 1995)

C. albicans – *CHS1,2*, chitin synthase (Gow, 1993); *EFG1*, enhanced filamentous growth (Stoldt *et al.*, 1997); *HYR1*, hyphally regulated gene (Bailey *et al.*, 1996); *HST7*, mitogen activated protein kinase components (Gow *et al.*, 1995); *RBI1*, RPG box binding factor 1 (Magee, 1997); *SAP1-7*, aspartyl proteinase gene (Hube *et al.*, 1994); *TUP1*, transcriptional regulator (Magee, 1997).

H. capsulatum – *HSP70*, *HSP83*, Heat shock protein; *TUB1*, α tubulin; *TUB2*, β-tubulin; *YPS3*, yeast phase specific gene (Maresca and Kobayashi, 1989).

M. recemosus – *CUP*, Copper metallothein gene (Cano-Canchola *et al.*, 1992)

U. maydis – *EG1*, filamentous growth gene (Bolker *et al.*, 1992).

P. brasiliensis – *HSP70*, heat shock protein (Da Silva, 1999).

Y. lipolytica – *XPR2*, alkaline protease (Madzak *et al.*, 1999)

[1]From Ghormade (2000), Gow (1995), Maresca and Kobayashi (1989), Orlowski (1991).

1.1.2 Factors influencing dimorphic transition

A large number of factors influence yeast -mycelium form transition (Table 1.3). These include: a) environmental factors such as temperature, pH, oxygen concentration or anaerobiosis with CO_2 or N_2, b) nutritional conditions (C:N ratio, presence of metal ion etc). In most of the dimorphic fungi, temperature change is the common factor which triggers yeast to mycelium transition. For instance, in case of *B. dermatitidis, B. poitrasii, H. capsulatum*, and *P. brasiliensis* incubation at 37 °C favours yeast-form growth whereas in *C. albicans* yeast formation is favoured at lower temperatures (25°C) (Cole and Sun, 1985; Ghormade and Deshpande, 2000; Maresca *et al.*,1994; San-Blas and San-Blas, 1983; Gow, 1995). In *C. albicans*, *N*-acetyl glucosamine and serum stimulated hyphal growth (Odds, 1988). Elevated temperatures, neutral pH and poor growth medium stimulated the growth of hyphal cells (Odds, 1988). In *Y. lipolytica* mycelium formation is favored by anaerobiosis (Zinjarde *et al.*, 1996). In *Ophiostoma ulmi*, dimorphic transition is regulated by controlled supply of nitrogen in the growth medium (Muthukumar and Nickerson, 1984). Ammonium ions, yeast extract and certain amino acids promote hyphal development (Kulkarni and Nickerson, 1981). In case of *Mycotypha africana*, pH of the growth medium affected the morphological outcome (Schulz *et al.*, 1972). The fungus predominantly grows as yeast between pH 5.8-6.5, while on the either side (pH below 4.5 or above 7.4) it grows as mycelium. In *B. poitrasii*, temperature, pH and glucose are important factors influencing dimorphic transition (Ghormade and Deshpande, 2000).

In *Mucor*, anaerobiosis and hexoses favor yeast form and even very low concentrations of O_2 promoted mycelial development (Orlowski, 1991). Hexose sugars were found to be required for the formation of yeast cells.

Table1.3: Factors influencing dimorphic transition in selected fungi

Fungal system	Factors influencing yeast to mycelium transition	References
Benjaminiella poitrasii	Decreasin temperature. Lower glucose.	Deshpande (1998).
Candida albicans	Elevated temperature. Serum. *N*-acetyglucosamine. Neutral pH. Nutrient poor growth medium.	Soll (1992). Gow (1995). Brown and Gow (1999)
Histoplasma capsulatum	Decreasing temperature. Theophylline. Acetyl-salicylic acid. Prostaglandin E1. Nerve growth factor.	Maresca and Kobayashi (1989). Maresca *et al.* (1994).
Mucor species	Favoured by aerobic conditions and EDTA. Inhibited by acriflavin or chloramphenicol, phenethyl alcohol, sodium fluoride, elevated glucose and CO_2 concentrations, antimycin A and KCN, dibutyryl cAMP, diamino butanone.	Stewart and Rogers (1978). Orlowski (1991). Gow (1995).
Paracoccidiodis brasiliensis	Decreasing temperature.	San-Blas and San-Blas (1983)
Saccharomyces cerevisiae	Nitrogen starvation. Exogenous cAMP or dibutyryl cAMP.	Gow (1995)
Yarrowia lipolytica	Anaerobic conditions	Zinjarde *et al.* (1998)

1.2 Fungal cell wall: Components, organisation and chemistry

The fungal cell wall is the outermost part of the cell and therefore provides the interface between the organism and its environment. The cell wall performs a diversity of functions such as determination of the shape and rigidity, protection from osmotic changes, maintenance of the intracellular concentrations of ions and solutes and the extracellular secretion of large molecules. The cell wall can also serve as a store of carbon reserves. Lastly, in certain parasitic and symbiotic fungi, cell wall plays an important role in establishing interaction with the host (Farkas, 1985; Peberdy, 1990).

1.2.1 Cell wall composition, structure and organisation

For the complete characterization of cell walls, it is important to know the chemical nature of individual wall components, their relative abundance, the spatial organisation of these components within the cell wall and the resultant architecture. Several reviews are available in the literature on fungal cell wall composition and architecture (Aronson, 1965; Bartnicki-Garcia, 1968; Farkas, 1985, 1990; Gooday, 1995; Peberdy, 1990, Wessels *et al.*, 1990).

Tables 1.4 and Table1.5 summarize the various cell wall components and their spatial organisation in fungi. The cell wall of the fungi is composed primarily of polysaccharides – both homo- and hetero-polymers. In some fungi proteins are also the significant components of the cell wall and frequently are associated with the polysaccharide components (Gooday, 1995). Lipids and melanins are the minor cell wall components.

Table 1.4: Main components of the fungal cell wall[1]

Component	Remarks
1. Aminopolysaccharides (Polymers of amino sugars)	
• Polymers of acetyl hexosamines	
o Chitin (Polymer of β–1,4 linked *N*-acetyl D-glucosamine)	Skeletal component. Crystalline. Occurs in a complex with R-glucan.
• Polymers of hexosamines	
o Chitosan (Polymer of β–1,4 linked D-glucosamine)	Skeletal component.
o Polymer of galactosamine	Binds polyphosphates
o Other polymers of glucosamine	Could link proteins and polysaccharides.
2. Nonaminopolysaccharides (Polymers of neutral sugars)	
• Glucans (Polymers composed of glucose but distinct from cellulose)	
o β–Glucans: R-glucan (β–1,3 glucan homopolymer comprised of β–1,3- and β–1,6-linked D-glucose)	Skeletal/ matrix element. Varying degrees of branching/ crystallinity. Some covalently linked to mannoproteins. Can link with chitin.
o α–Glucans: S-glucan (α–1,3 homopolymer of D-glucose) and Nigeran (α–1,3- and α–1,4-linked glucan)	Major component of outer matrix of the wall. Linear molecule. Microcystalline.
• Mannans (Polymers of mannose)	Matrix component. Exists as mannan-protein complex.
• Other neutral polysaccharides (Polymers of hexoses such as galactose, methyl pentoses such as fucose and rhamnose, and pentoses such as xylose)	Matrix component. Exist as complexes with proteins/ mannoproteins.
• Polyuronides (Polymers of uronic acids)	
3. Proteins	Probably exist as glycoproteins.
4. Other components	
• Lipids	
• Melanins	Dark brown to black pigments in wall distinct layers of spore walls.
• Inorganic constituents especially phosphates	
• Nucleic acid derivatives	

[1]From Aronson (1965), Peberdy (1990)

Table 1.5: Major polymers occurring in the fungal cell walls of various fungal classes[1]

Fungal class/ taxonomic group[2]	Cell wall polymers	
	Alkali insoluble (Fibrous/ Skeletal)	Alkali soluble (Gel-like/Matrix)
Ascomycota	R-Glucan, Chitin	Galactomannoproteins, S-Glucan
Basidiomycota	R-Glucan, Chitin	Xylomannoproteins, S-Glucan
Deuteromycetes	Chitin, Glucan[3]	Glucan[3]
Zygomycota	Chitin, Chitosan	Polyglucuronic acid, Glucurono-mannoproteins, Polyphosphate

[1]From Bartnicki-Garcia (1968), Farkas (1990), Gooday (1995); [2]Fungal classification based on Ainsworth and Bisby (1995); [3]Incompletely characterized. Probably, R-Glucan.

In the electron micrographs, walls usually appear to be composed of layers and often it is possible to assign a particular component to a particular layer (Figure 1.1). The shape- determining, more fibrillar polysaccharides, chitin, chitosan and chitin- glucan complexes make up the inner layer of the wall. These are also known as the structural components. These are embedded in more gel-like matrix polymers such as glucans and glycoproteins that make up the outer layer of the wall. These components are also known as the amorphous components. Different fungi possess a unique set of amorphous and crystalline components and this is of taxonomic significance as well.

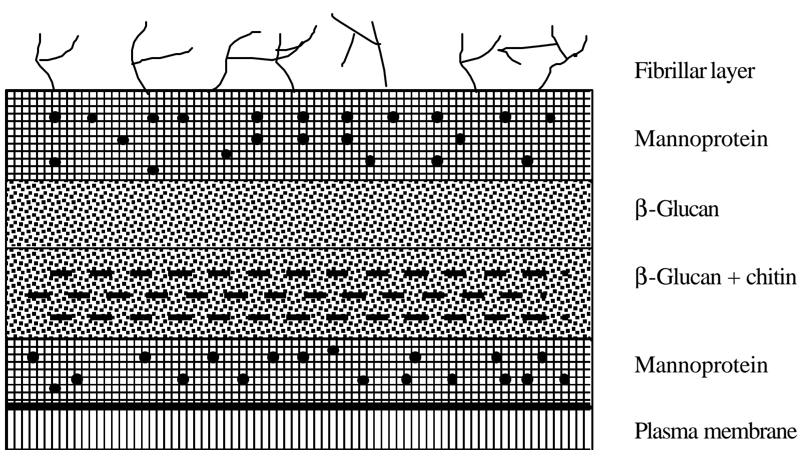


Figure 1.1 Cell Wall: Organisation of different components (Debona and Gordee, 1994)

1.2.1.1 Chitin and chitosan:

Chitin is the β(1-4)-linked polymer of *N*-acetyl glucosamine and chitosan, a deacetylated form of chitin, is the β(1-4)-linked polymer of glucosamine. Chitosan is formed by progressive deacetylation of chitin. In nature there is a great variety in degree of acetylation of these molecules. Chitin is abundant throughout the natural world. It occures as a structural polysaccharide in most invertebrates and many protists and is probably a universal components of fungal walls. Fungi such as fission yeasts, *Schizosaccharomyces* species, have been shown to have small amounts of chitin (Sietsma *et al.*, 1966; Sietsma and Wessels, 1990). The X-ray diffraction studies of chitinous material indicated that three different types,viz., α,β and γ-chitin are present in nature. The commonly found chitin in fungi is α-chitin, in which two *N*-acetyl glucosamine chains running in antiparallel direction form one unit cell. As compared to the other two types, α-chitin is more rigid (Gooday, 1979). In electron micrographs of chitin preparation, the microfibrils appear in a range of forms, such as short stubby ones from yeast walls and long interwoven ones from hyphal walls (Gow and Gooday, 1983). In the hyphae, septa typically are rich in chitin and have microfibrils arranged in a tangential fashion. Chitin does not occur by itself and its interactions with other components viz. peptides and glucan are of importance in the make up of the wall (Wessels, 1986,1990).

Chitosan occurs as a major component in the walls of zygomycetes. It is accompanied by anionic polymers rich in glucuronic acid and probably polyphosphates (Gooday, 1995).It is present as a minor component in the walls of other groups of fungi. It also occurs as a distinct layer in the ascospore wall of *Saccharomyces cerevisiae* (Briza *et al.*, 1990).

1.2.1.2 Glucans:

β-Linked glucans (R-glucans) are major constituents of most fungal walls. Walls of all members of ascomycetes and basidiomycetes contain β(1-3)-linked glucans with branches of one or more β(1-6) linked glucose residues (Wessels 1986, 1990). These branched glucans can form gels of interconnected triple helices. Covalent links with chitin microfibrils give them a major role in the structure of the mature wall. In *Schizophyllum commune*, mycelia become covered with a mucilage of β(1-3), β(1-6)-glucan which may gelatinise the entire culture medium. Microcrystalline α(1-3)-glucans (S-glucans) comprise a major component of the outer matrix of the wall (Wessels and Siestma, 1979).

1.2.1.3 Glycoproteins and proteins:

Glycoproteins, especially mannoproteins, galactomannoproteins and xylomannoproteins are major constituents of the matrix of many fungal walls. They may have various amounts of mannosyl-6-phosphoryl linkages. Glycoproteins containing galactosamine and/or *N*-acetylgalactosamine are reported from a range of fungal walls. In pathogenic fungi, such as *C. albicans* and *Aspergillus fumigatus*, they are important surface antigens. Most work on the structure of these molecules has been carried out in *S. cerevisiae* (Gooday, 1995).

Briza *et al.* (1990) reported that the outermost layer of ascospore walls of *S. cerevisiae* is a cross linked insoluble polymer of D, L di-tyrosine, glycine, alanine, glutamic acid and traces of glycine. This layer is closely associated and possibly covalently linked to the second outer layer consisting of chitosan. Mutant spores lacking the outermost di-tyrosine-rich layer are much less resistant as compared to wild type spores to adverse environmental conditions, suggesting that this outermost layer confers much of the resistance.

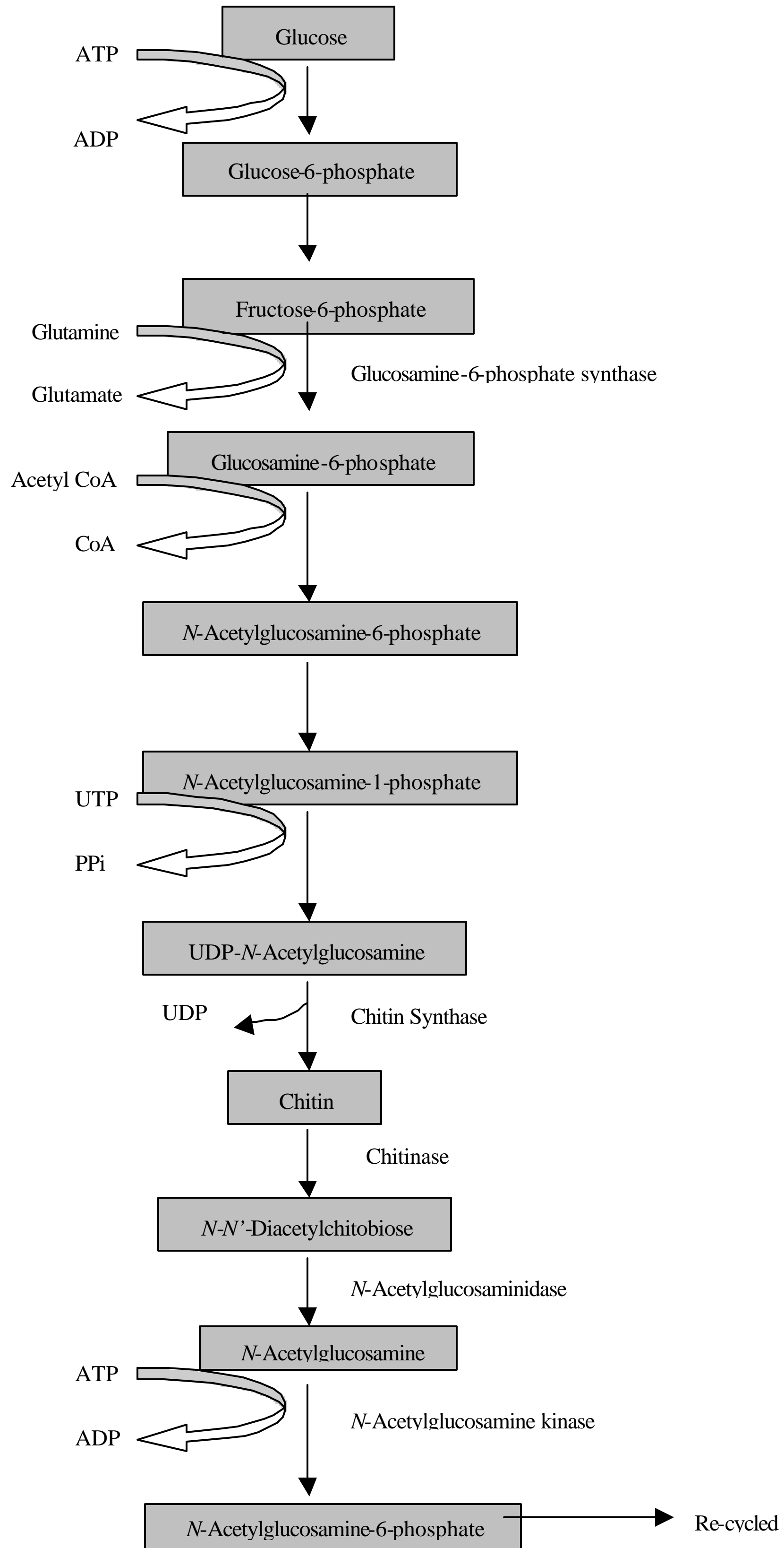
1.2.2 Chemistry of cell wall synthesis and degradation

Gooday (1995) has extensively reviewed cell wall biosynthesis, which occurs at three sites: cytoplasm, plasma membrane and the wall itself. Structural polymers, chitin, β(1-3)- and β(1-4)-linked glucans, are synthesized vectorially at the plasma membrane, by transmembrane synthases, accepting nucleotide sugar precursors from the cytosol and feeding the polymerised chains into the wall. Matrix polymers such as mannoproteins are synthesized in the cytoplasmic secretory pathway of endoplasmic reticulum through golgi vesicles to secretory vesicles. Wall assembly, involving activities such as covalent cross-linking of polymers and modifications such as deacetylation of chitin, takes place in the wall itself.

Several enzymes are involved in the synthesis and degradation of the various cell wall polymers. As in most of the fungi, chitin is the main structural component, its metabolism is considered to be important in cell wall growth. Bartnicki-Garcia (1973) proposed an integrated model for cell wall growth which describes co-ordinate regulation of chitin synthase and chitinase at the site of cell wall growth.

A pathway for chitin metabolism is shown schematically in Figure 1.2. Chitin is synthesized by chitin synthase which catalyses glycosidic bond formation from nucleotide sugar substrate, uridine diphospho-*N*-acetyl glucosamine. Glucosamine-6-phosphate synthase is the key enzyme of this pathway. This is the enzyme that connects the carbon and nitrogen metabolic pathways in the fungal cell. Three other enzymes are involved in chitin metabolism: chitinase, *N*-acetylglucosaminidase and *N*-acetylglucosoamine kinase. These three enzymes together provide a pathway for recycling of chitin or the digestion and utilization of exogenous chitin. After

Figure 1.2: Pathway for chitin metabolism
(Adapted from Gooday, 1995)



synthesis, chitin chains of the growing hyphal cells undergo important changes (like hydrogen boding between individual chains, formation of covalent links with glucans and modification to chitosan), the occurrence and extent of which differ in different fungi, giving different properties to the walls. The modification of chitin to chitosan is especially prominent in members of zygomycetes, which involves progressive deacetylation with chitin deacetylase to give a β(1-4)-linked polymer of glucosamine.

1.2.3 Cell wall and morphogenesis

Changes in the ultrastructural and chemical properties of the cell wall and consequent chnges in cell shape during morphogenesis are the most evident feature during morphogenesis. Therefore, an understanding of the changes in cell wall composition and structure during morphogensis seems necessary for a better understanding of the phenomenon of dimophism. One of the ways is to follow the cell wall synthesis during protoplast regeneration.

1.3 Protoplasts as a tool to study cell wall biosynthesis

Protoplasts are the cell wall-less structures bound by the cytoplasmic membrane. Protoplasts possess the ability to regenerate the cell wall when cultured on a suitable medium. This phenomenon enables the use of protoplast as ideal objects for *in vivo* cell wall synthesis studies.

1.3.1 Methods for protoplastation

Different enzymic and non-enzymic methods used for the isolation of protoplasts are described in Table 1.6. The most common and widely used method for protoplastation is the release of protoplasts using lytic enzyme systems. The choice of a suitable lytic enzyme system for a given fungus seems to depend upon the composition and structure of the cell wall. Reyes *et al.* (1984) reported use of

autolytic enzymes for the isolation of *Aspergillus nidulans, Botrytis cinerea, Mucor rouxii, Pencillium oxalicum* and *Schizophyllum commune* protoplasts.

Table 1.6: Different methods us ed for protoplast isolation from fungi

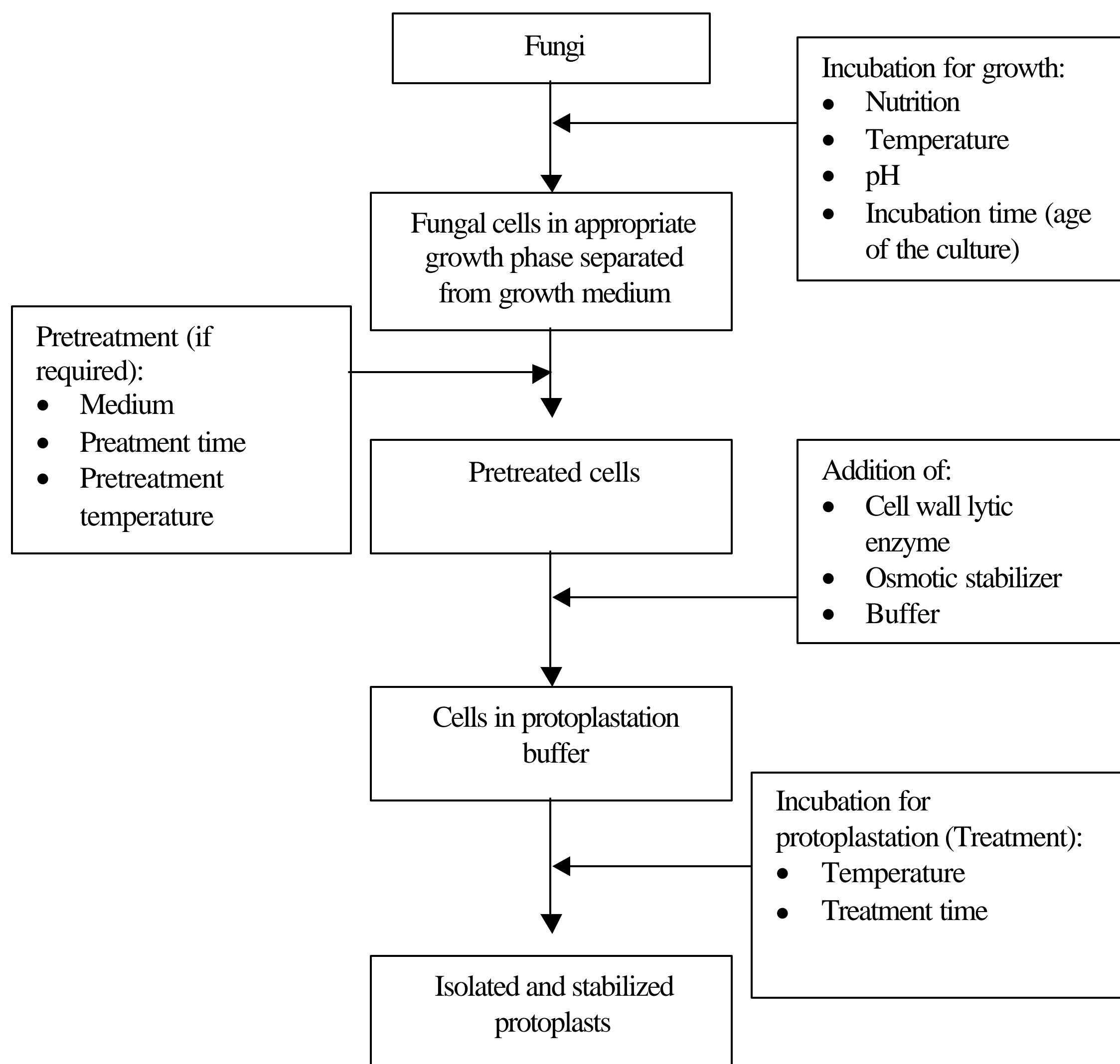
Method	Remarks
Cell wall degradation using enzymes	
• Use of lytic enzymes (commercial and inducible) to degrade cell wall	Most widely used method. Novozym 234, Sigma lysing enzyme, Zymolyase are the most commonly used preparations.
• Use of autolytic enzymes	Demonstrated in number of fungi such as *Aspergillus nidulans, Botrytis cinerea, Mucor rouxii, Pencillium oxalicum* and *Schizophyllum commune* (Reyes *et al.*, 1984).
Nonenzymatic release of protoplasts	
• Use of cell wall metabolism inhibitors	
• Use of 2-deoxy D-glucose and high concentrations of magnesium sulfate	
• Spontaneous growth of cell wall-less cells in suitable nutritional medium	

In *N. crassa*, protoplastation of conidia of the temperature sensitive *osmotic*-1 variant was possible during germination in the presence of sorbose and polyoxin B (Selitrennikoff *et al.*, 1981). *S. pombe* and yeast phase cells of *H. capsulatum* spontaneously produced protoplasts when grown in the presence of 2-deoxy D-glucose and $MgSO_4$ (Peberdy, 1979a). Protoplasts were spontaneously released from the tip of the conidial germ tube of *Entomophthora* when germinated in an insect tissue culture medium (Tyrrell and MacLeod, 1972).

1.3.2 Protoplastation using lytic enzyme system

The generic protocol for protoplastation using lytic enzyme systems is illustrated in Figure 1.3.

Figure 1.3: Generic protocol for protoplast isolation using lytic enzyme systems



The fungus is grown in a suitable growth medium and incubation conditions till an appropriate growth phase best suited for an optimum protoplast yield is reached. The fungal cells are then separated from the medium and subjected to cell wall lytic enzymes in the presence of an appropriate osmotic stabilizer under appropriate incubation conditions. In some case, a pre-treatment of the cells prior to exposure to a lytic enzyme is required in order to obtain protoplasts. The lytic

enzymes degrade the cell wall and expose the plasma membrane-bound fungal cytoplasm into the medium. The osmotic stabilizer then ensures the stability of these protoplasts in the medium. The protoplast yield is then monitored microscopically.

1.3.3 Factors influencing protoplast isolation using lytic enzyme systems

1.3.3.1 Culture conditions

Filamentous fungi and yeasts respond to various environmental and nutritious conditions in a variety of ways and hence nature of the growth medium and culture conditions is of prime importance in protoplast isolation. Experiments with *A. nidulans* have shown that protoplasts yields are higher from the mycelium growing on glucose salts medium than from mycelium growing on the same medium plus yeast extract (Peberdy, 1976). In *A. niger*, mycelium grown on mineral salts medium, supplemented with glucose and asparagine, released significantly more protoplasts per milligram of mycelial dry weight than mycelium grown on the same medium supplemented with malt extract or on malt extract alone (Musilkova and Fencl, 1968). While protoplastation from the yeast *Aureobasidium pullulans* was greatly enhanced when the cells were grown under anaerobic conditions (Finkelman *et al.*, 1980).

1.3.3.2 Age of culture

Protoplast yield was much greater for the mycelium in the exponential phase of growth (Peberdy, 1976). Cells from stationary or late stationary phase of growth gave a much reduced protoplast yield (Schwencke *et al.*, 1977). However in *P. chrysogenum*, large yields of protoplasts were obtained from cultures well into stationary phase and it was suggested that endogenous lytic activity may be involved in the process of protoplastation (Eyssen, 1977).

1.3.3.3 Pretreatment of organism

Pretreatment involves incubating the cells with appropriate thiol compound or detergent for different time interval before an enzymatic treatment, depending on organism and strain. The commonly used pre-treatment agents are: dithiothreitol (DTT), β-mercaptoethanol, EDTA, 2-deoxy D-glucose, sodium sulfite, Triton X-100, sodium dodecyl sulphate (SDS), sodium lauryl sulphate and an enzyme like pronase.

Usually, the fungal cell walls containing significant amounts of mannan and protein content (probably existing as mannoproteins) seem to be requiring a pre-treatment. Furthermore, pretreatment was reported more commonly in yeast-form cells than in mycelial-form cells for the dimorphic fungus, *M. rouxii*, which can be attributed to the higher mannan contents (Reyes *et al.*, 1983).

1.3.3.4 Lytic enzymes

Different enzyme preparations have been used singly or in combination for the protoplastation of different fungi. Usually these preparations exhibit activities such as chitinase, glucanase, protease, mannanase, etc. Commercial lytic enzyme systems have been used successfully more often for the case of fungi belonging to Ascomycetes, Basidiomycetes and Deuteromycetes. For the protoplastation of of zygomycetous fungi, effective enzyme preparations are not available commercially. One of the reasons could be qualitative differences in the cell wall composition of higher and lower phycomycetous fungi. Furthermore, it is also interesting that in dimorphic organisms, the two morphological forms require separate lytic enzyme systems in order to yield protoplasts.

In most cases, cell wall fragments or mycelium of the organism to be protoplasted has been used as the medium for growing the lytic enzyme producer organism (Reyes et al., 1983). Other media such as wheat bran (Toyama et al.,

1984a,b), ethanol extracted mushrooms (Waterfield and Sisler, 1988), mycelium or cell wall fragments of another organism (Dlugonski et al., 1984) and inducers such as glucosamine (Binding and Weber, 1974) have also been used. The most commonly used organisms for producing the lytic enzyme system are *Trichoderma* sp. and *Streptomyces* sp. Ocassionally, *Aspergillus* sp., *Bacillus* sp., *Penicillium* sp. and *Myxobacter* sp. have also been used.

1.3.3.5 Effect of osmotic stabiliser

The release of the fungal protoplast as an intact structure as well as its regeneration depends largely on the nature and osmolarity of the osmotic stabiliser used in the medium. A stimulatory effect of the osmotic stabiliser on the protoplast release was suggested (Rodriguez Aguirre *et al.*, 1964; Thomas and Davis, 1980). Musilkova and Fencl (1966) found that the $CaCl_2$ in the incubation medium was essential to obtain protoplasts from certain strains of *A. niger* and this was attributed to the stimulation of lytic enzyme activity by Ca^{2+} ions

A wide range of osmotic stabilisers including inorganic salts such as NH_4Cl, $(NH_4)_2SO_4$, $CaCl_2$, $MgCl_2$, $MgSO_4$, KCl, and NaCl, organic stabilisers such as glucose, sucrose, and sugar alcohols such as sorbitol and mannitol have been used in protoplastation. Citric acid and sodium citrate have also been used (Tully and Gilbert, 1985).

1.3.3.6 Treatment conditions

Temperature and pH affect the activities of the lytic enzymes and the effectiveness of the osmotic stabiliser used. Undesirably high temperatures may cause agglutination of certain organelles in isolated protoplasts and may render such protoplasts unsuitable for metabolic studies while low temperatures affect protoplast membrane stability (Kovac and Subik, 1970). The choice of the buffer and its

molarity depends upon the stability of the osmotic stabilisers and the stability of the enzymes in the system.

1.3.4 Regeneration of protoplasts

The sequence of events leading from protoplast to normal cell, capable of growth has been referred to in literature as either protoplast regeneration or protoplast reversion (Necas and Svoboda, 1985; Necas, 1980; Peberdy, 1979b). Cell wall synthesis is the key event in the process of protoplast regeneration. The phenomenon of regeneration serves as a model system for the study of various developmental processes. The regeneration of protoplasts is also a prerequisite for all the experiments employing hybridisation and genome manipulations in fungal cell.

Regeneration of the cell wall follows the same general pattern in all fungal systems and hence for the initial studies, yeast protoplasts were used as the model system (Svoboda and Necas, 1974). Protoplasts synthesise and release all the chemical constituents of the cell wall onto the surface. Some of these are produced in the quantities different from those found in the normal cell (Farkas and Svoboda, 1980). In some yeast species, aberrant glucans not present in the normal cell walls are incorporated in the regenerating wall (Kreger and Kopecka, 1975). The fibrillar and amorphous components are synthesised and transported independently irrespective of their differing chemical composition in different fungi (van der Valk and Wessels, 1976). The mode of wall material secretion onto the protoplast surface is still unclear. The synthesis of glucan and chitin macromolecules was associated with the plasma membrane (Vermeulen *et al.*, 1979). Glycoproteins, the main component of the matrix was thought to be discharged *via* exocytosis (Farkas, 1979). First structure assembled on the surface of regenerating protoplasts was reported to be the microfibrillar net composed either of glucan (Eddy and Williamson, 1959; Popov *et al.*, 1980), chitin

(Gibson *et al.*, 1976) or chitin and glucan (van der Valk and Wessels, 1976). For microfibril formation, two possible mechanisms were postulated (Necas and Svoboda, 1985). One assumes the self assembly of the macromolecules secreted on the protoplast surface. The second assumes that microfibrils are organised immediately after polymer synthesis occurs in particular enzyme complexes like chitosomes or gluconosomes (Bartnicki-Garcia, 1980; Necas and Svoboda, 1981). In the case of normal cell wall synthesis the existing wall operates as the epigenic information system. However, in the case of protoplasts cell wall synthesis occurs completely *de novo*. The only information determining the supramolecular structure of the wall is inherent in the secreted polymers. The self-assembly of these polymers results in the formation of the normal wall and is also controlled by the environmental conditions (Kreger and Kopecka, 1975; Necas, 1971).

The morphology of the protoplast growth and consequently the morphology of reversion depend on the relationship between an increase in cytoplasmic volume and the rate of wall regeneration. If the rate of wall regeneration and the increase in cytoplasmic volume are balanced, protoplasts revert rapidly, soon resuming normal morphogenesis and are usually isodiametrical. If wall regeneration proceeds at a low rate, protoplasts grow in an unisodiametric fashion; i.e., protoplasts of both yeast and mycelial fungi develop tubular, chainlike or other irregularly shaped formations that revert to cells after completion of wall regeneration. Thus by changing culture conditions, i.e., by modifying the rate and assembly of the wall, the growth and reversion morphology of the protoplasts can be greatly affected. Studies on the assembly of yeast protoplasts during regeneration showed that net structures of normal and protoplast cells were different and the difference could be due to the

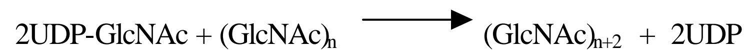
absence of β-1,6-glucan synthetase effected by the liquid medium for cultured protoplasts (Kreger and Kopecka, 1975).

1.4 Chitin synthases and their role in cell wall metabolism

Chitin is a major skeletal component in most of the fungal cell wall. Synthesis of chitin in the fungal cell is catalysed by an enzyme, chitin synthase. Various properties of chitin synthase, structure of chitin synthases and the regulation of these enzymes have been reviewed by Bulawa (1993), Gooday and Schofield (1995) and Nino-Vega (1997).

1.4.1 Properties and regulation of chitin synthases from fungi

Chitin synthase catalyses the following reaction:



$$2\text{UDP-GlcNAc} + (\text{GlcNAc})_n \longrightarrow (\text{GlcNAc})_{n+2} + 2\text{UDP}$$

Table 1.7 lists some biochemical properties of chitin synthases from selected fungi. The properties of chitin synthases from various sources appear to be very similar.

Chitin synthases are membrane bound enzymes and also are reported to be present in microvesicles known as chitosomes (Kamada *et al.*, 1991; Cabib, 1987). The enzyme is allosteric. The substrate uridine diphosphate *N*-acetyl glucosamine (UDP-GlcNAc), free GlcNAc and a range of other effectors activate chitin synthase. In growing hyphae chitin synthesis is highly polarized. All of it occurs at extreme hyphal apex, at branch points and in developing septa (Wessels, 1986; Hunsley and Gooday, 1974). In *Coprinus cinereus*, chitin is synthesized uniformly along the length of the vertically elongating cells. An unusual pattern of chitin deposition is shown by hyphae of *Allomyces macrogynus* that have been grown in oxygen deficient conditions. These hyphae are very narrow and respond to oxygen by their backward directed expansion of their chitin-rich wall (Youatt *et al.*, 1988). Thus it is clear that

Table 1.7: Biochemical properties of chitin synthases from fungi[1]

Organism	Optimum temperature, (°C)	Optimum pH	K_m for GlcNAc, mM	Optimum [Mg^{2+}], mM	Percent inhibition by UDP 0.5 mM	References
Allomyces macrogynus	30	7.8	ND[2]	ND[2]	ND[2]	Porter and Jaworski, 1966; Gooday, 1990
Benjaminiella poitrasii	ND[2]	7.4 (Y) 8.0 (M)	ND[2]	ND[2]	ND[2]	Deshpande *et al.*, 1997
Blastocladiella emersonii	20-25	8	3-4	6-20	ND[2]	Camargo *et al.*, 1967
Mucor rouxii	24-27	6.5	12.5	30	53	McMurrough and Bartnicki-Garcia, 1971; Muller *et al.*, 1981
Phycomyces blakesleeanus	28	6.5	ND[2]	20	75	Jan, 1974
Mortierella vinaceae	32	6	ND[2]	20	ND[2]	Peberdy and Moore, 1975
Saccharomyces cerevisiae	37	6.2	4.7	10	62	Keller and Cabib, 1971
Neurospora crassa	27	7.5	4.5	ND[2]	ND[2]	Glaser and Brown, 1957; Gow and Selitrennikoff, 1984
Coprinus cinereus	30	8	0.6	30	44	Gooday and de Rousset-Hall, 1975

[1]From Gooday (1977, 1990, 1995); [2] ND=Not determined.

chitin synthases must be tightly controlled in time and space. There are variety of manners in which it might be regulated. The possibilities include post-translational modification of chitin synthase polypeptides, regulation by availability of appropriate concentrations of allosteric activators and inhibitors and of cations, regulation by changes in the physical conformation of integral membrane polypeptides or associated molecules caused by stress (Gooday and Schofield, 1995).

Regulation by transcription and translation

The mRNA levels for chitin synthase genes have been estimated in some systems. Of the three chitin synthase genes identified in the dimorphic fungus *C. albicans*, the expression of both *CHS2* and *CHS3* increased during hyphal outgrowth, while *CHS1* is expressed at a lower level in both yeast and hyphal forms and showed little change or decrease in expression during hyphal outgrowth (Chen-Wu *et al.*, 1992; Sudoh *et al.*, 1993; Schofield, 1994; Gow *et al.*, 1995). Differential expression of chitin synthase genes was also observed in *P. brasiliensis* (Nino-Vega *et al.*, 2000), *P. blakesleeanus* (Miyazaki *et al.*, 1997) and *Mucor circinelloides* (Lopez-Matas *et al.*, 2000).

Other regulatory processes

A considerable interest has been found in the possibility that proteolytic activation of zymogenic enzyme molecules plays a major role in the activation of chitin synthases. In most of the *in vitro* studies it has been observed that enzyme activity can be increased, sometimes dramatically, by treatment with proteolytic enzymes such as trypsin or rennin, i.e., to varying extents the enzyme preparations are zymogenic (Cabib 1987). Chitin synthase preparations from hyphae of *A. nidulans* showed considerable activation by proteolytic enzymes. (Isaac *et al.,* 1978).

Genetic studies, involving selective gene disruptions, have allowed particular gene products to be studied in isolation. Thus in *S. cerevisiae*, chitin synthase I and II are mainly in zymogenic form in native extracts (i.e., stimulated by treatment with trypsin), while chitin synthase III appears to be fully active and is inactivated by trypsin (Sburlati and Cabib 1986; Orlean 1987; Cabib 1991; Bulawa 1993).

Nevertheless, until amino acid sequences of active enzymes are known, the mechanism(s) of proteolytic activation *in vitro* and their significance *in vivo*, if any, remains unclear (Ruiz-Herrera 1992).

Regulation through availability of substrate or allosteric effectors

Chitin synthases are activated by the monomer *N*-acetylglucosamine of chitin and its dimer (Rosset-Hall and Gooday 1975; Gooday 1977; Horsch and Rast 1993). It has been suggested that their localized production *via* the action of chitinases and *N*-acetyl-glucosaminidases on chitin in the growing wall could lead to localize activation of chitin synthases (Gooday 1979; Rast *et al.* 1991).

Regulation via cations

Chitin synthases require divalent cations such as magnesium, manganese, or cobalt for activity (Gooday 1979; Ruiz-Herrera 1992). Solubilized preparations of chitin synthase from *C. albicans* that was made in the absence of magnesium showed an absolute requirement for a metal cation for activity (Gooday 1979). The availability of Mg^{2+} could play a role in regulating the enzyme activity, unless there are localized mechanisms for maintaining a high concentration in its microenvironment. Enzymes differ in their responses to cations. Thus, chitin synthase II from *S. cerevisiae* was stimulated more by Co^{2+} than by Mg^{2+}, whereas for chitin synthases I and III, Mg^{2+} was favoured and Co^{2+} inhibited chitin synthase I (Sburlati and Cabib 1986; Orlean 1987; Cabib 1991; Bulawa 1993).

Martinez-Cadena and Ruiz-Herrera (1987) reported that incubation of chitosomes from *P. blakesleeanus* in the presence of Ca^{2+} for 2 h resulted in an indirect activation of chitin synthase. This activation was increased if calmodulin, a Ca-binding protein, was also present and this increase was inhibited by trifluoroperazine, a calmodulin inhibitor.

Effect of osmotic stress on activity of chitin synthase

Katz and Rosenberger (1971) reported the effect on chitin synthesis of subjecting hyphae of *A. nidulans* to osmotic shock. This treatment led to an increase in chitin synthesis (measured by autoradiography) in an uncontrolled fashion, more or less uniformly along the length of the hyphae. Gooday and Schofield (1995) have studied the effects of external osmotic pressure changes on chitin synthase activities of protoplasts of *C. albicans* and *S. cerevisiae.* These experiments with protoplasts suggest that when transferred to hypo-osmotic medium they react with an irreversible activation of latent chitin synthase.

Similar experiments were performed with yeast cells and hyphae of *C. albicans*, monokaryotic hyphae of *C. cinereus* and shmoos of *S. cerevisiae* (equal numbers of α and **a** cells pre-icubated together for 1.75 h, then transferred to media of low and high osmolalities for 1h). As for protoplasts, chitin synthase specific activities were higher in all preparations of intact cells growing in medium of low rather than high osmolality. Regulation of chitin synthase activity through osmotic stress was also demonstrated in *B. poitrasii* (Deshpande *et al.*, 1997).

Effect of specific chemicals leading to uncoordinated chitin deposition

Treatment of hyphae of *A. nidulans* with cycloheximide, a specific inhibitor of protein synthesis, led to uncontrolled increase in deposition of chitin approximately uniformly along the hyphae (Katz and Rosenberger, 1971; Sternlicht *et al.,* 1973).

Treatments with low concentrations of antifungal drugs which act by inhibiting ergosterol biosynthesis resulted in increases in deposition of chitin, in grossly aberrant fashion, especially in swollen areas of hyphae and in septal regions (Kerkenaar and Barug, 1984; Hector and Braun, 1987; Vanden Bossche, 1990). Treatment of *C. albicans* with monensin, a monovalent cation ionophore, also led to enormous aberrant wall and septal thickenings of chitin (Poli *et al.,* 1986).

The fluorescent brightener Calcofluor White and intercalating dye Congo red both interfere with crystallization of chitin. Treatment of growing cells of a range of fungi with Calcofluor White or Congo red resulted in a range of fungi with structures, associated with uncontrolled increase in chitin deposition (Pancaldi *et al.,* 1984 1988; Rico *et al.,* 1985; Roncero and Duran, 1985). Effects include pronouced bulges in the hyphae, at their apices but also irregularly along their hyphae. These effects may result from activation of chitin synthases where the wall is showing swelling because of the weakening of the chitin exoskeleton.

Effect of light

The photobiology of sporangiophores of *P. blakesleeanus* has been the subject of much study. These cells exhibit a transient growth response after illumination, which involves an intercalary increase in chitin deposition in the growing zone. Other phenomena accompany and perhaps are necessary for this growth response. This includes a stimulation of chitin synthase activity *in vivo* and *in vitro* (Jan, 1974; Herrera-Estrella and Ruiz-Herrera, 1983; Ruiz-Herrera, 1992), and a weakening of the cell wall in the growing zone (Ortega *et al.,* 1975). This stimulation of chitin synthase could be a result of turgor-driven membrane stress under the weakened wall.

1.4.2 Structure of chitin synthases

Information regarding the structure of the enzyme is restricted to predictions based on the primary nucleotide sequence. A characteristic three-domain pattern can be observed from hydrophobicity or hydrophilicity plots for the deduced amino acid sequences of the fungal chitin synthases of classes I, II and III (Bulawa *et al.*, 1986; Silverman, 1989; Yarden and Yanofsky, 1991; Chen-Wu *et al.*, 1992; Beth –Din and Yarden, 1994; Motoyama *et al.*, 1994; Mellado *et al.*, 1996). In general chitin synthases consists of a largely hydrophilic *N*-terminal domain, a mainly neutral central domain and a dominantly hydrophobic C-terminus. In the later domain, potential membrane-spanning segments can be identified which is in agreement with the localisation of the enzymes as intrinsic membrane proteins. Potential glycosylation site, a characteristc of membrane proteins, have also been identified (Bulawa *et al.*, 1986; Mellado *et al.*, 1996). Class IV chitin synthases differ from the other classes of chitin synthases in the presence of hydrophobic regions in the *N*-terminal domain (Valdivieso *et al.*, 1991. Two highly conserved domains (shown in Figure 1.4 as regions (a) and (b)) have been proposed as involved in the catalytic site of chitin synthases (Nagahashi *et al.*, 1995). The substitution of conserved amino acids (corresponding to *S. cerevisiae* Chs2 Asp^{562}, Gln^{601}, Arg^{604} and Trp^{605} and shown in Figure 1.4 as boxed regions) in those regions with alanine resulted in the loss of activity of *S.cerevisiae* chitin synthase 2. Even conservative substitution of these amino acids drastically decreased the activity of the enzyme (Nagahashi *et al.*, 1995).

Figure 1.4: Conserved regions in chitin synthase sequences. (Nagahashi *et al.*, 1995) Abbreviations: Sc, *Saccharomyces cerevisiae*; Ca, *Candida albicans*; Ro, *Rhizopus oligosporus*; Ad, *Aspergillus nidulans*

Horsch and Sowdhamini (1996) used the consensus sequence obtained from comparison of five different fungal chitin synthases on the putative catalytic region (classes I, II and III), for the prediction of secondary and three-dimensional structure. The secondary structure prediction gave an overall consensus consisting of 13 β-strands and 14 α-helices. Based on the alternating α-helices to β-strands, and on the coincidence of the predicted β–strands region with areas of hydrophobicity, they proposed a fold with buried β-strands. Comparisons of the secondary structure obtained for the consensus chitin synthase to the secondary structure of other proteins of known three-dimensional structre lead them to postulate an $(\alpha/\beta)_8$-barrel fold. In this model it was found that most of the conserved amino acids were placed at the C-terminal end of the β–sheet core structure. This is consistent with the observation that the active centre of a $(\alpha/\beta)_8$-barrel enzyme is located in this region (Horsch and Sowdhamini, 1996).

1.4.3 Chitin synthase genes and their classification

Chitin synthases in Saccharomyces cerevisiae :

Most of the studies on fungal chitin synthases have been done in *S. cerevisiae.* In this fungus, three chitin synthase enzymes have been identified *viz*., Chs1, Chs2 and Chs3. Each of these enzymes exhibited different properties. *In vitro* preparations of chitin synthases 1 and 2 are zymogenic and are stimulated by proteolytic enzymes such as trypsin (Cabib and Farkas, 1971; Sburlati and Cabib, 1986). Chitin synthase 3 was initially thought to be non-zymogenic. Recently however it is demonstrated to be zymogenic when isolated in the presence of excess substrate to protect against proteolytic activation during purification (Choi *et al.*, 1994).

The first chitin synthase gene isolated was the structural gene for *S. cerevisiae* Chitin synthase 1 (*CHS1*; Bulawa *et al.*, 1986). The *S. cerevisiae CHS1* gene product, chitin synthase 1 (the most abundant of the three enzymes in the yeast cell), acts in the repair of the cell wall during the process of cell separation of the mother and daughter cells (Cabib *et al.*, 1989). Disruption of this gene has no effect on chitin synthesis during normal growth, but mutants tend to leak contents from septum after release of the daughter cells when growing on the media of low pH (Bulawa *et al.*, 1986; Cabib *et al.*, 1989). A second structural chitin synthase gene (*CHS2*) was involved in the synthesis of primary septum of the bud. Mutants of *CHS2* were viable, although showing clustered growth with aberrant cell shapes and sizes (Silverman *et al.*, 1988). The *CHS3* gene product, chitin synthase 3, generated the bulk of the bud scar chitin and the thin chitin layer in the lateral cell wall (Sburlati and Cabib, 1986; Shaw *et al.*, 1991). Disruptants of *CHS3* showed lowered chitin content (5-10%) but they were still viable (Valdivieso *et al.*, 1991; Bulawa, 1992). Recent studies showed that it also contributed to the formation of a linkage between chitin and β–1,3-glucan (Kollar *et*

al., 1995), thereby increasing the rigidity of the cell wall. The *CAL3* and *CSD4/CAL2* gene products have been found to be also required for Chs3 activity in yeast (Bulawa, 1992; Choi *et al.*, 1994). However their functions are still unclear and are thought be the subunits of a multienzyme complex (Bulawa, 1993; Munro and Gow, 1995). Double mutants lacking either chitin synthases 1 and 2 or 1 and 3 are viable but the loss of all three products is lethal (Shaw *et al.*, 1991).

Chitin synthases in Candida albicans

C. albicans was the first dimorphic fungal human pathogen from which a chitin synthase gene was isolated (Au-Young and Robbins, 1990). In this organism, three chitin synthase genes were identified and cloned. These are *CHS1*, *CHS2* and *CHS3* (Au-Young and Robbins,1990; Chen-Wu *et al.*, 1992; Sudoh *et al.*, 1993). Disruption of *CHS2* indicated that it was not essential for the growth or virulence of the fungus (Gow *et al.*, 1994). Disruption of *CHS3* showed that this gene is not essential for the viability of the fungus. The product of this gene was responsible for 60-80% of the chitin content of both yeast and hyphal forms (Bulawa *et al.*, 1995; Mio *et al.*, 1996). Calcofluor staining of chs3 defective cells indicated that in yeast cells, this activity was required for the synthesis of the chitin rings present on the surface of the cell (Bulawa *et al.*, 1995). Double disruptant of *CHS2* and *CHS3* showed that *C. albicans* cells did not require these genes for viability. *CHS1* was thought to be involved in the septum formation. Attempts to produce homozygous chs1 –null mutant were not successful, suggesting that this gene might be essential for the viability of *C. albicans* (Mio *et al.*, 1996).

Chitin synthases in other fungi

Bowen *et al.* (1992), designed degenerate PCR primers by using two conserved amino acid sequences present in three different chitin synthases (*S.*

cerevisiae Chs1, Chs2 and *C. albicans* Chs1) and cloned 32 different fragments of chitin synthase genes from 15 fungal species. Their deduced amino acid sequences were compared and they could be classified into three classes viz., I, II, III. Recently a new class, class IV, was proposed for genes homologous to *CHS3* of *S. cerevisiae* and *C. albicans* (Motoyama *et al.*, 1994; Yanai *et al.*, 1994; Beth-Din *et al.*, 1996). Class V is another newly proposed class that thought to be necessary for the filamentous fungal growth.

1.5 Cell wall as a target for antifungal drugs

Opportunistic fungal infections are responsible for increased morbidity and mortality amongst AIDS and other patients immunocompromised as a result of cancer chemotherapy and organ transplantation. This alarming increase underscores an even greater medical need for the rapid diagnosis and effective treatment of these infections, although the therapy for human mycoses continues to improve (Walsh *et al.*, 1991; Khadori, 1989; Graybill, 1989; Edwards, 1991). Currently this therapy primarily consists of amphotericin B and the azoles (Debona and Gordee, 1994). These agents are not ideal because amphotericin B treatment is associated with severe side effects and the azoles possess fungistatic rather than fungicidal action. Current therapy is also limited by the lack of selective toxicity, in which the therapeutic target would be exclusively associated with the fungal cell. Fungi are complex organisms that share many biochemical targets with other eukaryotic cells. However, the cell wall is a unique organelle that fulfills the criterion for selective toxicity. This is the reason that the cell wall is a suitable target for action of antifungal drugs. One interesting approach to the development of cell-wall targeting antifungal drugs is the use of inhibitors of cell wall metabolic processes.

Chitin synthase inhibitors

Chitin synthase is the enzyme responsible for the catalysis of the last step in chitin biosynthesis. It is found in fungi but not in mammals and so a specific inhibitor might be expected to prevent growth of fungi, but should be non-toxic to the host. One group of compounds with this mode of action are the polyoxins. They are powerful specific antifungal antibiotics that have been used widely in Japan as agricultural fungicides. They were discovered in 1963 and are the metabolic product of *Streptomyces cacoi* var *asoensis* (Suzuki *et al.*, 1965). More recently nikkomycin, a closely related antibiotic was obtained from a strain of *Streptomyces tendae* (Dahn *et al.*, 1976).

Polyoxins and Nikkomycins are tripeptides and are very specific competitive inhibitors of chitin synthase, mimicking its substrate UDP-GlcNAc. Both polyoxins and nikkomycins were isolated as antifungal antibiotics and inhibit a wide range of different fungi (Gooday, 1979; 1989). Both groups are powerful competitive inhibitors of chitin synthase (Endo *et al.*, 1970; Muller *et al.*, 1981; Furter and Rast, 1985). Chitin synthase is an integral protein in the plasma membrane, accepting substrate from the cytosol. The polyoxins and nikkomycins require transport into the fungal cell for their inhibitory activity. Evidence for this comes from observations that the enzyme chitin synthase from a wide range of fungi is approximately equally susceptible to inhibition, but that the intact fungi show a very wide range of susceptibility (Gooday, 1989). The uptake of polyoxins and nikkomycins has been studied in detail for the human pathogen *C. albicans*. Payne and Shallow (1985) and McCarthy *et al.*, (1985) showed that strains and mutants resistant to polyoxin showed cross-resistance to nikkomycin. These authors and Yarden *et al.*, (1984) showed that mutants resistant to nikkomycin had changed patterns of peptide uptake. It is clear that in culture, under the right experimental conditions, inhibition of chitin synthase

by these antibiotics is a fungicidal activity. Neither polyoxins nor nikkomycins have any reported significant toxicity to vertebrates or higher plants (Isono *et al.*, 1967; Fiedler *et al.*, 1982; Adams and Gooday, 1983). They have no reported activity against any other enzyme system present in mammals involving UDP-GlcNAc, such as glycoprotein biosynthesis. This great specificity for chitin synthase reinforces the appeal of chitin synthase as the target. Efforts have been made to improve the antifungal potential of polyoxins and nikkomycins by producing semisynthetic or completely synthetic analogs. In particular a major aim has been to increase activity against human pathogenic fungi. To date, however, none of these analogs have been as active as the natural products (Gooday, 1989).

Chitinase inhibitors

Allosamidins are the first antibiotics specifically active against chitinases and are produced by *Streptomyces* sp no. 1713 (Sakuda *et al.*, 1986, 1987). Allosamidins are pseudotrisaccharides consisting of a disaccharide of *N*-acetylallosamine linked to a novel amino cyclitol derivative, allosamizoline. Allosamidin shows different activities against chitinase from different sources. It shows good activity against insect chitinase from *Bombyx mori*, poor activity against bacterial chitinase from *Streptomyces griseus* and *Serratia marcescens* and no detectable activity against plant chitinase from yam, lysozyme from egg white and insect β-*N*-acetyl glucosaminidase from *Bombyx mori*. Allosamidins are powerful against secreted chitinase from the fungi *N. crassa* and *A. nidulans* chitinase of nematode *Onchocerca gibsoni* (Gooday, 1989; Gooday *et al.*, 1988)

Glucan synthase inhibitors

The echinocandin lipopeptides and papulacandins inhibit glucan biosynthesis. Aculeacin A killed growing *S. cerevisiae* cells by causing leakage of intracellular

materials at the bud tip, the active site for cell wall biosynthesis (Mizoguchi *et al.*, 1977). Cilofungin exposure severely damaged the cell wall of *C. albicans*, resulting in deformed cells, some of which lysed (Drouhet *et al.*, 1990). Cilofungin was a non-competitive inhibitor of β–1,3-glucan synthase derived from *C. albicans* but had no effect on chitin synthase (Taft *et al.*, 1988).

Mannan binding antifungal antibiotics

The pradimicins and benanomicins are a unique group of antifungal antibiotics isolated from *Actinomadura hibisca* (Takeuchi *et al.*, 1988; Oki, 1992; Oki *et al.*, 1988) The antifungal action of these agents is not completely defined but it is suspected that the formation of an insoluble complex between pradimicins and yeast mannan in the presence of calcium may be involved in the antifungal effect (Ukei *et al.*, 1993).. Pradimicins A,B and C show a broad spectrum of *in vitro* antifungal activity against *Candida* sp., *Cryptococcus neoformans* and *Aspergillus* sp. Pradimicins are also active against systemic murine fungal infections (Oki *et al.*, 1990). Pradimicin A alters membrane permeability and this effect is Ca^{2+} dependant.

1.6 Present investigation and thesis organisation

In this PhD work, an attempt has been made to develop an improved understanding of the phenomenon of dimorphism in a model dimorphic fungus with a special focus on the role of the cell wall (and in particular, metabolism of cell wall polymers) in influencing morphogenesis.

1.6.1 *Benjaminiella poitrasii* as a model system

In this thesis, *Benjaminiella poitrasii*, a zygomycetous saprophytic fungus, has been used as a model system. There are several reasons that make *Benjaminiella poitrasii* a suitable model system for the studies on dimorphism. *Benjaminiella*

poitrasii readily undergoes dimorphic transition in response to relatively simple triggers such as temperature and glucose concentration. *B. poitrasii* shows temperature dependant dimorphism similar to pathogens like *H. capsulatum* and *P. brasiliensis* (Khale *et al.*, 1990). However, the transition in either direction in *B. poitrasii* is relatively rapid. The yeast – mycelium transition (60% in 6h) is obtained without the formation of intermediate (pseudo-) forms as observed in *Wangiella*, *Candida* etc. Hence it is easier to pinpoint the correlation of biochemical events with morphological change (Deshpande, 1998; Khale-Kumar and Deshpande, 1993; Khale and Deshpande, 1992; Khale *et al.*, 1990). Stable, monomorphic (yeast-form; Y-2 and Y-5) mutants of *B.poitrasii* are also available and the biochemical correlates of morphological outcome for these mutants have also been studied. Recently, for the first time, it has also been reported that that in *B. poitrasii* the asexual and sexual spores also respond to the dimorphism triggering conditions as vegetative cells do (Ghormade and Deshpande, 2000). Therefore, *B. poitrasii* holds promise as a complete system to screen antifungal agents.

Various aspects of dimorphism in *B. poitrasii* have been well studied and this body of literature is a useful backdrop for the work reported in this thesis. Khale *et al.* (1990) studied the mechanism of dimorphism in *B. poitrasii* and two stable yeast phase mutants. Khale and Deshpande (1992) studied the cell wall composition of mycelial phase (M), yeast phase (Y) and yeast-form mutants (Y-2, Y-5). Chitosan was abundant in M-phase (26.6%) whereas lesser amounts were present in Y-phase (17.3%). Although chitin was present as a smaller fraction of the total glucosaminoglycan in each of the different cell wall preparations, it was almost 3 times more prevalent in M-phase than the Y-phase. Existence of linkages between mannans and proteins, and glucans and glycosaminoglycans was also suggested.

In *B. poitrasii*, cell wall chitin contents decrease during mycelium-yeast transition. For chitin synthesis, both carbon and nitrogen pathways are brought together by the fungus *via* ammonia assimilation. The participating enzymes in ammonia assimilation are: glutamate dehydrogenase, glutamate synthase and glutamine synthase and the relative proportion of NADP and NAD-glutamate dehydrogenase measured as NADP-/NAD-GDH ratio were shown to have significance in dimorphic transition (Khale and Deshpande, 1992; Khale-Kumar and Deshpande, 1993). Other enzymes that contribute in cell wall morphogenesis are chitin synthase and chitinase. The yeast cells exhibited the presence of low native chitin synthase and high endo-chitinase, while mycelial cells showed high native chitin synthase and high *N*-acetyl glucosaminidase activity (Deshpande *et al.*, 1997; Ghormade *et al.*, 2000). All these activities are significantly correlated with morphological outcome and therefore are important in developing antifungal drugs (Deshpande, 1998).

1.6.2 Objectives of this study

The objectives of the present studies are as follows:

1. To isolate yeast and mycelial protoplasts, and study their regeneration under different dimorphism triggering conditions as well as in the presence of inhibitors like nikkomycin (chitin synthase inhibitor), tunicamycin (inhibitor of *N*-glycosylation of proteins) etc.
2. To undertake biochemical and molecular studies on chitin synthase(s), the enzyme involved in the synthesis of chitin, a main structural component of the cell wall, with respect to their regulation of and correlation with yeast-mycelium transition.

3. To evaluate chitin synthase as a possible target for the treatment of fungal infections.

1.6.3 Thesis organisation

The experiments presented in this PhD dissertation are organized into three main parts. The first part is a study on protoplast isolation and their regeneration under different dimorphism triggering conditions and in the presence of inhibitors (Chapter 3). The development of a novel lytic enzyme system for protoplastation of *B. poitrasii* is described in Section 3.1. Section 3.2 describes the studies on regeneration of protoplasts with a focus on examining the role of various cell wall components in the determination of morphological outcome.

The second part of this research effort includes biochemical studies of chitin synthase (the enzyme responsible for chitin biosynthesis) from *B. poitrasii* (Chapter 4). The first part of this chapter (Section 4.1) discusses biochemical properties of membrane bound chitin synthases from *B. poitrasii* and their regulation during dimorphic transition. The second part of this chapter (Section 4.2) describes the work on screening of chitin metabolism inhibitor producers.

The third and final part of this dissertation concerns molecular studies of chitin synthase genes of *B. poitrasii* and their differential regulation during growth and dimorphic transition (Chapter 5).

Chapter 2 describes the various materials and methods used during the course of this work. The thesis concludes with Chapter 6 which summarizes the salient features of this PhD research effort.

Chapter 2: Materials and Methods

2.1 Chemicals

The various chemicals used and their sources are listed in Table 2.1.

2.2 Organism and cultivation

2.2.1 Organisms and culture conditions

The dimorphic zygomycete, *Benjaminiella poitrasii,* was maintained on YPG (YP containing 1% glucose) agar (2%) slants at 28°C and was subcultured every 15 days. *B. poitrasii* was grown on YP (yeast extract, 0.3 %; peptone, 0.5%) medium containing different glucose concentrations. Sporangiospores (10^8) were inoculated in 50 ml of the YPG (0.5% glucose) medium and incubated at 37°C for 24 h in order to obtain an inoculum of yeast-form cells. This inoculum of yeast-form cells (10^6 cells) were then inoculated in 50ml of YP medium at 28°C for 16 h in order to obtain mycelial-form cells and YPG (0.5% glucose) at 37°C for 16 h in order to obtain yeast-form cells.

Rhizopus stolonifer and *Conidiobolus coronatus* used for protoplastation studies were maintained on Potato Dextrose Agar (PDA) slants at 28°C and were subcultured every 15 days. These organisms show only mycelium- form growth when inoculated with 10^8 spores in 100 ml YPG (1% glucose) medium after 18-20 h incubation at 28°C while shaking at 180 rpm.

Streptomyces sp MC1, a chitosanase producer, was maintained on MGYP (malt extract 0.3%, glucose 1%, yeast extract 0.3%, peptone 0.5%) medium slants and incubated at 28°C and was subcultured every 15 days.

Table 2.1: Sources of different chemicals used

Chemicals	Source
Agarose	Sigma Chemical Co., St. Louis, USA
Ampholytes	Pharmacia Chemicals, Uppsala, Sweden
Ampicillin	Sigma Chemical Co., St. Louis, USA
Benanomicin A&B	Gift from Dr. Daishiro Ikeda, Inst. Microbial Chem., Japan
Big-dye kit	Perkin Elmer, UK
Calcofluor white	Sigma Chemical Co., St. Louis, USA
Chitin	Sigma Chemical Co., St. Louis, USA
Chitosan	Sigma Chemical Co., St. Louis, USA
Chloroform	Fischer Scientific Co., UK
Competent *E. coli* cells	Promega, UK
Diethylpyrocarbonate	Sigma Chemical Co., St. Louis, USA
Digitonin	Sigma Chemical Co., St. Louis, USA
EDTA	Sigma Chemical Co., St. Louis, USA
Ethanol	Fischer Scientific Co., UK
$FeSO_4$	Qualigens, India
FITC-WGA	Sigma Chemical Co., St. Louis, USA
Formaldehyde	Fischer Scientific Co., UK
Glucono-δ-lactone	Sigma Chemical Co., St. Louis, USA
Glucose	Qualigens, India
Glucose estimation kit	Ranbaxy, India
Hybond-N nylon membranes	Amersham International, UK
Isopropanol	Fischer Scientific Co., UK
K_2HPO_4	Qualigens, India
KCl	Qualigens, India
KH_2PO_4	Qualigens, India
Lysing enzyme from *T. harzianum*	Sigma Chemical Co., St. Louis, USA
Lyticase	Sigma Chemical Co., St. Louis, USA
$MgSO_4$	Qualigens, India
Mini/ Midi/ Maxi prep plasmid isolation kit	Promega, UK
MOPS	Sigma Chemical Co., St. Louis, USA
N-Acetylglucosamine	Sigma Chemical Co., St. Louis, USA
NaCl	Qualigens, India
Nikkomycin	Sigma Chemical Co., St. Louis, USA
PCR primers	Oswal, UK
PCR, Solutions for	Promega, UK
Peptone (Bacto)	Difco Industries, Detroit, MI, USA
PGEM-T easy vector system	Promega, UK
Phenol	Fischer Scientific Co., UK
Phenyl methyl sulphonyl fluoride	Sigma Chemical Co., St. Louis, USA
POP	Sigma Chemical Co., St. Louis, USA
POPOP	Sigma Chemical Co., St. Louis, USA
Restriction enzymes	Promega, UK
RNAase free DNAase	Promega, UK
RT-PCR primers	Genosis, UK
RT-PCR, Solutions for	Amersham International, UK
Sorbitol	Qualigens, India
Sucrose	Qualigens, India
T4-DNA ligase	Promega, UK
Tetracyclin	Sigma Chemical Co., St. Louis, USA
Triton X-100	Sigma Chemical Co., St. Louis, USA

Trizma base (Tris)	Sigma Chemical Co., St. Louis, USA
Tunicamycin	Sigma Chemical Co., St. Louis, USA
Uridine diphosphate-[^{14}C] *N*-acetylglucosamine	Amersham International, UK
Verapamil	Sigma Chemical Co., St. Louis, USA
Yeast extract	Difco Industries, Detroit, MI, USA
Zymolyase 10T	Gift from Dr. Zita Lobo, TIFR, Mumbai, India

Escherichia coli (JM109 strain) was used as a host strain for all cloning experiments. Cells were taken from frozen glycerol stocks stored at -70°C, spread on LB plates (0.5% yeast extract, 1% tryptone, 1% NaCl, 1.5% agar) and incubated at 37°C until colonies were observed (16-18 h). For bacteria containing plasmids, the LB medium was supplemented with ampicillin (100 µg/ml). For small scale preparation of plasmids (Section 2.3.4.1), a single colony was inoculated into 5 ml LB medium containing 100 µg /ml ampicillin and grown for 16 h with shaking at 37°C. For medium scale plasmid preparation (Section 2.3.4.2), 5 ml culture was diluted 1:100 into 50 ml LB medium with ampicillin (100 µg/ml) and grown overnight. For permanent storage, bacterial cells were stored at -70°C in 50% (v/v) glycerol.

2.2.2 Yeast – mycelium transition in *B. poitrasii*

Yeast inoculum was grown in YP medium containing 0.5% glucose for 24h at 37°C and the transition was studied in YP medium at 28°C. Morphology was determined according to Chattaway *et al.* (1973). Aliquotes were taken from each sample just before harvesting and examined microscopically in a hemocytometer 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 M→ Y transition studies, mycelial strands were counted once for each hemocytometer square in which they appeared. During this transition, the 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 the total cell count.

The studies examining the effect of inhibitors on the yeast-mycelium transition were performed using yeast cells grown in YP medium containing 0.5% glucose for 24h at 37°C. These cells were incubated for 9h at 28°C in YP medium containing exogeneously added inhibitors and then percentage germ tube formation was assessed.

2.2.3 Determination of the growth curve and glucose utilization pattern under different conditions in *B. poitrasii*

The growth curve for *B. poitrasii* was studied under three different conditions: YP medium (without glucose) at 28°C, YPG medium (0.5% glucose) at 28°C and YPG medium (0.5% glucose) at 37°C. Yeast-form cells were obtained from a seven-day-old sporulating slant culture. Spores (10^8) were inoculated in 50 ml YP medium containing 0.5% glucose and incubated under shaking condition (180 rpm) at 37°C for 24 h. The same cells were used as an inoculum to obtain yeast and mycelium-form cells during different phases of growth and transition. Yeast-form cells were obtained by inoculating 4×10^8 yeast cells in 200 ml YP containing 0.5% glucose under shaking condition (180 rpm) at 37°C for 30 h. The culture broth was collected at 6h, 12h, 24h and 30h time points. For obtaining mycelium-form cells grown in the absence of glucose, 4X 10^5 yeast cells were inoculated in 200 ml YP medium and incubated under shaking condition (180 rpm) at 28°C for 30h. Samples were taken at 6h, 12h, 24h and 30 h. For obtaining mycelium- form cells grown in presence of glucose, 4 X10^5 yeast cells were inoculated in 200 ml YP medium containing 0.5% glucose and were incubated under shaking condition (180 rpm) at 28°C for 30h. Samples were collected at 6h, 12h, 24h and 30 h time points. For each time point, 50 ml sample was

used. The cells were collected by centrifugation (3000xg for 10 min), washed with distilled water and dried to constant weight. Cell growth was determined by dry weight estimations of the samples.

The amount of the glucose remaining in the culture broth during the growth of yeast and mycelium – form cells of *B. poitrasii* under two different conditions viz., YPG medium (0.5% glucose) at 28°C and YPG medium (0.5% glucose) at 37°C was determined at regular intervals of 3 h using the glucose estimation kit from Ranbaxy, India.

2.2.4 Production of chitosanase –rich cell wall lytic enzyme system

2.2.4.1 Screening of microorganisms for chitosanase production

Different bacterial and fungal cultures were tested for extracellular chitosanase production. The cultures were grown in a medium containing fungal cell walls as the sole carbon source under shaking condition (180 rpm) at 28°C for 96 h. The cell wall of yeast -form cells of *B. poitrasii* was isolated as described earlier (Khale and Deshpande 1992). The composition of the medium was (g/l): NH_4NO_3, 5; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; K_2HPO_4, 1; cell wall (wet wt.), 3. The pH of the medium was adjusted to 6.0.

2.2.4.2 Production of cell wall lytic enzymes

Streptomyces sp. MC1, a high chitosanase producer, was used for the production of cell wall lytic enzymes. Chitosanase production medium (10 ml) was inoculated with spores from 7 day old slant growth of *Streptomyces* sp. MC1 and was incubated under shaking condition (180 rpm) at 28°C for 24 h. This was used as the inoculum for 100ml medium and was incubated under shaking condition at 28°C for 24 h. The culture filtrate was centrifuged at 6800xg for 15 min. The supernatant was

concentrated 5 times by ultrafiltration using YM-3 membrane and was used for protoplast isolation.

2.2.5 Production of chitin synthase inhibitors

For the production of chitin synthase inhibitors, the cultures were grown in chitin synthase inhibitor production medium. The composition of the medium was (g/l): soyabean meal, 10; yeast extract, 10; mannitol, 15; starch, 5. The cultures were grown under shaking condition (180 rpm) at 28°C for 96 h. The supernatant was separated by centrifugation at 6800xg for 15 min and was used for further studies or otherwise stored at 8°C.

2.2.6 Growth conditions and harvesting of cells for isolation of total RNA from *B. poitrasii*

For RNA isolation, a seven-day-old sporulating slant culture was used. Spores (10^8) were inoculated in 50 ml YPG (0.5% glucose) and incubated under shaking condition (180 rpm) at 37°C for 24 h to obtain yeast-form cells which were used as an inoculum to obtain yeast and mycelium-form cells. Yeast-form cells were obtained by inoculating 10^8 yeast cells/50 ml YPG (0.5% glucose) under shaking condition (180 rpm) at 37°C for 72 h. The culture broth was collected at 6h, 24h and 72h time points. For obtaining mycelium-form cells grown in the absence of glucose, 10^5 yeast cells were inoculated in 50 ml YP medium and incubated under shaking condition (180 rpm) at 28°C for 24h. Samples were taken at 6h and 24 h. For obtaining mycelium-form cells grown in presence of glucose, 10^5 yeast cells were inoculated in 50 ml YPG (0.5% glucose) and were incubated under shaking condition (180 rpm) at 28°C for 24h. A single sample was collected at the end of 24h. Cells collected from each sample were then processed for RNA isolation.

The cells from the samples collected at each time point were harvested by centrifugation at 5800×g for 10 min. The cells were directly used for RNA isolation and if required for later use, were stored at -70°C.

2.2.7 Hyphal tip bursting test

To obtain actively growing tips, *B. poitrasii* spores were inoculated on YPG (1% glucose) agar plates and these plates were incubated at 28°C for 16-18h to obtain actively growing hyphal tips. Hyphal tip elongation of *B. poitrasii* was approximately 1.5div/ 2min. The culture filtrate (10µl) of the potential antifungal organism was added to the plates along with sorbitol (0.6M). The bursting of the hyphal tips was monitored microscopically up to 3 h. The hyphal tips that burst in a stipulated time were counted to find % HTB.

2.2.8 Isolation of protoplasts

The mycelial- and yeast- cells, grown as mentioned earlier, were separated and washed with potassium phosphate buffer (PB, 0.2 M, pH 5.8) containing KCl (0.6 M). The lytic enzyme mixture was filter- sterilized using a 0.45µ filter (Millipore Intertech Corporation Inc., USA). The washed mycelial biomass (250 mg wet weight) was incubated with 5 ml of the cell wall lytic enzyme mixture (containing 0.6 M KCl) in a 25 ml conical flask and was incubated on a reciprocating water-bath shaker (75 strokes/min) at 28°C for 5h.

Washed yeast cells (10^8 in 5 ml) were pretreated with a 10 mg /ml protease solution in PB for 30 min at 28 °C. The cells were then washed once with PBE (PB containing 2 mM EDTA) and then treated with a solution containing dithiothreitol (50 mM) and β-mercaptoethanol (10 mM) in the PBE at 37°C for 1h. The cells were washed 5 times with the PBE and then directly suspended in the cell wall lysing

enzyme mixture [*Streptomyces* sp. MC1 preparation (5 ml), mentioned above, plus Sigma lysing enzyme, 5mg /ml, zymolyase (2 mg /ml) and lyticase (2 mg /ml)] containing 0.6 M KCl.

The protoplasts were separated from the lysing enzyme by low speed centrifugation, at 500xg for 15 min. The protoplast pellet was then washed with PB containing 0.6 M KCl, centrifuged and was then resuspended in the same stabilized buffer. The integrity of the isolated protoplasts was checked by vital staining using 0.1% (w/v) eosin in a stabilized buffer and the yield was determined using haemocytometer.

2.2.9 Regeneration of protoplasts

The protoplast regeneration was studied on osmotically stabilised (0.6M KCl) YP and YPG (5% glucose) media solidified with agar (1.5%) at 28°C and 37°C. The effect of different concentrations of the inhibitors indicated appropriately in Section 3.2.2.3 (nikkomycin, tunicamycin, benanomicin A and B, phenyl methyl sulphonyl fluoride (PMSF), glucono-δ -lactone, verapamil, cycloheximide, H-7 and trifluoperazine) on protoplast regeneration was studied.

2.3 Biochemical methods

2.3.1 Isolation of cell wall

All the operations were conducted between 0-4°C. The cells were harvested on Whatman filter paper No.1 and then purity of samples was checked using light microscopy. The cells were washed and resuspended in cold distilled water and were broken by mechanical shaking with glass beads (0.45-0.5 mm) in a Braun homogeniser. Breakage of the 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 1500xg for

10 min and were washed in cold distilled water (5-6 times) until the supernatent became clear. The pellet was then resuspended in cold distilled water and cytoplasmic materials and membranes adhering to the cell walls were removed using the following two steps: the resuspended pellet was sonicated twice for 30 s each in an ice bath. After centrifugation at 1500xg for 10 min, the cell wall fragments were washed in cold sodium chloride (NaCl) with decreasing concentrations (5%, 2%, 1%) successively, twice each. A final wash was given in cold distilled water. The cell wall fraction was used in the medium on the wet weight basis.

2.3.2 Isolation and solubilisation of cell membrane

All the manipulations were carried out at 4°C, unless otherwise stated. Cells were harvested by centrifugation (3000 x g, 5 min). One gram fresh weight of the pellet was washed twice with TM (50 mM Tris-HCl, pH 7.5; 2.5mM $MgCl_2$) containing 1 mM EDTA, resuspended in TM buffer (1 ml for 250 mg wet weight) and homogenised in Braun's homogeniser (4 runs of 15 s each). The homogenate was centrifuged (2000xg, 5 min) to remove cell wall debris and then at 100000xg for 40 min to seperate the mixed membrane fraction and cytosol. The membrane pellet was resuspended in 250 µl TM buffer containing 30% glycerol. For digitonin solublisation, the mixed membrane fraction was resuspended in 1% digitonin in TM buffer, incubated at 0°C for 30 min, and centrifuged at 165000xg for 1h. The supernatent was separated and was used as the digitonin solubilised fraction.

2.3.3 Isolation of genomic DNA from *B. poitrasii*

Yeast cells of *B. poitrasii* were used for the isolation of genomic DNA. The yeast cells were harvested by filtration through Whatmann No. 1 paper and washed extensively with sterile water. The cells were immediately frozen in liquid N_2. The

frozen cells were stored at -80°C until use. The cells were crushed in liquid N_2 in mortar and pestle and then transferred (1g) to 10 ml of extraction buffer (Tris pH 8.0, 10 mM; NaCl 100 mM; Na-EDTA 1mM; SDS 1%; DTT 50 mM; β–ME 1%) and mixed by inversion 2-3 times. This was followed by three phenol: chloroform (1:1) and two chloroform extractions. The DNA was precipitated using 0.8 volumes of isopropanol. The pellet was then washed with absolute ethanol, air-dried and resuspended in TE (Tris pH 8.0, 10 mM; Na- EDTA 1 mM).

2.3.4 Plasmid DNA isolation from *E. coli*

2.3.4.1 Small scale plasmid DNA isolation

This method was used for rapid analysis of *E. coli* transformants and is a modification of the alkalline lysis method by Sambrook *et al.* (1989). Cells were grown for 16 h in 5 ml LB-ampicillin as described in Section 2.2.1. A 1.5 ml portion of culture was transferred into a 1.5 ml Eppendorf tube and spun down at 11600xg for 30 s in a microcentrifuge. The pellet was resuspended in 100 µl ice-cold solution consisting of 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0. After the addition of 200 µl freshly prepared 0.2 M NaOH, 1 % sodium dodecyl sulfate (SDS), the suspension was mixed by inversion and placed on ice. A 150 µl of stock solution containing 60 ml potassium acetate (5M), 11.5 ml glacial acetic acid and 28.5 ml distilled water was added and the tube vortexed in an inverted position for 10 s to disperse the solution through the viscous lysate. The tube was then kept on ice for 3-5 min. The lysate was spun at 11600xg for 5 min and the supernatant was transferred to a clean eppendorf tube. An equal volume of phenol/chloroform (1:1) saturated TE, was added after vortexing. The suspension was then centrifuged at 11600xg for 2 min. The aqueous phase was removed and the DNA was precipitated with two volumes of 96% ethanol at room temperature for 2 min. The DNA was pelleted by centrifugation

at 11600xg for 5 min. The pellet was washed with 70% ethanol and dried at 37°C for 10 min. The DNA was redissolved in 50 µl TE containing DNAase – free pancreatic RNAase (20 µg/ml).

2.3.4.2 Medium scale plasmid DNA isolation

In order to obtain plasmid DNA for sequencing and subcloning, QIAGEN columns (QIAGEN Ltd., Surrey, UK) were used. The protocol was adopted from the manufacturer's instructions. A QIAGEN tip-100 typically yields upto 100 µg supercoiled DNA for a high copy number plasmid from 50 ml LB culture. The cells were grown for 12-16 h as described in 2.2.1, harvested by centrifugation at 3000xg, 4°C for 10 min and resuspended in a solution containing 100 mg/ml RNase A, 50 mM Tris-HCl, 10 ml 200 mM NaOH, 1% SDS, mixed gently by inversion and incubated at room temperature for 5 min. The lysate was neutralised by the addition of 4 ml potassium acetate (3 M, pH 5.5) and left on ice for 15 min. To remove cell debris, the lysate was centrifuged at 30000xg for 30 min at 4°C. A QIAGEN tip-100 column was equilibrated by applying 4 ml 750 mM NaCl, 50 mM MOPS (3-N-morpholinopropanesulphonic acid), 15% ethanol, 0.15% Triton X-100, pH 7.0. The cleared lysate was then loaded onto the column and allowed to empty by gravity flow. The column was washed twice with 10 ml NaCl (1M), 50 mM MOPS, 15 % ethanol, pH 7.0 for the removal of any residual RNA from the resin and to disrupt non-specific interactions, if any. The DNA was eluted from the column with 5 ml NaCl (1.25M), 50 mM Tris-HCl, 15% ethanol pH 8.5, followed by precipitation with 3.5 ml isopropanol at room temperature. The precipitated DNA was centrifuged at 30000xg, 4°C for 30 min, and the pellet was resuspended in 400 µl TE. The resuspended DNA was transferred to a 1.5 ml Eppendorf microcentrifuge tube and reprecipitated by adding 0.1 volume sodium acetate (3M, pH 5.25) and 2 volumes of 95% ethanol. The

DNA was pelleted by centrifugation at 11600xg for 5 min at room temperature. The pellet was washed with 1ml 70 % ethanol and dried for 10 min at 37°C. Finally the pellet was dissolved in 100µl TE.

2.3.5 Isolation of total RNA from *B. poitrasii*

RNA was isolated according to method described by Brown (1994). The isolated RNA was treated with the RNAase-free DNAase from Promega as per the manufacturer's instructions, and was suspended in water and stored at -80° C till use. All the solutions and plastic-ware required for RNA isolation were made RNAase free by treating it with 0.1% diethyl pyrocarbonate (DEPC) overnight followed by autoclaving.

2.3.6 Polymerase chain reaction (PCR) and RT-PCR

Degenerate PCR primers directed towards conserved regions in chitin synthase were used for the PCR amplification of the genomic DNA. The primers were: **CSF (F1)**: 5'-CTG AAG CTT AZN ATG TAY AAY GAR GA-3'; **CS3F (F2)**: 5'-TGG GGA TCC GC5 WW5 CAR GT5 TTY GAR TA-3'; **CS3F2 (F3)**: 5'-TGG GGA TCC GC5 WW5 CAR GT5 TAY GAR TA-3'; **ZCHS1 (F4)**: 5'-GWA CWA TGM ATG GTG T5A TG-3'; **ZCHS2 (F5)**: 5'-ATY AAY TCH CAY CGY TGG-3'; **CSR (R1)**: 5'-GTT CTC GAG YTT RTA YTC RAA RTT YTC-3'; **CS3R (R2)**: 5'-ATA GAA TTC TT5 ATC CA5 CK5 CK5 CKY TG-3'. For amplification of class I-III chitin synthases, the primer sets F1and R1, F4 and R1, F5 and R1 were used. The primer set F1 and R1 was designed according to Bowen *et al.* (1992) while F4 and R1 was designed according to Miyazaki *et al.* (1997). The primer set F5 and R1 were designed for amplification of the conserved regions in the chitin synthases from zygomycetous fungi. For amplification of class IV-V chitin synthases primer sets F2 and R2, F3 and R2 were used. The primer set F2 and R2 was designed according to

Mellado *et al.* (Mellado *et al.,* 1995) , while the primer set F3 and R2 was designed according to Nino-Vega *et al.* (1998).

The PCR reactions were performed in 100µl volume containing reaction buffer (without $MgCl_2$), forward and reverse primers (1µM of each, obtained from Oswal, UK), 1.5 mM $MgCl_2$, 200µM of each dNTPs and 1 unit of Taq polymerase (all procured from Promega, UK) and 100-150 ng of genomic DNA. The amplification was carried out using the thermal cycler, programmed for 1 cycle of 94°C for 5 min, followed by 30 cycles each of 94°C for 30 s, 50°C for 1 min and 72°C for 2 min. This was followed by the final cycle of 72°C for 10 min. The products obtained at the end of the PCR reactions were run on the gel to determine the existence of the amplified fragments of the desired length.

Primers used for RT-PCR reactions were designed to be specific towards the cloned chitin synthase gene fragments. The primers were: **B1-F**: 5'-CTG ATG GTT GGA AAA AGG TC -3'; B1-R: 5'-CAC GAG CGG ATT CAG CAG CAG -3'; **B6-F**: 5'-CGT TCA AAA ACA TGG GGA GC-3'; **B6-R**: 5'-GAT TGA TAT CAA ATG ATT TC-3'; B28-F: 5'-GGA CCT ATT ATC CAG CCC-3'; **B28-R**: 5'-GCA GCT ACA AGC GGG TTA AG-3'; **B29-F**: 5'-CGT TGA TCC CCA TGT ATG-3'; **B29-R**: 5'-CAC TAA AGG ATT AAG AAG AG-3'; **A1-F**: 5'-GTA TCA AGG CCC CCA AGG GTC CCG-3'; **A1-R**: 5'-CAC CAT AAA AGT ATC TGG C-3'; **A2-F**: 5'-CAT CTC GCA TCA CCA GTC C-3'; **A2-R**: 5'-GTG TCT GGA ACC ACC GTC TTG-3'; **A29-F**: 5'-CGG GGG CAT TGG TCA TAC AGC-3'; **A29-R**: 5'-CGC ACC ATC TCG CCA AGG CC-3'; **A30-F**: 5'-CGC ATC ATT TGG CAA AAG GC-3'; **A30-R**: 5'-GCA AGA CTT TAA ATT CAT C-3'.

RT-PCR reactions were carried out using Ready-To-Go RT-PCR beads from Amersham (UK) and the reactions were carried out according to manufacturer's

instructions. Primer sets used for expression study of the individual gene were as follows: B1-F and B1-R for Bp*CHS*1, B6-F and B6-R for Bp*CHS*2, B28-F and B28-R for Bp*CHS*3, B29-F and B29-R for Bp*CHS*4, A1-F and A1-R for Bp*CHS*5, A2-F and A2-R for Bp*CHS*6, A29-F and A29-R for Bp*CHS*7and A30-F and A30-R for Bp*CHS*8. For each reaction 1µg of DNA free RNA was used and was converted to cDNA by reverse transciptase action, during first cycle of RT-PCR reaction. Amplification of the cDNA obtained was carried out using the PCR conditions described earlier in this Section. The amplification products were then run on the agarose gel and the expression levels of different genes were compared semi-quantitatively based upon the intensity of the bands observed after ethidium bromide staining of the gel.

2.3.7 Cloning of DNA

Cloning of the PCR amplified fragments was done in two steps: (a) ligation reaction and (b) transformation. Ligation reactions were performed at 4°C using T4 DNA ligase from Promega according to manufacturer's instructions. For transformation reactions, *E. coli* JM109 competent cells from Promega were used. The transformation reactions were also performed according to manufacturer's instructions. The transformed cells were plated on LB media containing X-gal (40 µg/ml), IPTG (0.5mM) and ampicillin (100 µg/ml) and incubated at 37°C for 24h. After incubation, white colonies on the plates were identified as those containing recombinant DNA.

2.3.8 Digestion of DNA with restriction enzymes

Restriction digestions were performed according to manufacturer's recommendations. Typically, 1-5 units of enzyme were used for digestion of 1 µg of DNA in a total volume of 30 µl at 37°C in 1 h. For digestion of large amount of DNA,

the reaction volume was scaled up proportionally, and digests were incubated at 37°C overnight.

2.3.9 Southern blot analysis

In order to analyse DNA (either genomic or cloned) by hybridization, it was first digested with a restriction enzyme. The digested DNA was then separated by agarose gel electrophoresis and visualised under ultraviolet light. The DNA was transferred to a nylon membrane (Hybond-N, Amersham International Plc.) by vacuum blotting. Vacuum transference was carried out using a VacuGene XL vacuum blotting system (Pharmacia LKB Biotechnologies, Herts, UK), at a pressure of 50 mbar and following a modification from the manufacturers conditions for the treatment of the gel. First, depurination solution (0.25 M HCl) was poured on the gel. After 20 min the solution was removed and the denaturation solution (1.5 M NaCl plus, 0.5 M NaOH) poured on the gel and left for 20 min. The denaturation solution was then removed and neutralized with a solution (1M Tris.HCl, 2M NaCl pH 5.0) and left for 20 min. Finally, the solution was removed and blotter chamber filled up with 20 X SSC (3M NaCl, 300 mM Tri-sodium citrate pH 7.5) and left for 8 h.

At the end of the transfer, positions of the wells were marked with a needle. The membrane was washed in 2 X SSC for 5 min and allowed to dry on a pad of filter paper for 30 min. The DNA was visualised on a UV Foto1/ Prep transilluminator and the positions of DNA molecular weight markers were noted. The DNA was then fixed by baking at 80°C for 2 h and used for hybridizations.

Southern blot analysis employed the standard methods (Sambrook *et al.*, 1989). Chromosomal DNA (1µg) was digested using 1U of different restriction enzymes. Six different restriction enzymes viz. Eco RI, PstI, Xba I, Xho I, Bam HI and Hind III were used separately. Approximately 20 µg of the restriction digests of

the chromosomal DNA were run on 1% agarose gels using TAE buffer (Sambrook *et al.*, 1989). The gels were then denatured and blotted onto nylon membranes (Hybond N; Amersham, UK). To obtain the probes for the Southern hybridisations, the fragments cloned in pGEM-T easy vector were treated with EcoRI. The product of the reaction was run on the gel and the small fragment generated was used as the probe after purifying it from the gel. The probes were labelled with [α-^{32}P] dCTP using Ready-To-Go DNA labeling beads following the manufacturer's instructions.

2.3.10 Southern hybridization

The hybridization procedure consisted of prehybridization and hybridization reactions followed by post-hybridization washes. The prehybrization mixture consisted of 0.5M Na_3PO_4 (pH 7.2), 1mM EDTA and 7% SDS. The prehybridization reactions were carried out at 65 °C for 3-4 h. The hybridization mix was prepared by adding radiolabelled probe to the fresh prehybridization mix. Hydridization was carried out overnight at 65 °C. Post-hybridization washes were carried out in three steps. These included, (a) 2 X SSC at 65 °C for 15 min, (b) 2 X SSC containing 0.1% SDS at 65 °C for 15 min and (c) 1 X SSC containing 0.1% SDS at 65 °C for 15 min.

The filters were sealed in polyethylene bags and exposed to KODAK x-ray film at –70 °C for 12-24 h. The film was then developed with KODAK M-35 X-Omat processor.

2.3.11 DNA sequencing reactions

The PCR products were cleaned using Wizard plus PCR purification system and products were cloned into pGEM-T easy vector system from Promega, according to manufacturer’s instructions. Plasmids were isolated from the clones containing the desired fragments using the Midi Prep Kit for plasmid isolation from Promega, UK. These plasmids were sequenced using the Big Dye Kit (Perkin Elmer, Worington,

U.K.) and run on an ABI 377 automated sequencer from Perkin - Elmer. The sequences obtained were then subjected to FASTA3 searches (Pearson, 1990) and were grouped into different categories based on the maximum homology shown to a known chitin synthase gene from the database.

2.3.12 Determination of protein

2.3.12.1 Lowry method

Protein concentration was determined using Folin-Ciocalteau reagent as described by Lowry *et al.* (1951). Crystalline bovine serum albumin, fraction V (Sigma Chemical Company, St. Louis, USA) was used as standard.

2.3.12.2 Optical method

The protein concentrations were determined by measuring the absorbance of the sample at 260nm, 280nm and 340nm. The protein concentrations (mg/ml) were determined using the formula:

$4/7[2.3(OD_{280nm}-OD_{340nm})-(OD_{260nm}-OD_{340nm})]$

2.3.13 Determination of nucleic acid

2.3.13.1 Optical method

Absorbance measurements were done at 260 nm in a spectrophotometer (LKB Biochem Ltd, Cambridge, UK), using a 1 cm quartz cuvette. For DNA samples, the concentration was estimated assuming that $A_{260} = 1$ corresponds to 50 µg/ml of double stranded DNA. For RNA samples, $A_{260} = 1$ was assumed to correspond to 40 mg /ml (Sambrook *et al.*, 1989).

2.3.13.2 Ethidium bromide method

For DNA samples containing either low concentrations of DNA (< 50 ng /ml) or impurities, concentration was estimated by agarose gel electrophoresis (Section 2.4.5) and compared with DNA molecular weight markers of known concentration.

2.3.14 Determination of glucose

Inoculum was grown as mentioned in the Section 2.2.3 and cells were inoculated in YPG (0.5% glucose) and incubated at 28°C and 37°C. The samples were taken at 0,3,6,9,12,24h for estimation of unutilised glucose of the culture broth during the growth of yeast and mycelium – form cells in YPG (0.5% glucose). Glucose concentration was determined using the glucose estimation kit from Ranbaxy Laboratories, India. The working solution was prepared by dissolving 0.4g of enzyme powder in 100 ml distilled water and adding 1.7 ml phenol before use. The assay was run by taking 1ml of working solution and 10µl of either blank, standard or test sample was added to it. The same was mixed and incubated at RT for 30 min. The absorbance was read at 505nm.

2.3.15 Enzyme assays

2.3.15.1 Chitinase

A reaction mixture containing 1ml phosphoric acid swollen chitin (7 mg /ml), 1 ml of 0.05M acetate buffer, pH 5.0 and 1 ml of suitably diluted enzyme was incubated at 50 °C for 1h. Reducing sugars were estimated as *N*- acetylglucosamine equivalents by Somogyi-Nelson method (Nelson, 1944; Somogyi, 1952). One international unit of enzyme is defined as the amount of enzyme that produces 1µmole of the product per minute under the assay conditions.

2.3.15.2 Chitosanase

The chitosanase activity was estimated using acid swollen chitosan as the substrate. The reaction mixture contained: 1 ml acid swollen chitosan (10 mg /ml), 1 ml acetate buffer (50 mM, pH 5.0) and 1 ml of the suitably diluted enzyme. The reaction was carried out at 50° C for 1h. The reducing sugars were estimated as glucosamine equivalent using the Somogyi – Nelson method (Nelson, 1944; Somogyi,

1952). One international unit of enzyme activity was defined as the amount of the enzyme that liberated 1µ mole of glucosamine per min.

2.3.15.3 β-1, 3-Glucanase

An aliquot of enzyme solution (0.5 ml) was mixed with 0.5 ml of 1% laminarin prepared in 0.05M acetate buffer, pH 5.0 and incubated at 50^0C for 30 min. Reducing sugars were estimated as glucose equivalents by Somogyi-Nelson method (Nelson, 1944; Somogyi, 1952). One international unit of enzyme activity was defined as the amount of enzyme that liberated 1µmole of glucose per minute from laminarin under above conditions.

2.3.15.4 Mannanase

Mannanase activity was measured using modified method of McCleary (1978). The activity was measured using yeast mannan as the substrate. Suitably diluted enzyme (0.5 ml) was mixed with 0.5 ml of 1% (w/v) yeast mannan prepared in potassium phosphate buffer (0.2M, pH 5.8) and was incubated at 50°C for 1h. The reducing sugar liberated in the reaction was estimated using the Somogyi-Nelson method (Nelson, 1944; Somogyi, 1952). One international unit of enzyme activity was defined as the amount of enzyme that liberated 1µmole of mannose per minute from laminarin under above conditions.

2.3.15.5 Protease

Protease activity was measured using Hammerstein casein as a substrate. The reaction mixture (1ml) contained an aliquot of suitably diluted enzyme solution, 10mg Hammerstein casein and 0.2M of sodium carbonate buffer, pH 9.7. The reaction was carried out at 40 ^{0}C for 10 min and was terminated by the addition of 3 ml TCA (2.6 ml, 5% w/v TCA plus 0.4 ml, 3.3N HCl). The absorbance of the TCA soluble fraction was measured at 280 nm. One internationalunit of enzyme activity was equivalent to

that amount of enzyme that liberated 1µmole of tyrosine per minute under assay conditions.

2.3.15.6 Chitin synthase

Each assay mixture was 50 µl containing 5 µl of enzyme preparation and final concentration of Tris-HCl (50 mM, pH8.0), $MgCl_2$ (10 mM), *N*-acetylglucosamine (25mM), UDP-^{14}C-GlcNAc (1mM) containing 25nCi UDP-^{14}C-GlcNAc. The assay mixture was incubated at 37°C for 1h and the reaction was terminated by the addition of 66% (v/v) ethanol. The assay mixture was filtered through glass microfibre filters (presoaked with 10% TCA; Whatman GF/C, 2.5 cm) on a Millipore Manifold system. The reaction tubes washed twice with 1 ml 1% Triton X-100 and the filters were washed with 4 ml 66% ethanol. The filters were dried at 80°C for 8h and transferred to scintillation vials containing 3 ml scintillation fluid (4 g PPO (2,5-diphenyloxazole) and 0.1 g POPOP 91,4-bis[5-phenyl-2-oxazoyl]-benzene) in 1000 ml toluene) for radioactive counting. The total and specific activities were calculated as described earlier (Gooday and de Rousset-Hall, 1975). Chitin synthase activity was expressed as pkat and is defined as the amount of enzyme that produces 1pmole of the product per second under the assay conditions. The results showed variation in the specific activities between experiments. The data presented are therefore the average of three sets of triplicate experiments, unless otherwise stated.

2.4 Analytical methods

2.4.1 Identification of *Streptomyces* sp.

The diaminopimelic acid isomers in the cell wall and the whole-cell hydrolysates were analyzed by TLC. The identification of *Streptomyces* sp. was done according to Staneck and Roberts (1974).

2.4.2 Fluorescence microscopy

Fluorescent marker, fluorescein isothiocyanate (FITC), tagged with lectins specific to cell wall polymers was used to confirm the synthesis of the cell wall during regeneration. FITC-WGA (0.5 mg/ml in 0.6 M $CaCl_2$ in 20mM Tris-HCl buffer, pH 7.0) and calcofluor white (0.05 mg/ml in 150mM NaCl in 20mM Tris-HCl buffer, pH 7.0) were used for staining as described by Mormeneo *et al.* (1996). The stained cells were incubated in dark for 30 min and then washed with buffer to remove excess stain. The cells stained with FITC-labelled lectin were observed under the I3 filter with an excitation range of 450-490 nm. A-filter was used to observe cells stained with calcofluor white with an excitation range of 340-380 nm. The fluorescence was observed by epifluorescence microscope (Leitz Labor Lux, Germany). A Wild MPS 32 camera unit was used to record fluorescence and LM photographs.

2.4.3 Vital staining

The viability of the isolated protoplasts was determined using 1% eosin solution.

2.4.4 Isoelectric focusing

For isoelectric focusing for 1% digitonin solubilised membrane pellet of mycelium and yeast cells was used. The isoelectric focussing was carried out using preparative isoelectric focusing unit from Bio-Rad, using ampholytes in the pH range of 3-10. The isoelectric focussing was carried out till the current observed reached a constant value. At the end of focusing, 20 fractions of 3ml each were collected Chitin synthase activity and pH for each fraction was determined.

2.4.5 Agarose gel electrophoresis

Agarose gels were prepared by melting agarose in TAE buffer (Tris-acetate, 40 mM; EDTA, 1mM; pH 8.0). To cast and run the gel, Bio-Rad Mini, Midi or Maxi

electrophoresis cells were used (Bio-Rad laboratories Ltd, Herts, UK). These were connected to a Pharmacia LKB GPS 200/400 power supply (Pharmacia Biotechnologies Ltd, Herts, UK). The concentration of agarose varied according to the size range of DNA molecules being separated. These were: uncut DNA, greater than 8kb (0.5-0.8%); 8–0.5 kb (0.8–1.2%); 2–0.4 kb (1.2 – 1.6%) and less than 1kb fragment (2%).

The solidified gel was placed into the electrophoresis cell. TAE buffer was poured onto the gel upto approximately 5 mm over the gel. DNA samples were loaded into the wells after being mixed with 10 % (v/v) gel loading buffer (bromophenol blue, 0.25%; xylene cyanol, 0.25%; Ficoll -type 400, 15%). As molecular weight markers, 200 ng of a HindIII digest of bacteriophage lambda DNA (Promega) was used. DNA samples were electrophoresed through the gel at 5 V/cm for 2 h and stained in a bath with ethidium bromide (5µg/ml, 15 min). The DNA was visualised under ultraviolet light on a Foto/Prep 1 transluminator.

2.4.6 Formaldehyde gel electrophoresis

RNA samples were checked for integrity using formaldehyde gel electrophoresis. Total RNA was denatured by mixing with 3 times the MMF solution (formamide, 50%; formaldehyde, 6%; 1 X MOPS (3-N-morpholinopropanesulphonic acid, 20 mM; sodium acetate, 5mM; EDTA, 1mM; pH7.0); 0.8 µg /ml ethidium bromide). The mixture was heated at 65°C for 15 min and put on ice. Formaldehyde loading buffer (10X) was added before loading onto a 25 cm length, 1.2% agarose gel containing 1X MOPS with recirculation of the buffer by a peristaltic pump at 1.25 V/cm for 20 h. After electrophoresis, RNA was visualised under UV and a photograph of the gel was taken.

2.4.7 Analysis of DNA sequence data

DNA and deduced amino acid sequences were analysed using the Genetics Computer Group package (Madison, WI, USA). Protein homologies were identified using the FASTA3 program (www2.ebi.ac.uk/fasta3) and phylogenetic trees were constructed by the neighbour joining method included in the Clustal W program (Thompson *et al.,* 1994).

2.4.8 Phylogenetic analysis

Phylogenetic analyses were performed by using the deduced amino acid sequences of PCR fragments corresponding to eight *B. poitrasii* CHS genes identified in this study. They were compared to the amino acid sequences of several fungal chitin synthases retrieved from Gen Bank Database. The analyses were carried out with the computer programs available in the Phylogeny Inference Package (PHYLIP) (version 3.5p by Felsenstein, 1993).

The analysis was done by the bootstrap method using parsimony. Bootstrapping is a statistical method used to evaluate the confidence level of the phylogenetic estimate by random sampling of the data (Felsenstein, 1985). A total of 1000 bootstrap replicates for each group were obtained using the computer program "seqboot" (Felsenstein, 1993). A phylogeny estimate for each replicate was obtained by the program "Protpars" (Felsenstein, 1993) which infers an unrooted phylogeny from protein sequences by parsimony using a method intermediate between the approaches of Eck and Dayhoff (1966) and Fitch (1971). This method counts only those nucleotide changes that change the amino acid, on the assumption that silent changes (those that do not change the amino acid) are more easily accomplished. A consensus tree was obtained for out of the 1000 phylogenies using the program "Consense" (Felsenstein, 1993) which finds the majority rule consensus tree.

Chapter 3: Isolation and regeneration of protoplasts from *Benjaminiella poitrasii*

3.1 Chitosanase-rich enzyme lytic preparation for protoplastation

3.1.1 Introduction

There has been significant interest in the literature in experiments involving protoplasts (cell wall-less structures bound by cell membrane) of fungi (Section 1.3). Protoplasts have been used in the strain improvement studies (Peberdy, 1990), cell wall structure analysis (Wessels *et al.*, 1990) and studies on the contribution of cell wall architecture in morphogenesis (Section 3.2). In all these experiments, an essential prerequisite is to be able to obtain viable protoplasts in sufficient numbers. This requires an appropriate lysing enzyme system.

In most fungi, isolation of protoplasts (protoplastation) is achievable easily using commercially available enzyme preparations which are rich in chitinase and β-1,3-glucanase activities (Section 1.3). However the same preparations often do not yield protoplasts for zygomycetous fungi (Section 1.3). This is because the cell wall of zygomycetous fungi contains more amount of deacetylated chitin, chitosan, as the skeletal component (Bartnicki - Garcia, 1968).

In this Section, the production and characterization of a chitosanase-rich cell wall lytic enzyme preparation which was used to produce protoplasts of three different zygomycetous fungi viz., *B. poitrasii*, *Rhizopus stolonifer*, and *Conidiobolus coronatus* has been reported *B. poitrasii* was chosen since it was the model system for studies on dimorphism. *Conidiobolus* sp and *Rhizopus* sp are useful due to their commercial importance. Further more, the cell walls of *B. poitrasii* and *Rhizopus* sp. show significant differences in concentrations of chitin and non-aminopolysaccharide polymers.

3.1.2 Results

3.1.2.1 Screening for chitosanase producers and production of lysing enzyme mixture

The chitosanase activity of the commercial enzymes, viz. Sigma lysing enzyme, Zymolyase and Lyticase was tested. The chitosanase and other hydrolytic enzyme activities of these enzyme preparations are shown in Table 3.1. While Zymolyase and Lyticase did not show any detectable chitosanase activity, Sigma lysing enzyme showed the presence of chitosanase activity ($5.49 X 10^{-4}$ IU/ mg protein).

Table 3.1 Hydrolytic enzyme activities of commercial lysing enzyme preparations

Lysing Enyme Preparation	Hydrolytic enzyme activities (IU/mg protein)				
	Chitinase	Chitosanase	β-1,3-Glucanase	Mannanase	Proteinase
Sigma Lysing Enzyme	5.49×10^{-4}	1.2	3.26×10^{-2}	3.2×10^{3}	1.23×10^{-1}
Zymolyase	ND	2×10^{-2}	1.53	4.1×10^{1}	ND
Lyticase	ND	ND	1.1	2.7×10^{2}	2.3×10^{3}

ND, not detected

Bacterial cultures (*Bacillus* sp1, *Bacillus* sp2 and actinomycetes) and a fungal culture (*Myrothecium verrucaria*) were tested for chitosanase production. Among the tested isolates, actinomycete culture MC1 showed high levels of chitosanase production (Table 3.2). As compared to the Sigma lysing enzyme MC1 produced almost 2000 times more specific chitosanase activity.

The MC1 strain formed spores in long chains. The *meso*-diaminopimelic acid was the predominant isomer in the cell walls of the aerial mycelium and galactose was

Table 3.2 Screening of microorganisms for extracellular chitosanase producers

Culture[1]	Chitosanase activity (IU/ml)
Streptomyces sp MC1	0.2160
Bacillus sp1	0.0040
Bacillus sp 3	0.0012
Myrothecium verrucaria	0.0065

[1] All the cultures were grown as described under Materials and Methods

the predominant sugar in the whole cell hydrolysate. The MC1 strain therefore has been identified as *Streptomyces* sp. *Streptomyces* No 6 was used for protoplastation of zygomycetous fungi (Peberdy, 1989; Ramirez-Leon and Ruiz-Herrera, 1972). However, the chitosanase production of *Streptomyces* No 6 on *B. poitrasii* cell wall was 2.7 times lower than *Streptomyces* sp MC1.

3.1.2.2 Properties of chitosanase-rich lysing enzyme mixture

The other hydrolytic enzyme activities in the chitosanase-rich lysing enzyme preparation were also measured (Table 3.3).

Properties of the enzyme preparation such as stability, optimum pH, optimum temperature and the isoelectric point were also measured and are shown in Table 3.3. The enzyme preparation was found to be stable for 12h at 28 °C and retained 95% of the initial chitosanase activity. The preparation was stable at 4°C. It retained > 90 % of the initial activity upto 15 days.

Table 3.3: Properties of chitosanase-rich lytic enzyme mixture prepared using Streptomyces sp. MC1

Enzymes	Specific activity, IU/mg
• Chitosanase	1.074 ± 0.4
• Chitinase	0.42 ± 0.06
• Protease	0.012 ± 0.003
• β-1,3-Glucanase	0.0053 ± 0.0012
• Mannanase	0.0045 ± 0.0009
Biochemical characteristics of chitosanase preparation	
• Optimum pH	5.0
• Optimum temperature, °C	50°C
• Isoelectric point	8.5
• Stability	
o at 4°C after 15 days	90% (% residual activity)
o at 28°C after 12 h	95% (% residual activity)

3.1.2.3 Use in protoplastation of zygomycetous fungi

The chitosanase-rich lysing enzyme system was then tested for its use in protoplastation of mycelium-form cells of three members of zygomycetes viz., *B. poitrasii*, *Conidiobolus* sp. and *Rhizopus* sp. Optimal protoplast yield was obtained at 28°C after 5h incubation for *B. poitrasii* when 0.6 M KCl was used as the osmotic stabiliser. For *Conidiobolus* sp and *Rhizopus* sp, the optimal protoplast yield were obtained at 28°C after 5h incubation when 0.6 M $MgSO_4$ and 0.6M NaCl, respectively were used as the osmotic stabilizers. The isolation frequencies for these three organisms in the presence of different osmotic stabilisers are as shown in the Table 3.4.

Table 3.4 Effect of the osmotic stabilizer on the isolation of protoplast for different organisms

Name	Yield (Number of protoplasts/ml)		
	0.6M NaCl	0.6M KCl	0.6M $MgSO_4$
Benjaminiella poitrasii	$1 (\pm 0.1) \times 10^{2}$	$5 (\pm 0.4) \times 10^{4}$	$1 (\pm 0.4) \times 10^{3}$
Conidiobolus sp.	$8 (\pm 1.8) \times 10^{4}$	$4 (\pm 0.9) \times 10^{4}$	$1 (\pm 0.3) \times 10^{5}$
Rhizopus sp.	$6 (\pm 1.2) \times 10^{4}$	$2 (\pm 0.4) \times 10^{4}$	$3 (\pm 0.6) \times 10^{4}$

3.1.3 Discussion

Chitosan is an important constituent cell wall polymer in zygomycetes. Furthermore, there is an evidence in the literature that chitosan in the cell walls of zygomycetes needs to be degraded in order to release protoplasts (Reyes *et al*, 1984). Therefore, any lysing enzyme system for protoplastation of members of the zygomycetes fungal class needs to have chitosanase as one of its constituents.

In the literature, *Streptomyces* sp. (an actinomycete) was used most commonly as the producer organism of the lysing enzyme preparation for protoplastation of zygomycetes (Peberdy, 1989). *Trichoderma* sp, *Pencillium* sp and *Myxobacter* sp were used for the purpose. *M. verrucaria* has been shown in the past to be a good producer of chitinase (Vyas and Deshpande, 1989). In this context, bacterial cultures (*Bacillus* sp1, *Bacillus* sp2), a fungal culture (*M. verrucaria*) and a soil actinomycete isolate were screened for chitosanase production. The actinomycete isolate, later identified as St*reptomyces* sp MC1, was found to be a better producer of chitosanase (Table 3.2).

The properties of chitosanase in several preparations produced using various organisms have been reviewed by Somashekar and Joseph (1996). The optimum pH and temperatures for chitosanase from *Streptomyces* No 6 (Somashekar and Joseph,

1996) and *Streptomyces* sp. MC1 are similar. The chitosanase activity in the preparation from *Streptomyces* sp. MC1 was found to be 2.7 times higher than that for *Streptomyces* No.6 under identical conditions of growth.

A chitosanase-rich lysing enzyme mixture was then prepared using the extracellular culture broth of *Streptomyces* sp MC1 grown in a medium containing the cell walls of *B. poitrasii* yeast form cell walls. Apart from chitosanase, the lysing enzyme preparation also showed significant chitinase activity and lower β-glucanase, mannanase and proteinase activities. This combination of lysing enzymes was found to be useful for protoplastation of different zygomycetous fungi - *B. poitrasii*, *Conidiobolus* sp and *Rhizopus* sp. Thus, a lysing enzyme preparation that is successful in protoplastation of these three organisms may have broader applicability to other useful zygomycetes as well.

The chitosanase-rich lysing enzyme preparation prepared in this work was found to successfully release protoplasts from all three organisms. The enzyme preparation was also found to be stable at 28° C. The stability of the preparation might be an asset while developing the preparation on a commercial scale.

3.2 Isolation and regeneration of protoplasts in *Benjaminiella poitrasii*

3.2.1 Introduction

The morphology of fungi is decided mainly by their cell wall components, such as chitin, glucan, mannan, and protein and their arrangement and the sequence of their synthesis with respect to each other (Khale and Deshpande, 1992; Peberdy, 1989). Role of proteins and actin cytoskeleton in the cell wall organisation in *Saccharomyces cerevisiae* has been extensively studied (Kaptyen *et al.*, 1999; Gabriel and Kopecka, 1995) and the role of cell wall assembly in fungal morphogenesis has been known (Sietsma and Wessels, 1991). For instance, Pardo *et al.* (1999) studied the pattern of protein secretion during protoplast regeneration in *Saccharomyces cerevisiae* to understand the mechanism of cell wall synthesis. Protoplast regeneration was used as an approach to understand the synthesis of cell wall components under different conditions and the studies are mainly carried out with respect to the chemical composition. (Elorza *et al.*, 1983). However the relative importance of individual cell wall components in the ultimate morphology is still not clear. In this regard, one of the approaches that can be used is protoplast regeneration and microscopic observations during protoplast regeneration to understand the morphogenesis process (Woestemeyer and Woestemeyer, 1998). The isolation of the protoplasts from the mycelial- and yeast- form cells of dimorphic fungi such as *Mucor rouxii* (Reyes *et al.*, 1983) and *C. albicans* (Elorza *et al.*, 1983) were reported but adequate studies were not carried out to understand the dimorphism, *per se.*

Protoplast isolation technique is being followed since a very long time but it has mainly remained an empirical method and therefore, the standardization with a new system is always necessary (Woestemeyer and Woestemeyer, 1998). The present

investigations deal with the isolation of protoplasts from the yeast and mycelial cells of *B. poitrasii* and their regeneration under dimorphism triggering conditions. Attempts were made to identify the relative importance of the different components of the cell wall in determining the morphological outcome during protoplast regeneration in the presence of different enzyme inhibitors.

3.2.2 Results

3.2.2.1 Isolation of protoplasts from mycelium – form cells and yeast-form cells of *B. poitrasii*

The extracellular broth of *Streptomyces* MC1 grown on *B. poitrasii* yeast-form cell walls was used singly or in combination with commercial enzymes for protoplast isolation from the *B. poitrasii* mycelium and yeast cells. For optimum yeast cell protoplastation, pretreatment with protease and a mixture of the reducing agents, such as BME and DTT was found to be essential. Further optimization studies to identify suitable osmotic stabilizers, incubation conditions and age of the culture were carried out.

Among the inorganic stabilizers tested (Table 3.5), the yields of protoplasts from both, mycelium and yeast, in the presence of 0.6 M NaCl (1130 mOs) were low ($1 \pm 0.1 \times 10^2$ /ml and $2 \pm 0.2 \times 10^3$ /ml, respectively). Though the mycelial protoplast yield was low ($1 \pm 0.4 \times 10^3$ /ml), 0.6M $MgSO_4$ (645 mOs) supported protoplastation of the yeast-form cells ($5 \pm 0.2 \times 10^4$ /ml). The 0.6 M KCl (1120 mOs) was useful to obtain protoplasts from yeast ($4 \pm 0.3 \times 10^4$ /ml) as well as mycelium – form cells ($5 \pm 0.4 \times 10^4$ /ml). However, the mixture of 0.48 M KCl and 0.12 M $MgSO_4$ (1025 mOs of the mixture) gave the maximum protoplast yield of $2 \pm 0.3 \times 10^6$ /ml and $3 \pm 0.4 \times 10^7$ /ml for both mycelial and yeast cells, respectively. The organic stabilizers *viz.* 0.6 M sucrose (740 mOs) or sorbitol (754 mOs) did not support protoplast formation from

Table 3.5: Effect of the osmotic stabilizer on the isolation of protoplasts of *B. poitrasii.*

Osmotic stabiliser	Yield (Number of protoplasts/ml)	
	Mycelium cells	Yeast cells
NaCl, 0.6 M	$1\pm0.1 \times 10^2$	$2\pm0.2 \times 10^3$
KCl, 0.6 M	$5\pm0.4 \times 10^4$	$4\pm0.3 \times 10^4$
$MgSO_4$, 0.6 M	$1\pm0.4 \times 10^3$	$5\pm0.2 \times 10^4$
KCl, 0.48M + $MgSO_4$, 0.12M	$2\pm0.3 \times 10^6$	$3\pm0.4 \times 10^7$
Sorbitol, 0.6 M	ND	ND
Sucrose, 0.6 M	ND	ND

ND, not detected

either of the cells. For further protoplast isolation experiments, the mixture of KCl (0.48 M) and $MgSO_4$ (0.12M) was used.

Protoplast yields were checked at different time intervals (Table 3.6). The maximum yields ($2 \pm 0.3 \times 10^6$ /ml and $3 \pm 0.4 \times 10^7$ /ml for mycelial and yeast cells, respectively) were observed after 5h incubation. The yields were 2-6 times less after 10 h of incubation. The age of the fungus also affected protoplastation. Optimum yields were obtained for the 16h old cells. Though the 24h old mycelium and yeast-form cells showed appreciable protoplast formation, the older cells were found to be resistant to enzymatic hydrolysis (data not shown).

Table 3.6: Optimization of the incubation time for the protoplastation

Incubation time (h)	Yield (Number of protoplasts/ml)	
	Mycelium	Yeast cells
1	$1 \pm 0.2 \times 10^2$	$7 \pm 0.3 \times 10^2$
2	$5 \pm 0.4 \times 10^2$	$6 \pm 0.3 \times 10^3$
3	$2 \pm 0.2 \times 10^3$	$8 \pm 0.5 \times 10^4$
4	$6 \pm 0.4 \times 10^4$	$9 \pm 0.4 \times 10^5$
5	$2 \pm 0.3 \times 10^6$	$3 \pm 0.4 \times 10^7$
10	$1 \pm 0.2 \times 10^6$	$5 \pm 0.3 \times 10^6$

3.2.2.2 Regeneration of protoplasts under mycelium and yeast favoring conditions

In both cases, protoplasts regenerated into an irregular mass for the first 10-12h irrespective of the incubation conditions (Figure 3.1a, c, e, g). Staining with calcofluor white (non-specific fluorescent dye for polysaccharides; data not shown) and fluorescein isothiocyanate-wheat germ agglutinin (FITC-WGA, *N*-acetylglucosamine specific fluorescent dye) revealed that the irregular mass possessed cell wall synthesized in non-regulated manner (Figure 3.2a,b). In response to the incubation conditions, *viz*. YP, 28 °C (mycelium favoring) and YPG, 37 °C (yeast favouring), this mass further developed into mycelial or yeast colonies, respectively in 36 h (Figure. 3.1b, d, f, h).

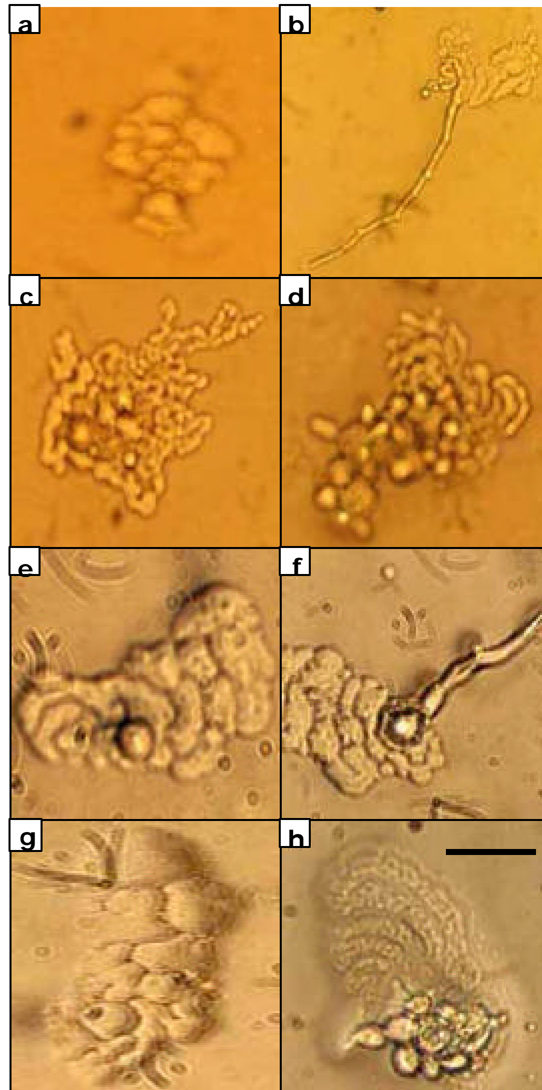


Figure 3.1: Regeneration of protoplasts of *Benjaminiella poitrasii*. (a) Mycelial-form cells, YP, 28° C, 12 h; (b) Mycelial- form cells, YP, 28° C, 36 h; (c) Mycelial-form cells, YPG (5% glucose), 37°C,12 h; (d) Mycelial- form cells, YPG (5% glucose), 37° C, 36 h; (e)Yeast- form cells, YP, 28° C, 12 h; (f) Yeast- form cells, YP, 28° C, 36 h; (g) Yeast-form cells, YPG (5% glucose), 37° C,12 h. (h) Yeast- form cells, YPG (5% glucose), 37° C, 36 h. All the photographs taken at 250X magnification. The bar corresponds to 50 μ.

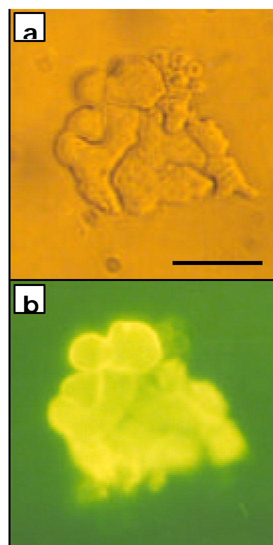


Figure 3.2: Staining of the initial irregular mass formed (12h growth) during protoplast regeneration using FITC-WGA (a)Light microscopy (b)Fluorescence microscopy. Photographs taken at 250X magnification. The bar corresponds to 50 µm

3.2.2.3 Effect of different inhibitors on the regeneration of mycelial protoplasts of *B. poitrasii*

As protoplasts from both types of cells behaved in a similar manner during regeneration (Figure 3.1), the effect of different inhibitors was studied using mycelial protoplasts. The concentrations of the inhibitors were such that they did not affect growth but delayed yeast-mycelium transition. The inhibitors which affect signal transduction such as verapamil (0.2-8 mM, Ca^{++} channel blocker), trifluoroperazine (5-20 µM, Ca-calmodulin inhibitor) and H-7 (30-70 µg, cAMP-dependent protein kinase inhibitor) did not show any appreciable effect on the morphological outcome during regeneration after 36 h. In the presence of cycloheximide (protein synthesis inhibitor) the irregular mass did not differentiate into either of the forms up to 36 h (Figure. 3.3 a, b) as observed in the respective media (Figure 3.1 b, d) (Table 3.7). The presence of nikkomycin (chitin synthase inhibitor) affected

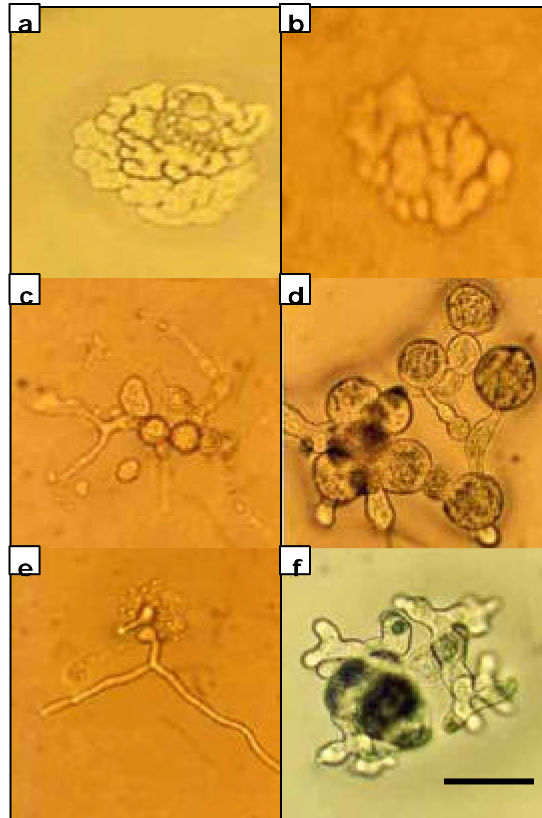


Figure 3.3. Regeneration of mycelial protoplasts of *Benjaminiella poitrasii* in the presence of inhibitors. (a) YP, cycloheximide (3-20 µM), 28° C, 36 h; (b) YPG (5% glucose), cycloheximide (3-20 µM), 37° C, 36 h; (c) YP, nikkomycin (5-20 µM), 28° C, 36 h; (d) YPG (5% glucose), nikkomycin (5-20 µM), 37° C, 36 h; (e) YP, PMSF (0.5-5.5 mM), 28° C, 36 h; (f) YPG (5% glucose), PMSF (0.5-5.5 mM), 37° C, 36 h,. All the photographs taken at 250X magnification. The bar corresponds to 50 µ..

Table 3.7. Effect of different cell wall metabolism inhibitors on the regeneration of mycelial protoplasts of *B. poitrasii*[1,2]

Inhibitor	Morphological outcome after 36 h			
	28°C		37°C	
	YP	YPG	YP	YPG
None	M	Y	M	Y
Cycloheximide, 3-20µ M	I	I	I	I
Nikkomycin, 5-20 µM	D	Y	D	Y
Tunicamycin, 2-50µM	M	Y	M	Y
Benanomycin A, 3-30µM	M	Y	M	Y
Benanomycin B, 1-15 µM	M	Y	M	Y
Phenyl methyl sulphonyl fluoride, 0.5-5.5 mM	M	D	M	D
Glucono-δ-lactone, 2-30 mM	Y	Y	Y	Y

[1] The inhibitor was present in 75 µl of the osmotically stabilised PB. The inhibitor solution was applied to the microscopic slide (7.5 x 2.5 cm) coated with 2 ml regeneration medium as indicated. (YP or YPG(5% glucose))and kept at room temperature for 15 min. The mycelial protoplasts (80-100) were used per slide for regeneration.

[2]Abreviations: Y: Yeast cells, M: Mycelium, NR: No Regeneration within 36h, I: Irregular mass, D: Deformed cells

protoplast regeneration under mycelium favoring conditions (Figure. 3.3c) but not under yeast favoring conditions (Figure. 3.3d). Other inhibitors that affect cell wall polysaccharide synthesis, directly or indirectly, tunicamycin (inhibitor of *N*-glycosylation of proteins) and benanomicin A and B (mannan synthase inhibitors) did not affect the regeneration process (Table 3.7). The addition of phenyl methyl sulfonyl fluoride (PMSF, serine proteinase inhibitor) added during regeneration did not affect protoplast regeneration under mycelium favoring conditions (Figure 3.3e), but deformed growth was observed under yeast favoring conditions (Figure 3.3f). Interestingly, in the presence of glucono-δ-lactone (glycosidase inhibitor) only yeast-type colonies were developed even under the mycelium favoring condition (Table 3.7).

3.2.3 Discussion

In the literature, protoplasts from zygomycetous fungi like *M. rouxii* (Reyes *et al.*, 1983), *M. rouxii* IM 80 (Ramirez-Leon and Ruiz- Herrera, 1972), *M. racemosus* (Laska and Borgia, 1980), *Mortierella ramanniana* (Matsunobu *et al.*, 1996), *Rhizopus nigricans* (Gabriel, 1968) and *Phycomyces* sp (Binding and Weber, 1974) were obtained using enzymes from different sources showing appreciable chitosanase activity. For protoplast isolation from *B. poitrasii* different commercial preparations were tested, but none of them was suitable for obtaining high yields. The *Streptomyces* sp. MC1, used in the present studies, produced high chitosanase activity and appreciable levels of other cell wall hydrolytic enzyme activities, too.

The quantitative differences in the cell wall components, such as chitin/chitosan and the matrix component, mannan of yeast and mycelial forms of dimorphic fungi were well documented (Gow, 1995; Sypherd *et al.*, 1978). In the case of *B. poitrasii*, mannan and protein contents are reported to be higher in the yeast cells

as compared to mycelial cells (Khale and Deshpande, 1992). Therefore, to isolate protoplasts from yeast-form cells pretreatment with protease, DTT and BME, and supplementation of the lysing enzyme system with protease and mannanase rich commercial preparations was found to be necessary.

It has been reported that organic compounds, such as sugars and sugar alcohols, stabilize yeast protoplastation and inorganic stabilizers favour protoplastation in the filamentous fungi (Davis, 1985). Protoplastation of both yeast and mycelium -form cells of *C. albicans* was favoured in the presence 1.0 M mannitol (Braun and Calderone, 1978). However, protoplastation of germ tube forming yeast cells of *C. albicans* (Elorza *et al.*, 1983) and yeast and mycelial forms of *M. rouxii* (Reyes *et al.*, 1983) with high yields in the presence of inorganic stabilizer (0.6 M KCl) were also reported. Similarly, in the case of *B. poitrasii*, 0.6 M KCl or a mixture of 0.48 M KCl plus 0.12 M $MgSO_4$ was suitable for isolation of the protoplasts from mycelium- as well as yeast-form cells (Table 3.5). The osmolality of the inorganic as well as organic stabilizers did not affect the yield of protoplasts. The presence of 0.6 M $MgSO_4$ (645 mOs)supported protoplastation in *B. poitrasii* while in the presence of 0.6 M sucrose (740 mOs) with comparable osmolality, protoplastation was not observed. The yields obtained in the presence of 0.6M NaCl, 0.6M KCl (1120 mOs) and mixture of 0.48 M KCl and 0.12 M $MgSO_4$ (1025 mOs) were different though all had comparable osmolality.

In *B. poitrasii*, protoplasts regenerated to form an irregular mass in the initial 10-12 h (Figure 3.1a, c, e, g). As observed in case of *S. cerevisiae* (Eddy and Williamson, 1959), *M. rouxii* (Reyes *et al.*, 1983) and *C. albicans* (Elorza *et al.*, 1983) formation of an irregular mass during regeneration was reported. In general, protoplasts of filamentous fungi regenerate by producing germ tubes while protoplasts

of yeasts regenerate by budding (Kelkar *et al.*, 1990). The formation of irregular mass during regeneration of *S. cerevisiae* was correlated to the slow rate of cell wall synthesis as compared to that of the cell cycle (Necas and Svoboda, 1985). It is also possible that due to the dimorphic nature of *S. cerevisiae, M. rouxii, C. albicans* and *B. poitrasii*, the response to the environmental conditions with respect to the cell wall deposition pattern is delayed. This observation suggests that the initial cell wall synthesis is required for the determination of cell wall deposition patterns (Szaniszlo, 1985) and hence the morphological outcome in the dimorphic fungi. Interestingly, the protoplasts from both mycelium and yeast-form cells regenerated similarly, indicating that the regeneration process is independent of the initial biochemical state of the cell.

To assess the presence or absence of wall residues on the protoplasts UV fluorescent dye, such as calcofluor white was used (Davis, 1985). The protoplast staining with both calcofluor white and FITC-WGA revealed the absence of cell wall residues. The presence of chitin in the cell wall of irregular mass was confirmed through FITC-WGA staining (Figure 3.2). Effect of different cell growth inhibitors on the regeneration of protoplasts from the mycelium-form cells showed that in the presence of cycloheximide an irregular mass was observed under all regeneration conditions even after 36h incubation (Figure 3.3a, b). This could be due the inability of the protoplasts to form the cell wall components under these conditions. However, Dermastia and Komel (1993) reported that the effect of hygromycin B, a protein synthesis inhibitor, was more pronounced on the hyphal growth of *Cochliobolus lunatus* than the protoplast regeneration that was correlated to the antagonistic effect of osmotic stabilizer added during regeneration. In the case of fission yeast, *Schizosaccharomyces pombe*, staurosporine, a phospholipid/calcium-dependent protein kinase inhibitor affected the glucan synthesis in the cell wall of a yeast

morphology mutant sts5, suggesting the involvement of protein kinase pathway in the synthesis, secretion and distribution of cell wall components. (Konomi *et al.*, 1999). Previously, the effect of signal transduction inhibitors, such as verapamil and H-7 on the yeast-mycelium transition in *B. poitrasii* has been reported (Deshpande *et al.*, 1997). However addition of H-7, a cAMP- dependent protein kinase inhibitor did not show any appreciable effect on the morphological outcome during protoplast regeneration.

Adams *et al.* (1993) studied the co-ordinate regulation of chitin synthase and chitinase in fungi. It has been reported that as the chitin contents are relatively higher in the mycelial cells than the yeast-form cells, the *N*-acetylglucosaminidase (a glycosidase) activity was found to be necessary for the supply of GlcNAc during mycelium formation in *C. albicans* (Sullivan *et al.*, 1984) and *B. poitrasii* (Ghormade *et al.*, 2000). Addition of glucono-δ- lactone, a glycosidase inhibitor, decreased *N*-acetylglucosaminidase activity to a large extent (50-75%, Table 3.7) while the endo-chitinase activity remained unaffected. The protoplasts regenerated in the yeast-form cells under all conditions of regeneration. It can be suggested that the absence of mycelial growth can be correlated to the inhibition of glycosidase activity and the necessary GlcNAc for yeast cell growth is supplied by the action of endo-chitinase (Ghormade *et al.*, 2000).

Elorza *et al.* (1994) reported that both the mycelial and the yeast-form cell protoplasts of *C. albicans* were produced initially by a chitin network followed by the incorporation of glucan and mannoproteins in the cell walls. It has been reported that the effect of tunicamycin depended on the timing of addition to the regeneration process (Peberdy, 1990). In case of *C. albicans* addition at the start of regeneration affected the level of all cell wall polymers while mannoprotein and the alkali-

insoluble glucan synthesis was hampered if added during regeneration (Elorza *et al.*, 1987). In the case of *B. poitrasii,* addition of tunicamycin and benanomicin A and B at the start of regeneration did not affect the typical response of the protoplasts to the environmental conditions (Table 3.7). It can be suggested that the role of *N*-glycosylation of proteins and mannan components could be secondary in the determination of morphology. Watanabe *et al.* (1997) reported that the susceptibility of the *Saccharomyces* protoplasts to the benanomicin A was dependent on the presence of glucose in the regeneration medium. However, such effect was not observed in *B. poitrasii.*

It can be suggested that in *B.poitrasii*, the initial synthesis of cell wall could be a prerequisite in deciding the morphology of the yeast- and mycelial-forms. Moreover, the inhibitor studies showed that chitin metabolism *viz*. synthesis, degradation and its regulation, contributed significantly in determining the final morphological outcome during protoplast regeneration in *B. poitrasii.*

Chapter 4: Biochemical studies on chitin synthases from *Benjaminiella poitrasii*

4.1 Biochemical characteristics of chitin synthases from *Benjaminiella poitrasii*

4.1.1 Introduction

Yeast – mycelium transition in dimorphic fungi is accompanied by differential synthesis of cell wall components, leading to significant quantitative changes in the chemical composition of the cell wall. For instance, the amount of chitin present in the mycelium – form cell walls of *B. poitrasii* is three times higher than that in the yeast – form cells (Khale and Deshpande, 1992). In view of protoplast regeneration studies in the presence of various inhibitors involved directly/ indirectly in cell wall metabolism, it has been suggested that chitin metabolism *viz.* synthesis, degradation and its regulation, contributed significantly in determining the final morphological outcome in *B. poitrasii* (Chapter 3).

Various biochemical and biophysical processes have been reported to be involved in the regulation of chitin synthesis. These include: proteolytic activation (Arroyo-Begovich and Ruiz-Herrera, 1979; Deshpande *et al.*, 1997; Ruiz-Herrera and Bartinicki-Garcia, 1976), involvement of chitinases (Rast *et al.*, 1991), Ca-calmodulin dependant activation (Martinez-Cadena and Ruiz-Herrera, 1987; Suresh and Subramanyam, 1997), chitin synthesis inhibitors (Craig *et al.*, 1981) and osmotic stress (Deshpande *et al.*, 1997; Gooday and Schofield, 1995). The studies on chitin synthases in *B. poitrasii* with respect to the subcellular localization of chitin synthases, *in vitro* trypsinization and the effect of hypo- and hyper-osmotic environmnent on the membrane bound chitin synthases have already been conducted (Deshpande *et al.*, 1997; Khale et al., 1990). In the present chapter, studies exploring different biochemical characteristics of chitin synthases of *B. poitrasii* and their regulation have been described.

4.1.2 Results

4.1.2.1 Chitin synthase activity during yeast-mycelium transition

Yeast – mycelium transition was carried out in YP medium at 28°C using yeast cells grown at 37 °C in YPG (0.5% glucose) medium for 24 h. The chitin synthase activity of mixed membrane fraction was estimated at different time intervals. The chitin synthase activity increased 5 folds after first 6h during yeast-mycelium transition (51$\pm$4 % germ tube formation), after which it remained practically unchanged. The chitin synthase activity in the mixed membrane fraction of yeast-form cells was 0.45 $\pm$ 0.25 pkat/mg protein whereas the chitin synthase activity in the mixed membrane fraction of mycelium-form cells was 2.10 $\pm$ 0.83 pkat/mg protein after 24 h.

4.1.2.2 Biochemical characterization of membrane-bound chitin synthases from yeast and mycelium-form cells

Effect of metal ions

The effect of 10 mM metal ions (Mg^{2+}, Mn^{2+} and Co^{2+}) on the chitin synthase activities from mixed membrane fraction of yeast and mycelium-form cells of *B. poitrasii* was studied. The results are shown in Table 4.1. The chitin synthase activity in the absence of any metal ion was very low (0.33 $\pm$ 0.18 and 1.7 $\pm$ 0.55 pkat/mg protein for yeast and mycelium mixed membrane fractions, respectively). The addition of Mn^{2+} (10mM) and Co^{2+}(10mM) increased the yeast chitin synthase activity by 2.5 and 4 fold (0.845 $\pm$ 0.44 pkat/mg and 1.32 $\pm$ 0.72 pkat/mg) respectively and 1.5 fold (2.55 $\pm$ 0.83 pkat/mg) for the mycelial mixed membrane fraction. In the presence of Mg^{2+} (10mM) the activity of mycelial mixed membrane

fraction increased 1.2 fold while that of yeast mixed membrane fraction increased 1.3 times (Table 4.1). The chitin synthase activity was estimated in the presence of Mg^{2+} in further experiments.

Table 4.1: Effect of metal ions (10mM) on chitin synthase activity

Metal ion	Chitin synthase activity (pkat/ mg protein)	
	Yeast chitin synthases	Mycelium chitin synthases
Without metal ions	0.33 ± 0.18	1.7 ± 0.55
Mg^{2+}	0.45 ± 0.25	2.1± 0.69
Mn^{2+}	0.85 + 0.44	2.5+ 0.75
Co^{2+}	1.32 ± 0.72	2.7± 0.75

Results are average ± SD of 3 sets of triplicate experiments.

Effect of different additives on chitin synthase activity

Effect of H-7, a cAMP-dependant protein kinase inhibitor and TFP, a Ca-calmodulin inhibitor on the chitin synthase activity was studied. As shown in Table 4.2, the addition of H-7 (200 µM), an inhibitor of cAMP-dependant protein kinases, did not have any significant effect on the chitin synthase activity of mycelium-form cells but reduced chitin synthase activity of yeast-form cells by 30%. On the other hand, TFP (40 µM) completely inhibited chitin synthase activity in both yeast- and mycelium-form cells.

Chitin synthase activity from mixed membrane fraction of yeast and mycelium-form cells of *B.poitrasii* was also determined in the presence of 8µM nikkomycin (a competitive inhibitor of chitin synthase). The addition of nikkomycin led to a 3-4 fold decrease in chitin synthase activities of yeast and mycelium mixed membrane fraction (Table 4.2).

To determine the effect of membrane solubilizers on chitin synthase activity, mixed membrane fraction from yeast and mycelium-form cells was solublised in 1% (w/v) digitonin as described in the Section 2.3.2. The chitin synthase acivities were determined in the digitonin-solublized membrane from yeast and mycelium-form cells. Mixed membrane fraction not solubilised in digitonin was used as the control. The results of this study are shown in Table 4.2. Digitonin solubilisation led to a 200 fold and 8 fold increase in chitin synthase activity from both yeast as well as mycelium-membrane fractions respectively. The addition of Triton X-100 and Tween 80 at similar concentrations also showed considerable solubilisation of the chitin synthases in yeast- and mycelium-form cells.

Table 4.2: Effect of different additives on chitin synthase activity of *B. poitrasii*

Medium	Chitin synthase activity, pkat/mg protein	
	Yeast membrane fraction	Mycelium membrane fraction
• Control	0.45 ± 0.25	2.10 ± 0.69
• H-7 (200 µM)	0.34 ± 0.07	2.3 ± 0.63
• TFP (40 µM)	ND[1]	ND[1]
• Nikkomycin (8 µM)	0.11 ± 0.06	0.72 ± 0.61
• Digitonin (1% w/v)	131.69 ± 3.42	36.35 ± 8.79
• Triton X-100(1%w/v)	61.16 ± 23.89	22.71 ± 1.18
• Tween 80 (1% w/v)	18.29 ± 8.64	6.22 ± 3.20

[1]ND=not detected.

4.1.2.3 Isoelectric focusing for chitin synthases from yeast and mycelium- form cells

Isoelectric focusing was done for 1% (w/v) digitonin solubilised membrane fraction from yeast as well as mycelium-form cells. The isoelectric focusing was done in the pH range 3-10 using a preparative scale isoelectric focusing unit from Bio-Rad

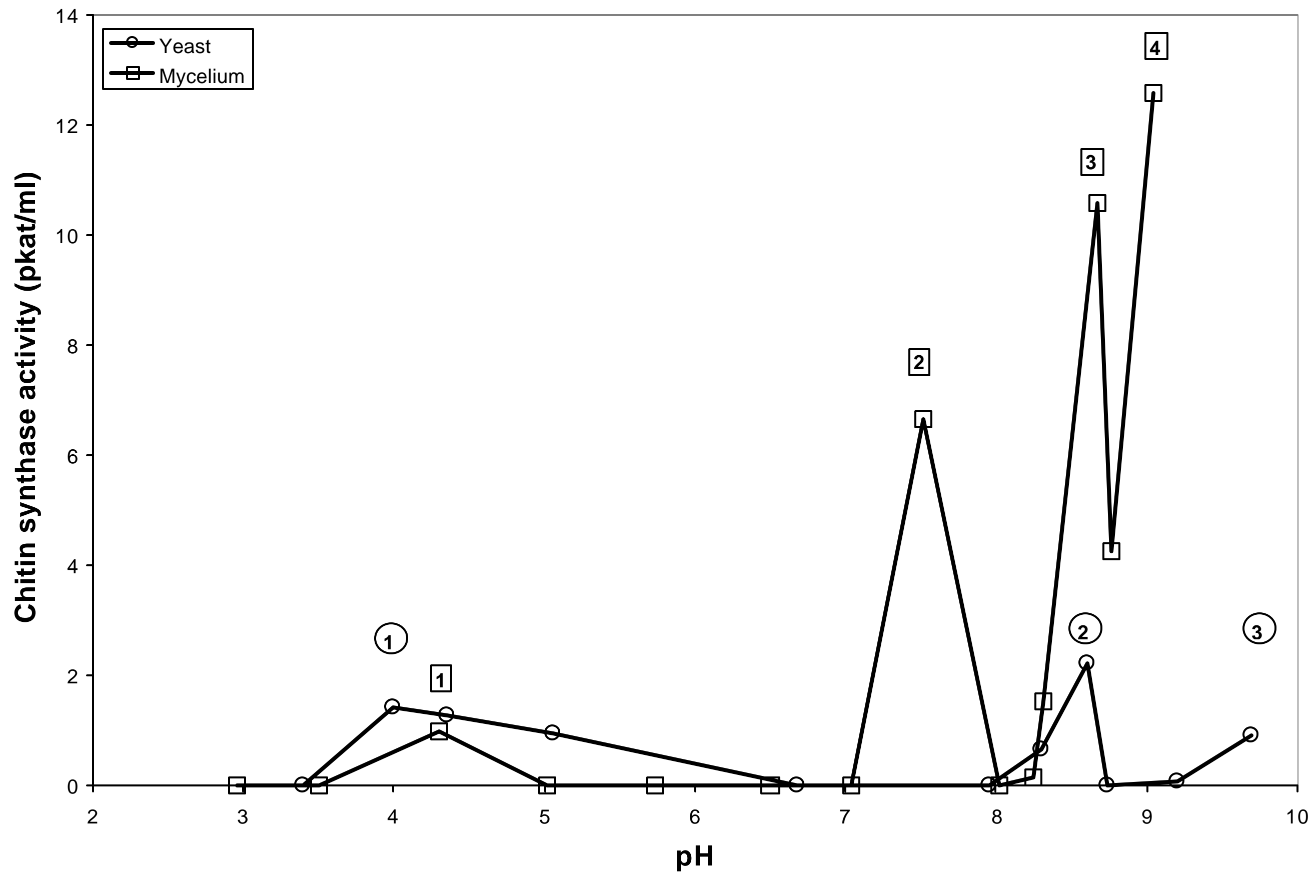


Figure4.1: Comparison of yeast and mycelium chitin synthases through isoelectric focussing (O) Yeast, ( ) Mycelium

as described in Section 2.4.4. After 4 h, twenty fractions were collected and analyzed. The pH and chitin synthase activity were measured for each fraction. The results are shown in Figure 4.1. Isoelectric focusing for yeast-form membrane fraction showed 3 peaks while that for mycelium-form membrane fraction showed 4 peaks. For the yeast-form membrane fraction, peaks were observed at the pH values 3.5-6.0, 4.5-5.0 and 8.0-9.0. For the mycelium-form membrane fraction, the peaks were observed at 3.5-5.0, 7.0-8.0, 8.0- 9.0 and > 9.0.

4.1.3 Discussion

The role of chitin synthase in morphology determination in *S. cerevisiae* (Bulawa, 1993), *C. albicans* (Munro *et al.*, 1998), *A. nidulans* (Aufauvre-Brown *et al.,* 1996) and *N. crassa* (Yarden and Yanofsky, 1991) was reported in the literature. In *B. poitrasii*, chitin synthesis was found to influence morphological outcome during protoplast regeneration (Section 3.2).

Detailed studies in our lab on the subcellular localization of chitin synthase in *B. poitrasii* showed that most of the chitin synthase activity was associated with the cell wall and the membrane (Khale, 1990). In this context, the biochemical properties of native chitin synthase activity in mixed membrane fractions of yeast- and mycelium-form cells were studied. Yeast to mycelium transition was triggered in *B. poitrasii* by changing the temperature and glucose concentrations. During this transition the chitin synthase activity was found to increase five folds. Similar results were reported for the case of *Candida albicans* (Munro *et al.*, 1998).

Chitin synthases of yeast- and mycelium-form cells of *B. poitrasii* were activated in the presence of 10 mM metal ions (Mg^{2+}, Mn^{2+} and Co^{2+}). For both yeast-form cells and mycelium-form cells, the activation was in the order $Co^{2+} > Mn^{2+} >$

Mg^{2+}. This difference in the activation behaviour is probably due to the existence of different types of chitin synthases in the mixed membrane fractions in different proportions. Differences in the activation behaviour of different chitin synthases by different metal ions have been reported for the case of *S. cerevisiae* (Bulawa, 1993). For instance, it was found for the case of *S. cerevisiae* that chitin synthase type I is activated by Mg^{2+} but inhibited by Co^{2+}. It was also reported that the activation of chitin synthase for chitin synthase type II was in the order $Co^{2+} > Mg^{2+}$ while for chitin synthase type III it was in the order $Mg^{2+} > Co^{2+}$.

Several second messenger systems have been implicated in dimorphic transition and hence, it would be interesting to examine the signal transduction systems that influence chitin synthase activity. Studies in the literature have demonstrated that addition of an exogenous second messenger, such as cAMP or its precursors or analogues, can elicit a dimorphic response (Chattaway et al., 1981; Khale and Deshpande, 1993; Sabie and Gadd, 1992). Antagonism of a second messenger system by addition of calmodulin inhibitors has also been shown to retard dimorphic transition (Sabie and Gadd, 1989). In the present study, the complete inhibition of chitin synthase activity in the presence of TFP (a Calcium calmodulin inhibitor) indicated that primary signal transduction mechanism associated with chitin synthase activity involved Calcium calmodulin dependant kinases. Ca-Calmodulin was shown to be important in the regulation of chitin synthase activity from *Phycomyces blackesleeanus* and *Neurospora crassa* (Martinez-Cadena and Ruiz-Herrera, 1987; Suresh and Subramanyam, 1997). However, further experimentation with the purified enzymes is necessary to ascertain the role of phosphorylation in the activation of chitin synthases in *B. poitrasii*, if any.

Chitin synthase is a membrane bound enzyme and hence, the use of membrane solubilisers is expected to release the enzyme into solution where many more active sites become available for reaction. The effect of various saponins on chitin synthase activity was studied for the case of *Absidia glauca* (Machida and Saito, 1993), *B. poitrasii* (Deshpande *et al.*, 1997), *C. albicans* (Gozalbo *et al.*, 1985) and *C. cinereus* (Gooday and deRousset-Hall, 1975). Machida and Saito (1993) studied the solubilisation behaviour of nearly 40 different detergents for the case of *A. glauca* and discovered that only 2 of the 40 detergents *viz*, digitonin and n-heptyl β-D-thioglucoside could solubilize chitin synthase. Digitonin solubilized 70% of the microsomal chitin synthase activity. Gooday and deRousset-Hall (1975) observed a transient increase in chitin synthase activity on addition of digitonin followed by a loss of activity. This complex behaviour was attributed to the fact that digitonin acts in part as a detergent and partly by complexing with membrane sterols (Gooday and de Rousset-Hall, 1975).

In this study, membrane solubilisation of mixed membrane fractions of yeast- and mycelium- form cells using digitonin, Triton X-100 and Tween 80 resulted in very high levels of activation of the chitin synthases. However, similar membrane solubilization experiments with digitonin on mixed membrane fractions of yeast-form cells of *B. poitrasii* performed earlier (Deshpande *et al.*, 1997) did not show any appreciable solubilisation of native chitin synthase activity.

The effect of nikkomycin on chitin synthase activity in yeast- and mycelium-form cells of *B. poitrasii* was also studied. Nikkomycin is a known competitive inhibitor of chitin synthase. The addition of nikkomycin led to a decrease in chitin synthase activities for both yeast- and mycelium-form chitin synthases as expected.

The isoelectric focussing experiment with digitonin solubilized mixed membrane fractions (MMF) of yeast- and mycelium-form cells resolved chitin synthase activities into 3 peaks for yeast MMF and 4 peaks for mycelium MMF. The multiple peaks observed for the isoelectric focussing could be due to the mutiplicity of chitin synthases in *B. poitrasii*. Multiplicity of chitin synthases has also been observed for the case of *S. cerevisiae* (Bulawa, 1993) where three distinct chitin synthase activities corresponding to chitin synthase types I, II and III have been identified.

4.2 *Benjaminiella poitrasii*: A model system to screen chitin synthase inhibitors

4.2.1 Introduction

Antifungal agents for human mycoses that target the cell wall do not show as many side effects as other antifungal agents. Among the various targets within the cell wall, the enzymes engaged in the metabolism of the cell wall polymers such as chitin (which is not found in the host human cells) are preferred targets. Therefore, an attractive approach for the development of antifungal agents is to identify suitable inhibitors of enzymes involved in chitin metabolism such as chitin synthase and chitinase.

For the screening of potential antifungal compounds and/ or microorganisms that produce such compounds, different tests have been suggested in the literature. The tests include hyphal tip bursting (Zhu and Gooday, 1992; Ghormade, 2000), inhibition of yeast to mycelium transition (Davila *et al.*, 1986; Frost, 1995; Ghormade, 2000), inhibition of spore germination (Gooday *et al.*, 1976; Zhu and Gooday, 1992; Ghormade, 2000), retarded mycelial growth (Gooday *et al.*, 1976; Davila *et al.*, 1986; Smith *et al.*, 1990; Zhu and Gooday, 1992), inhibition of fungal growth (Tariq and Devlin, 1996; Watanabe *et al.*, 1997), retarded rate of protoplast regeneration (Gooday *et al.*, 1976), efficacy of sorbitol protection (Frost, 1995), inhibition of chitin synthase activity (Brillinger, 1979; Kobinata *et al.*, 1980; Binks, 1990; Sakurai, 1999) and inhibition of chitinase activity (Sakuda, 1996; Ghormade, 2000). Various potential antifungal compounds have been examined in the literature for their efficacy using various model organisms including *B. poitrasii* (Ghormade, 2000), *Botrytis cinerea* (Smith *et al.*, 1990; Zhu and Gooday, 1992), *C. albicans* (Frost, 1995), *Coprinus cinereus* (Gooday *et al.*, 1976; Brillinger, 1979), *Fusarium*

graminearum (Binks, 1990), *M. rouxii* (Zhu and Gooday, 1992), *P. brasiliensis* (Davila *et al.*, 1986) and *Trichoderma viride* (Gooday *et al.*, 1976).

Ghormade (2000) used *B. poitrasii* as a model system to evaluate inhibitors targeting the enzyme chitinase (that degrades chitin). Observations relating to Y → M transition, spore germination, hyphal tip bursting and direct estimation of chitinase activity were used to screen suitable chitinase inhibitors. The focus of this investigation, however, was on evaluating inhibitors for their ability to inhibit chitin synthase activity. Both cell-free extracellular broths of fungal and bacterial cultures containing potential inhibitors and commercial inhibitors such as Benanomycin A, Benanomycin B, glucono-δ-lactone, nikkomycin, tunicamycin, H-7, TFP, verapamil, cycloheximide and isophthalic acid were investigated for their effectiveness in inhibiting chitin synthase. These inhibitors were also examined for their effectiveness in causing hyphal tip bursting and in inhibiting yeast-mycelium transition. Two fungal culture filtrates, showing inhibition of chitin synthase activity from *B. poitrasii* mixed membrane fraction, were also tested for their effectiveness in inhibiting chitin synthase activity in selected pathogenic fungi.

4.2.2 Results

Cell-free extracellular broths of fungal and bacterial cultures grown on an inhibitor production medium containing soyabean meal, starch, yeast extract and mannitol (Section 2.2.5) and commercial inhibitors were screened for their effectiveness in inhibition of the cell wall metabolism processes using three different screening tests. The bacterial cultures used were *Bacillus* sp101, *Bacillus* sp102 and *Streptomyces* sp. The fungal cultures were *Chaetomium* sp, *Sclerotium* sp and *Volutella* sp. The commercial inhibitors chosen for this study included (a) inhibitors

of the enzymes involved in the synthesis of cell wall components viz., Benanomycin A, Benanomycin B (both mannan synthase inhibitors), glucono-δ-lactone (*N* -acetyl glucosaminidase inhibitor), nikkomycin (chitin synthase inhibitor) and tunicamycin (inhibitor of *N*-glycosylation of proteins – a process involved in the incorporation of proteins within the cell wall), (b) inhibitors such as H-7 (cAMP dependent protein kinase inhibitor), TFP (Ca- calmodulin inhibitor) and verapamil (Ca^{2+} channel blocker), cycloheximide (protein synthesis inhibitor) and isophthalic acid (an inhibitor of NAD-GDH, an important enzyme of the nitrogen metabolic pathway which is important in chitin metabolism) .

The three screening tests used in this study were a) hyphal tip-bursting, b) observations on germ tube formation during yeast-mycelium transition and c) measurements of chitin synthase activity from yeast- and mycelium-form cells of *B. poitrasii*. In the hyphal tip bursting (HTB) test, the inhibitor was added to the plates having 16-18 h old mycelia (hyphae) of *B. poitrasii* and the hyphal tips (total vs burst tips) were counted and stated in terms of percentage hyphal tips burst as described in Section 2.2.7. In the second test, *B. poitrasii* cells were observed during Y → M transition after 6-8h incubation (Section 2.2.2) and percentage of cells forming germ tubes were counted. In the third approach, chitin synthase activity was estimated for yeast and mycelium membrane fractions (isolated as described in Section 2.3.2) as per Section 2.3.15.6.

4.2.2.1 Hyphal tip bursting test

The extracellular broth of different bacterial and fungal cultures grown on an inhibitor production medium was tested for cell wall metabolism inhibitors using the hyphal tip bursting test (HTB). The number of burst hyphae (reported as percent

bursting) and the time required for the earliest instances of bursting were noted (Table 4.3). The control used for this reaction was the 0.6M sorbitol in water. Of the various cultures used, bursting of the hyphae was observed in the presence of culture filtrates of *Bacillus* sp 101, *Bacillus* sp 102, *Chaetomium* sp and *Streptomyces* sp. All these four cultures showed the bursing of *B. poitrasii* hyphae within 3-5 min of application. The percentage of burst hyphae was higher (50-80%) for the culture filtrates of *Bacillus* sp 102 and *Chaetomium* sp. The bursting of *B. poitrasii* hyphae was not observed for the culture filtrates of *Sclerotium* sp and *Volutella* sp.

Hyphal tip bursting test was also performed for different commercial inhibitors of cell wall metabolic processes. Of the different inhibitors studied, hyphal tip bursting was observed in the presence of verapamil (48 $\pm$ 4% within 5 min), nikkomycin (73 $\pm$ 4% within 7-8 min) and H-7 (48 $\pm$ 4% within 15 min). While bursting in hyphae occurred most rapidly in the case of verapamil, the number of burst hyphae was highest in the case of nikkomycin. The other inhibitors tested did not show any bursting of *B. poitrasii* hyphae. The culture filtrates of *Bacillus* sp 102 and *Chaetomium* sp were stronger promoters of hyphal tip bursting than nikkomycin at the concentrations studied.

Table 4.3. Screening for inhibitors of cell wall metabolism[1]

Inhibitor preparation	Hyphal tip bursting	
	Burst hyphae (%)	Time for start of bursting (min)
Control	NB	-
Extracellular broth of bacterial and fungal cultures		
Bacillus sp101	53 ± 4	3-5
Bacillus sp102	83 ± 4	3-5
Chaetomium sp	75 ± 7	3-5
Sclerotium sp	NB	NA
Streptomyces sp	45 ± 7	3-5
Volutella sp	NB	NA
Inhibitors		
Benanomycin A (30 μM)	NB	NA
Benanomycin B (15 μM)	NB	NA
Cycloheximide (20 μM)	NB	NA
Glucono-δ-lactone (30 mM)	NB	NA
H-7 (100 μM)	48 ± 4	15
Isophthalic acid (60 mM)	NB	NA
Nikkomycin (20 μM)	73 ± 4	7-8
Phenyl Methyl Sulphonyl Fluoride (5 mM)	NB	NA
Trifluoroperizine (20 μM)	NB	NA
Tunicamycin (50 μM)	NB	NA
Verapamil (10 mM)	48 ± 4	5

[1]NB=No burst hyphae.

4.2.2.2 Germ tube formation in the presence of cell wall metabolism inhibitors

The effect of different extracellular culture broths and commercial inhibitors on germ-tube formation during Y→ M transition is shown in Table 4.4. The inhibition of germ tube formation was highest (25-30%) in the presence of the extracellular culture broth of *Bacillus* sp102, *Chaetomium* sp and *Volutella* sp. The inhibition of germ tube formation was lesser in the presence of the culture filtrates of *Bacillus* sp 101, *Sclerotium* sp and *Streptomyces* sp.

All the inhibitors used showed inhibition of germ tube formation to a smaller or greater extent. Among the different inhibitors studied, germ tube formation was not observed at all in the presence of glucono-δ–lactone, TFP, tunicamycin and verapamil. Germ tube formation was inhibited to a large extent in the presence of isophthalic acid, PMSF and nikkomycin. Germ tube formation was inhibited to a very small extent in the presence of Benanomycin A, Benanomycin B, cycloheximide and H-7 (Table 4.4).

Table 4.4: The effect of chitin synthase inhibitor producers on yeast-mycelium transition in *B. poitrasii*

Inhibitor preparation	Germ tube formation (%)	Inhibition (%)
Control	82 ± 3	0
Extracellular broth of bacterial and fungal cultures		
Bacillus sp101	73 ± 15	11
Bacillus sp102	60 ± 21	26
Chaetomium sp	58 ± 12	29
Sclerotium sp	61 ± 20	25
Streptomyces sp	71 ± 4	13
Volutella sp	63 ± 5	23
Inhibitors		
Benanomycin A (30 µM)	58 ± 17	29
Benanomycin B (15 µM)	66 ± 13	19
Cycloheximide (20 µM)	79 ± 30	4
Glucono-δ-lactone (30 mM)	ND	100
H-7 (100 µM)	66 ± 17	19
Isophthalic acid (60 mM)	22 ± 8	73
Nikkomycin (20µM)	32 ± 4	61
Phenyl Methyl Sulphonyl Fluoride (5 mM)	35 ± 15	58
Trifluoroperizine (20 µM)	ND	100
Tunicamycin (50 µM)	ND	100
Verapamil (10 mM)	ND	100

ND = Not detected (no germ tube formation)

4.2.2.3 Chitin synthase activities in the presence of cell wall metabolism inhibitors

Chitin synthase activities of yeast and mycelium- form membrane fractions of *B. poitrasii* were studied in the presence of extracellular broth of different bacterial and fungal cultures. Chitin synthase activities of yeast and mycelial membrane fractions in the presence of commercial inhibitors viz, H-7, nikkomycin and TFP had been done previously as part of the study described in Section 4.1. The chitin synthase activities determined in the absence of the addition of extracellular broth of cultures or inhibitors served as the control. The results are shown in Table 4.5.

Chitin synthase activity from yeast-form cells increased in the presence of the extracellular broth of all the six fungal and bacterial cultures. The chitin synthase activity from the mycelium-form cells was inhibited in the presence of *Chaetomium* sp., *Sclerotium* sp. and *Volutella* sp. while it was activated in the presence of culture broth of *Bacillus* sp 101, *Bacillus* sp102 and *Streptomyces* sp. The activity of chitin synthase from mycelium-form cells was not detected when the culture broth of *Sclerotium* sp was used. The activity of chitin synthase from yeast- and mycelium-form cells of *B. poitrasii* was not affected by H-7 and PMSF. Nikkomycin and TFP inhibited chitin synthase activity from both yeast- and mycelium-form cells. The chitin synthase activity was not detected in the presence of TFP for both yeast- and mycelium- form cells. The inhibition due to nikkomycin was more for chitin synthase from mycelium-form cells.

Table 4.5. Screening for chitin synthase inhibitors

Culture filtrate of	Chitin synthase activity, pkat/mg	
	Yeast membrane fraction	Mycelium membrane fraction
Control	0.45 ± 0.25	2.10 ± 0.83
Extracellular broth of bacterial and fungal cultures		
Bacillus sp101	1.34 ± 0.34	2.54 ± 0.71
Bacillus sp102	32.5 ± 6.01	6.91± 0.14
Chaetomium sp	1.91 ± 0.29	1.60 ± 0.47
Sclerotium sp	1.48 ± 0.01	ND
Streptomyces sp	0.69 ± 0.25	2.2 ± 0.66
Volutella sp	3.82 ± 0.84	0.41 ± 0.30
Inhibitors		
H-7 (200 µM)	0.34 ± 0.07	2.3 ± 0.58
Nikkomycin (8 µM)	0.11 ± 0.06	0.72 ± 0.61
Trifluoroperizine (20 µM)	ND	ND

ND=not detected

4.2.2.4 Effect of culture filtrates on chitin synthase activity from pathogenic fungi

Since the extracellular culture broths of *Chaetomium* sp and *Volutella* sp showed inhibition of chitin synthase activity of the mycelial chitin synthase in *B. poitrasii* and also inhibited germ tube formation, these were taken up further evaluation as potential inhibitors of chitin synthase activity in selected human and

plant pathogenic fungi. The effect of the extracellular culture broths of *Chaetomium* sp and *Volutella* sp on the activity of chitin synthase from mixed membrane fractions of pathogenic fungi *C. albicans*, *S. rolfsii* and *A. niger* was studied. Mixed membrane fractions from pathogenic fungi were isolated according to the method described in Section 2.3.2. The chitin synthase activity in this fraction was determined according to the Section 2.3.15.6. The activity of chitin synthase from mixed membrane fractions from these fungi measured in the absence of the extracellular culture broth was used as the control. The chitin synthase activity from *C. albicans* was inhibited (67%) by the extracellular broth of *Chaetomium* sp while it was activated by the extracellular culture broth of *Volutella* sp. Chitin synthase activity from *S. rolfsii* remained unchanged in the presence of the extracellular culture broth of *Chaetomium* sp and *Volutella* sp. The chitin synthase activity in *A. niger* was inhibited (76%) by the extracellular broth of *Volutella* sp. but was unaffected by the extracellular culture broth of *Chaetomium* sp.

Table 4.6: Chitin synthase activity from pathogenic fungi in the presence of potential chitin synthase inhibitors

Culture filtrate of-	Chitin synthase activities from – (pkat/mg)		
	Candida albicans	*Sclerotiunm rolfsii*	*Aspergillus niger*
Control	0.172 ± 0.005	0.027 ± 0.002	0.037 ± 0.014
Chaetomium sp	0.063 ± 0.014	0.036 ± 0.004	0.036 ± 0.004
Volutella sp	0.431 ± 0.178	0.034 ± 0.006	0.009 ± 0.002

4.2.3 Discussion

In the literature, several inhibitors of cell wall metabolism were studied using different screening tests and model/test organisms. Antifungal antibiotic polyoxin D was found to prevent the elongation of stipes of fruit bodies of *C. cinereus* and showed almost total autolysis (Gooday *et al.* 1976). It powerfully inhibited particulate and solubilized preparations of chitin synthase from stipe tissue (Gooday *et al.* 1976). The effect of polyoxin D on germination, morphological development and biosynthesis of cell wall of *T. viride* was also studied. Polyoxin D showed 40-60% inhibition of germination. Mycelium growing in presence of polyoxin D became irregular and lost its rigidity, showing several bulges on the hyphae. Regeneration of protoplasts were less affected by polyoxin D. The protoplasts regenerated slowly in the presence of polyoxin D but the percentage of regeneration was more than 80% (Gooday *et al.* 1976).

Brillinger (1979) used chitin synthase from *C. cinereus* as a model for screening substances with insecticidal properties. Kobinata *et al.* (1980) studied neopolyoxins A,B, C which are potent chitin synthase inhibitors. The antifungal activity was determined in terms of the minimum inhibitory concentrations (MIC) for the culture. Davila *et al.* (1986) reported that papulacandin B inhibited glucan synthase in *P. brasiliensis*. Papulacandin B inhibited mycelial growth and the yeast-mycelium transition but did not affect yeast morphology or growth. Binks (1990) found that membrane bound chitin synthases from *F. graminearum* grown in presence of ediphenphos caused reduction in the *in vivo* incorporation in the [^{3}H] GlcNAc into chitin. Inhibitors of ornithine and argenine decarboxylases reduced growth of the fungus *B. cinerea* cultured on Czapek dox agar (Smith *et al.* 1990). Zhu and Gooday (1992) studied the effect of nikkomycin and echinocandin on differentiated and

undifferentiated mycelia of *B. cinerea* and *M. rouxii* in terms of spore germination and hyphal growth. Wenke *et al.* (1993) found that chitin synthase inhibitors pseurotin A and 8-0-dimethyl pseurotin A isolated from submerged cultures of *A. fumigatus* inhibited both the membrane bound and solubilized forms of chitin synthase. Frost (1995) used whole cells of *C. albicans* to identify the inhibitors towards cell wall synthesis and assembly. The author used efficacy of sorbitol protection and inhibition of cell morphogenesis in the presence of these inhibitors as the indicators of novel antifungal agents. Watanabe *et al.* (1997) discovered that Benanomycin A inhibited growth of a wide range of pathogenic fungi. Tariq and Devlin (1996) reported that nikkomycin Z showed selective toxicity against chitinous fungi that were parasitic in or on eukaryotic host organisms. Sakuda (1996) used various chitinase inhibitors such as allosomidin for the control of insects. More recently, various chitin synthase inhibitors were screened using chitin synthase defective mutants and *in vitro* chitin synthesis estimation (Sakurai *et al.*, 1999).

Three screening tests were used in this study: a) hyphal tip-bursting, b) observations on germ tube formation during yeast-mycelium transition and c) measurements of chitin synthase activity from yeast- and mycelium-form cells of *B. poitrasii*. The bursting of the hyphal tip indicated that either a) the cell wall synthesis at the growing tip was inhibited or b) the cell wall degradation/ dissolution was promoted in a relative sense by one or more components of the inhibitor preparation. While the HTB tests directly indicated the influence of an inhibitor preparation on cell wall metabolic processes, it is difficult to correlate the role of the inhibitor with any one cell wall metabolic process. Attributing the exact role of the inhibitor to any one or more cell wall metabolic processes required the use of complementary tests (like the observations on germ tube formation and chitin synthase activity) along with

HTB. None the less, the HTB test was a useful and simple screening test indicating the efficacy of the inhibitor during mycelium phase growth.

Of the various cultures used, bursting of the hyphae was observed only in the presence of culture filtrates of *Bacillus* sp 101, *Bacillus* sp 102, *Chaetomium* sp and *Streptomyces* sp within 3-5 min of exposure with the culture filtrates of *Bacillus* sp 102 and *Chaetomium* sp showing much higher numbers of burst hyphae. The extracellular culture broth of *Bacillus* sp101 and *Volutella* sp showed the highest levels of inhibition of germ tube formation while the inhibition of germ tube formation was lesser in the presence of the culture filtrates of *Bacillus* sp 102, *Sclerotium* sp and *Streptomyces* sp. The chitin synthase activity from the mycelium-form cells was inhibited in the presence of *Chaetomium* sp., *Sclerotium* sp. and *Volutella* sp. while it was activated in the presence of culture broth of *Bacillus* sp 101, *Bacillus* sp102 and *Streptomyces* sp with the highest levels of inhibition being seen for the case of the culture broth of *Sclerotium* sp.

The inhibitor preparation based on *Bacillus* sp 101 did not contain inhibitors for chitin synthase (Table 4.5) or chitinase (Ghormade, 2000). However, the preparation showed low levels of hyphal tip bursting and inhibition of germ tube formation which could be due to the presence of other components in the filtrate. *Bacillus* sp 102 has been shown to produce chitinase (both endochitinase and *N*-acetyl glucosaminidase) inhibitors (Ghormade, 2000) which could be the probable reason for high levels of hyphal tip bursting. *Chaetomium* sp, *Sclerotium* sp and *Volutella* sp showed inhibition of chitin synthase in the mycelium-form cells but not in the yeast-form cells which was probably because these culture filtrates contained chitin synthase inhibitors of chitin synthase types seen in mycelium forms.

Among the various commercial inhibitors tested, nikkomycin, H-7 and varapamil showed hyphal tip bursting. The number of burst hyphae was highest in the case of nikkomycin. Nikkomycin and TFP showed high levels of inhibition of activity of chitin synthase from both yeast- and mycelium-form cells in the stationary phase. TFP showed the highest levels of inhibition. H-7 showed inhibition of yeast-form chitin synthase but activation of mycelium-form chitin synthase. All the screened inhibitors, inhibited germ tube formation. High levels of inhibition of germ tube formation were seen for the case of glucono-δ-lactone, isophthalic acid, nikkomycin, PMSF, TFP, tunicamycin and verapamil.

Since the extracellular culture broths of *Chaetomium* sp and *Volutella* sp showed inhibition of chitin synthase activity of the mycelial chitin synthase in *B. poitrasii* and also inhibited germ tube formation, these were taken up further evaluation as potential inhibitors of chitin synthase activity in selected pathogenic fungi – *C. albicans*, *S. rolfsii* and *A. niger*. The chitin synthase activity from *C. albicans* was inhibited by the extracellular broth of *Chaetomium* sp while the chitin synthase activity in *A. niger* was inhibited by the extracellular broth of *Volutella* sp.

Thus, in this study, various inhibitor preparations were successfully screened for their efficacy in inhibition of various cell wall metabolic processes and in particular chitin synthase activity using *B. poitrasii* as the model system. Extracellular broths of two fungal cultures were shortlisted based on these screening experiments in order to test for inhibition of chitin synthase activity in pathogenic fungi. Inhibitor preparations that successfully inhibited activity of chitin synthases in *C. albicans* and *A. niger* were identified.

Chapter 5: Molecular studies on chitin synthases from *Benjaminiella poitrasii*

5.1 Introduction

The cell wall of zygomycetous fungi like *B. poitrasii* contains chitin and chitosan (Bartnicki-Garcia, 1968; Khale and Deshpande, 1992; Ruiz-Herrera, 1992). Chitin, which is the polymer of *N*-acetyl glucosamine is synthesized in the cell through the action of chitin synthase (Cabib, 1993). Chitosan is a deacetylated form of chitin and is derived from chitin by the action of chitin deacetylase (Ruiz-Herrera, 1992). Chemical analyses of *B. poitrasii* cell wall also showed differences in the relative proportion of chitin, chitosan and other neutral polysaccharides between yeast and mycelium-form cells (Khale and Deshpande, 1992); therefore, it is reasonable to expect that chitin synthase plays an important role in morphology determination in *B. poitrasii*. The role of chitin synthase in morphology determination in *S. cerevisiae* (Bulawa, 1993), *C. albicans* (Munro *et al*., 2001), *A. fumigatus* (Aufauvre-Brown *et al*., 1997), *A. nidulans* (Borgia *et al*., 1996; Specht *et al*., 1996) and *N. crassa* (Yarden and Yanofsky, 1991) is well studied. The biochemical studies of membrane-bound chitin synthases (Chapter 4) showed different properties in yeast- and mycelium-form cells of *B. poitrasii*, thereby indicating that a possible role for chitin synthases in morphogenesis.

The chitin synthase (*CHS*) genes of a variety of fungi have been well studied and have been reported to be present in the multiple forms (Section 1.4.3). For instance, five *CHS* genes were reported in *S. cerevisiae* (Bulawa, 1993), four *CHS* genes were reported in *C. albicans* (Munro *et al.*, 1998; Trilla *et al.*, 1997) while ten *CHS* genes were reported in *Phycomyces blakesleeanus* (Miyazaki *et al.*, 1997) These genes have been classified into five classes based on the differences in the conserved regions (Bowen *et al.,* 1992; Mellado *et al.,* 1995; Bulawa, 1993; Cid, 1995). The differential expression of the *CHS* genes during morphogenesis has also been studied

for the case of *C. albicans* (Munro *et al.,* 1998), *Paracoccidiodis brasiliensis* (Nino-Vega *et al.,* 1999) and *P. blackesleeanus* (Miyazaki *et al*, 1997). In this study, studies on identification of CHS genes from a dimorphic, zygomycete *B. poitrasii* are reported. The genes have been sequenced and analysed for their relatedness to other fungal CHS genes. The differential expression of *CHS* genes during different stages of growth and dimorphic transition in *B. poitrasii* under different temperature and nutritional conditions has been reported.

5.2 Results

5.2.1 PCR amplification of chitin synthase genes from *B. poitrasii* genome

The PCR amplification of the genomic DNA of *B. poitrasii* was done using the five primer sets described in Section 2.3.6. Based on the conserved regions in the chitin synthase genes of different fungi, the primers were designed. The primer set F1-R1 was designed according to Bowen *et al.* (1992). The PCR amplification using this primer set did not give any detectable product. In order to amplify class I-III CHS genes two primer sets F4 - R1 and F5 - R1 were designed. The primer set F4 - R1 were designed according to Miyazaki *et al.* (1997). The amplified product was run on an agarose gel and a single band of 250 bp size was observed. The primer set F5 - R1 was designed considering the 5' upstream conserved region in the CHS sequence of zygomycetes. The PCR amplification using this primer set gave a single band of 600 bp when the PCR product was run on the gel.

In order to amplify CHS genes belonging to class IV and V, the degenerate PCR primers designed by Mellado *et al.* (1995) were used. The primer sets F2 - R2 and F3 - R2 were used for PCR. The products obtained from the PCR amplification were run on an agarose gel. The product of F2-R2 reaction was a single band of ~ 350

bp in size while for the primer F3-R2, two bands ~ 400 bp and ~ 450 bp were observed on the gel.

5.2.2 Cloning of PCR-amplified products, sequence determination and analysis

The purified PCR products were cloned in pGEM-T easy vector system. Around 400 recombinant colonies were obtained which were then sequenced using the Big Dye Kit as described in Section 2.3.11. The sequences thus obtained were used as the query for the FASTA3 searches. Based on the results of the searches, the clones were divided into 8 different groups and a representative from each group was used for further analysis. The unique sequences of these clones were labeled as *BpCHS1-8*. The deduced amino acid sequences for these fragments were compared with the other sequences from the database. Table 5.1 shows these chitin synthase gene fragments and the corresponding known chitin synthase genes in the database that show maximum homology. *BpCHS1-4* were homologous to the chitin synthases from class I-III while *BpCHS*5-8 were homologous to the chitin synthases from class IV-V.

The amino acid sequences for the cloned *CHS* gene fragments is shown in the Figure 5.1. The deduced amino acid sequences of *BpCHS* fragments showed a high degree of similarity to the other CHS in the database (Table 5.1). *BpCHS1* showed 87.56 % similarity to the *CHS2* gene of *M. circinelloides. BpCHS2* showed 87.5 % similarity to *CHS1* of *Rhizopus oligosporus. BpCHS3* showed 88.88 % similarity to *CHS 1* of *Rhizomucor racemosus*. *BpCHS4* showed 92.59 % similarity to *CHS2* gene of *Rhizopus oligosporus.* All these four CHS gene fragments belong to CHS class I-III chitin synthases. *BpCHS5* showed 84.37 % similarity to *CHSD* of *A. nidulans*. *BpCHS6* was 81.6% similar to CHS gene from *Glomus versiforme*. *BpCHS7* gene was 83.49% similar to *P. blakesleeanus CHS10* gene. *BpCHS8* showed 86.29 % similarity

to CHS gene of *Gigaspora margarita*. All these CHS gene fragments showed maximum similarity to chitin synthases from other zygomycetes except for *BpCHS5* that is homologous to *A. nidulans CHS3*.

Table 5.1: Comparison of different chitin synthase gene fragments

Clone	Homologues chitin synthase genes			
	Gene	Accession number	Similarity	CHS class
*Bp*CHS1	*Mucor circinelloides* CHS2	q 01138	87.56%	-
*Bp*CHS2	*Rhizopus oligosporus* CHS1	p 30594	87.50%	Class I
*Bp*CHS3	*Rhizomucor racemosus* CHS1	q 12632	88.88%	Class II
*Bp*CHS4	*Rhizopus oligosporus* CHS2	p 30595	92.59%	Class II
*Bp*CHS5	*Aspergillus nidulans* CHSD	p 78611	84.37%	Class V
*Bp*CHS6	*Glomus versiforme* CHS	o 93962	81.60%	Class IV
*Bp*CHS7	*Phycomyces blackesleeanus* CHS10	o 74123	83.49%	Class IV
*Bp*CHS8	*Gigaspora margarita* CHS	o 94211	86.29%	-

For the taxonomic and phylogenetic study by the amino acid and sequence comparison of *CHS*, an analysis of the the deduced amino acid sequences of *BpCHS* was carried out with the amino acid sequences of other chitin synthases from Genbank using neighbour-joining method included in the Clustal W program. For the analysis, chitin synthase class II sequences from different fungi were used. The phylogenetic tree has been shown in Figure 5.2. The chitin synthase sequences, each of zygomycetes, ascomycetes and deuteromycetes were found to be clustered separately.

Figure 5.1: Pile-ups of the amino acid seque nces

BpCHS class I-III

```
1                                                     50
Bpchs1  TMHGVMKNIA HLCSRARSKV WNTDGWKKVV VCIVXDGHKK CDPRVMDVLT
Bpchs3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Bpchs4  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Bpchs2  ~~~~~~~~~~ ~~~SRDRSKT WGASGWKKVV VCIVSDGRTK IHPRTLSVLA

        51                                                100
Bpchs1  TIGVYQKGVA KNMVNDKPVQ AHLFEYTTQV SVDSDLKVRG AEKGVVPVQI
Bpchs3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Bpchs4  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Bpchs2  AMGVYQDGLA KNIVNGKPVT AHIYEHTTQL SVDSDMNFRG SEKGVVPVQV

        101                                               150
Bpchs1  LFCLKEKNAK KINSHRWFFN AFGPILQPNI CVLLDVGTRP GNSSIYQLWK
Bpchs3  ~~~~~~~~~~ ~INSHRWFFN AFGPIIQPNV CILLDVGTRP GNSSIYQLWK
Bpchs4  ~~~~~~~~~~ ~INSHRWFFQ AFGPIVDPHV CVLIDVGTKP GGTSIYHLWK
Bpchs2  LFCLKEKNQK KINSHRWFFQ AFGPLLRPNV CVLLDVRTKP GGKSIYSLWK

        151                                       193
Bpchs1  SFDTNENVGG ACGEICAMKG TACVDLLLNP LVAAQNFEFK LEN
Bpchs3  VFHRNPLVGG ACGEIRAMTG IA.GHQLLNP LVAAQNFEYK LEN
Bpchs4  AFDINSNIAG ACGEIRAMAG TAGV.ALLNP LVAAQNFEYK LEN
Bpchs2  SFDINRNVAG ACGEIKAMLG TAG~~~~~~~ ~~~~~~~~~~ ~~~
```

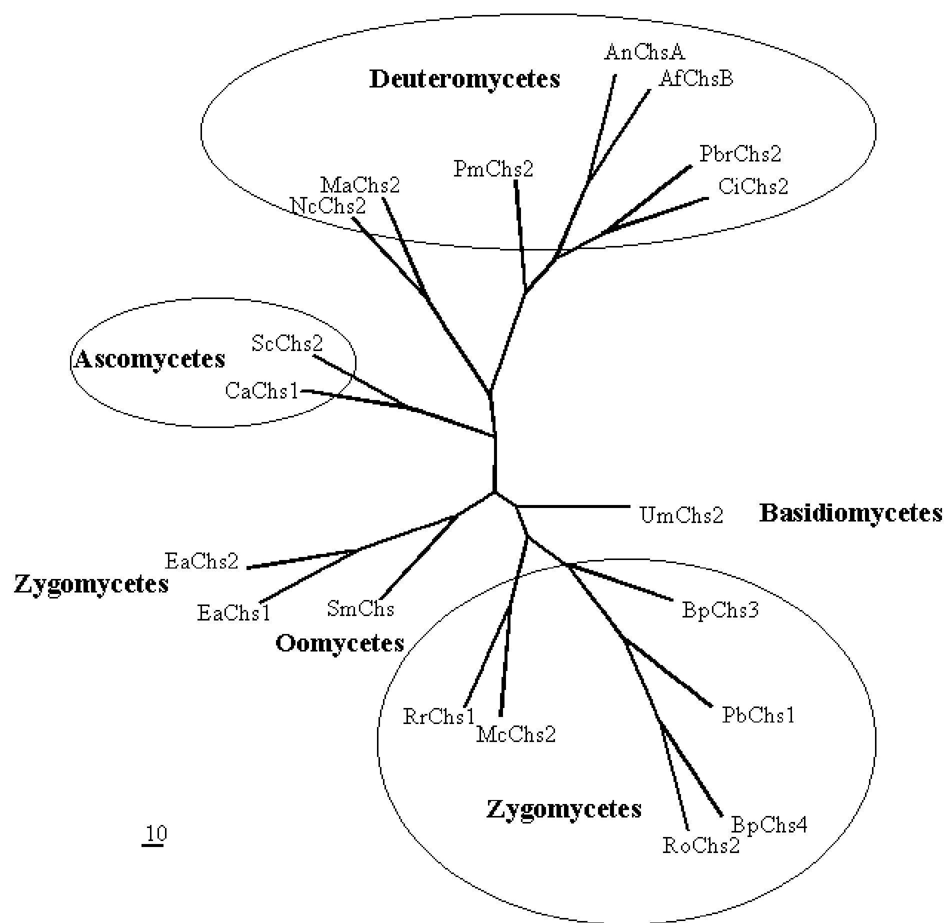
BpCHS class IV-V

```
1                                                     50
Bpchs5  WGSAKQVFEY YISHHQSKAF ESVFGGVTCL PGCFRMYRIK APKGPDGYWV
Bpchs6  WGSALQVFEY YISHHQSKAF ESVFGGVTCL PGCFCMYRVK APKGTQGHWV
Bpchs8  WGSALQVYEY FISHHLAKGF EAVFGGVTCL PGCFCMYRLK ARKG.DGDWV
Bpchs7  ~~~~~~~~~~ YISHHLAKAF ESLFGSVTCL PGCLCMYRVR STTRNQ....

       51                                                100
Bpchs5  PILANPDIVE RYSENIVDTL HKKNLLLLGE DRYLSTLMLR TFPNRKMVFV
Bpchs6  PILANPDIIE RYSENVVDTL HKKNLLLLGE DRHLSTLMLK TFPRRKMLFV
Bpchs8  PIITKPEIVQ EYSSNTIDTL HQKNLLLLGE DRFLTTLMLR NFPFRKMVFT
Bpchs7  PLLVSNQVIK DYAENQVDTL HKKNLLHLGE DRYLTTLILK HFPNYKTKFT

       101                          128
Bpchs5 PQAVCKTVVP DTFMVLLSQR RRWIKNSI
Bpchs6 PQAVCKTVVP DTFGVLLSQS RRWIKNS~
Bpchs8 PQAICKTIXP DEFKVLLSQR RSWIKNS~
Bpchs7 PDAGCMTNAP DQWPVLLSQS RRWIKNSI
```

Figure 5.2: Phylogenetic tree to show the relatedness between *CHS* genes of *B. poitrasii* and other organisms (figure generated using Clustal W program)[1]



[1]Abbreviations used: AnChsA: *A. nidulans* ChsA; AfChsB: *A. fumigatus* ChsB; PbrChs2: *P. brasiliensis* Chs2; CiChs2: *Coccidiodis immitis* Chs2; UmChs2: *Ustilago maydis* Chs2; BpChs3: *B. poitrasii* Chs3; PbChs1: *P. blakesleeanus* Chs1; BpChs4: *B. poitrasii* Chs4; RoChs2: RoChs2: Rhizopus oligosporus Chs2; McChs2: *Mucor circinelloides* Chs2; RrChs1: *Rhizomucor racemosus* Chs1; SmChs: *Saprolegnia monoica* Chs; EaChs1: *Entomophaga aulicae* Chs1; EaChs2: *Entomophaga aulicae* Chs2; CaChs1: *C. albicans* Chs1; ScChs2: *S. cerevisiae* Chs2; NcChs2: *N. crassa* Chs2; MaChs2: *M. anisopliae* Chs2; PmChs2: *Penicillium marneffei* Chs2

5.2.3 Southern analysis and the organisation of CHS genes in the genome

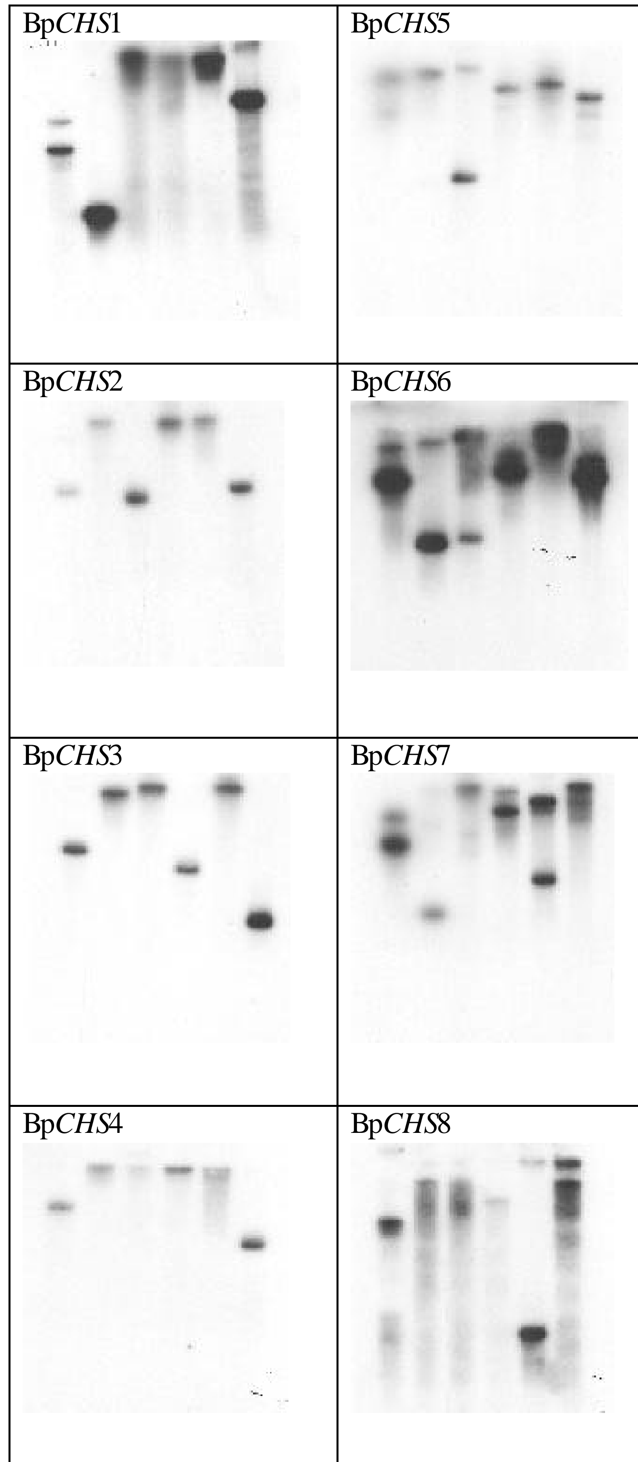
Organisation of chitin synthase genes in the genome of *B. poitrasii* was studied using Southern blot analysis (Section 2.3.9 and 2.3.10). The genomic DNA was restriction digested using six different restriction enzymes viz., HindIII, XhoI, BamHI, XbaI, PstI and EcoRI as described in Section 2.3.8. For this analysis, cloned CHS gene fragments were used as probes. As seen in the Figure 5.3, the Southern blot pattern obtained for each gene fragment is different. Also Southern blots for each of these genes showed the presence of a single major band. In the case of *BpCHS6*, *BpCHS7* and *BpCHS8*, one or two minor bands were also present.

5.2.4 RT-PCR for studying expression of chitin synthase genes

Differential expression of *CHS* genes during growth and dimorphic transition under different nutritional and temperature conditions were studied through RT-PCR. The 24 h old yeast-form cells grown at 37°C and YPG (0.5% glucose) medium were used as an inoculum. To obtain yeast form cells and mycelium form cells, 10^8 cells /50 ml and 10^5 cells /50 ml medium, respectively, were used as the inoculum. Three different nutritional and environmental conditions were studied in order to induce different growth and/or transition conditions: a) Budding yeast growth at 37°C and YPG (0.5% glucose) medium, b) Y → M transition at 28°C and YP (no glucose) medium and c) Y → M transition at 28°C and YPG (0.5% glucose) medium.

Figure 5.3 Southern analysis of *CHS* gene fragments

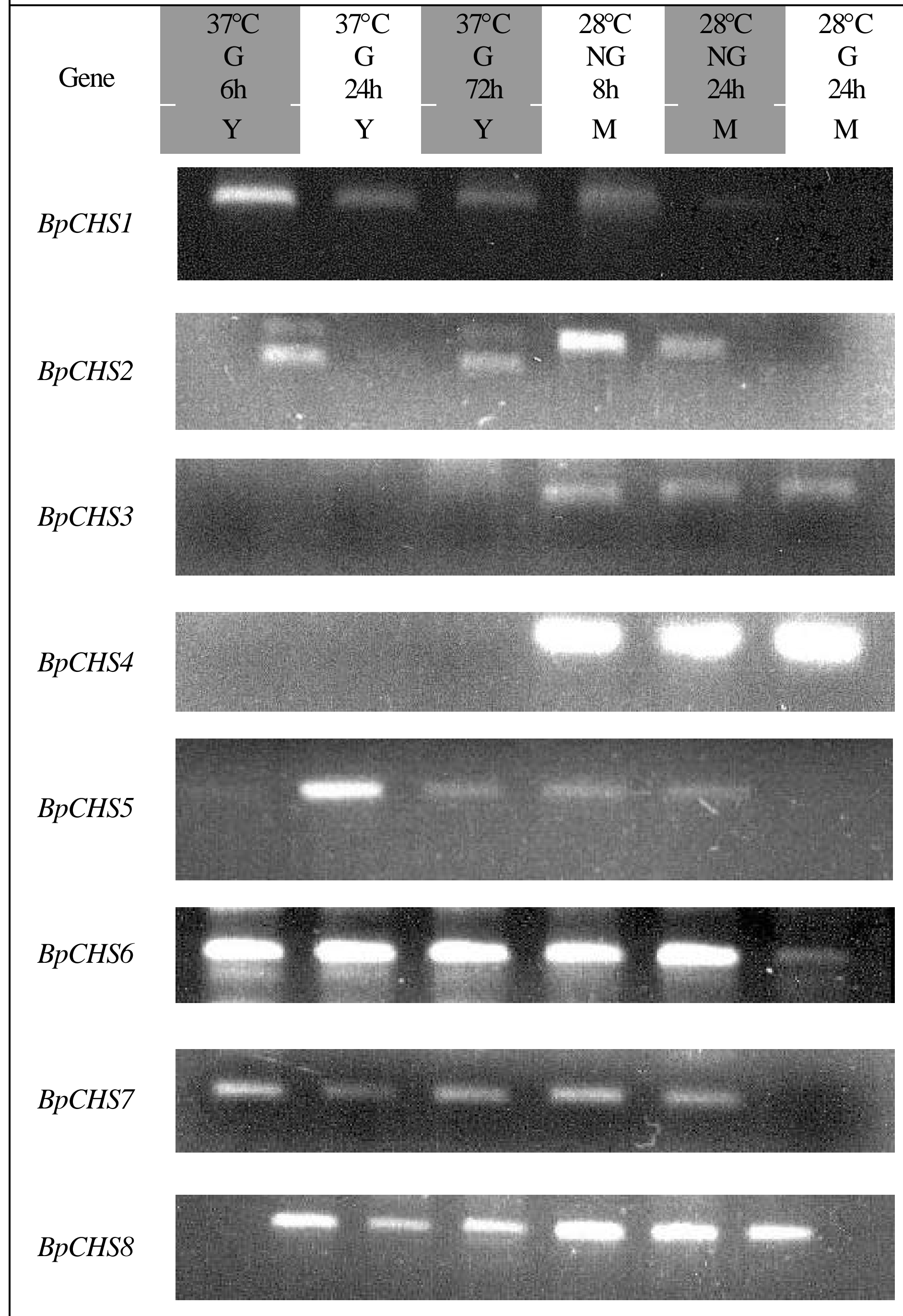
(Note: Lanes 1-6 for each southern are: Hind III, XhoI, BamH1, XbaI, PstI and EcoR)



The cells grown under the above mentioned different conditions were harvested at different time points and the RNA was extracted as described in Section 2.2.6. The extracted RNA was then used to carry out RT-PCR reactions as described in the Section 2.3.6. The products of RT-PCR were run on an agarose gel. The presence of RT-PCR reaction products on the gel corresponding to particular genes indicated the transcription of that gene. The results of the study are shown in Figure 5.4. Expression of different gene products was compared through the visual observation of ethidium bromide stained gel.

BpCHS1 showed high levels of expression only during the early stages of yeast phase growth at 37°C in YP (0.5% glucose) medium. Its expression was poor under all other conditions studied. *BpCHS2* was most prominent during the exponential phase of the Y → M transition at 28°C in YP (no glucose) medium. A moderate level of expression of BpCHS2 was observed in the early stages of yeast phase growth at 37°C in YP (0.5% glucose) medium and the stationary phase at 28°C in YP (no glucose) medium. *BpCHS3* and *BpCHS4* genes were expressed only during mycelium phase growth. *BpCHS3* was expressed in moderate levels while *BpCHS4* was expressed in high levels in both the presence and absence of glucose. *BpCHS5* was expressed in significant levels only in the early stationary phase during yeast phase growth. *BpCHS6* is expressed at high levels in all cases except for Y→ M transition in the presence of glucose where it is expressed in low levels. *BpCHS7* gene is expressed in low to moderate levels in all cases except for Y→ M transition in the presence of glucose where it is not expressed at all. *BpCHS8* was expressed under all conditions studied at moderate to high levels.

Figure 5.4 Fxpression of chitin synthase genes



5.3 Discussion

In this study, eight distinct 200-600 bp long gene sequences were cloned by PCR amplification of *B. poitrasii* genome using primers directed towards conserved regions of chitin synthase genes. The primer set F1-R1, used previously for amplification of class I-III chitin synthases of various fungi (Bowen *et al.*, 1992), didn't yield any product. Similar observations were made by Miyazaki *et al.* (1993) for the case of *P. blackesleeanus*. This was probably due to the presence of an intron in the conserved region of the genome of zygomycetes. Hence another two primer sets were designed so as to avoid this intron region. These primer sets F4-R1 and F5-R1 amplified chitin synthase gene fragments homologous to class I-II chitin synthases. For class IV-V chitin synthases, primer sets F2-R2 and F3-R2 were used. The primer set F3 differs from F2 only in one nucleotide base, but these two primer sets are found to amplify totally different CHS in some filamentous fungi (Din *et al.*, 1996).

Multiple chitin synthase (CHS) genes have been observed in the literature for several fungi (Bowen *et al.,* 1992; Mellado *et al.,* 1995; Bulawa, 1993; Cid, 1995). For instance, five *CHS* genes have been reported in *S. cerevisiae*, four *CHS* genes have been reported in *C. albicans* while ten *CHS* genes have been reported in *P. blakesleeanus.* However, this is the first time that as many as eight distinct chitin synthase genes have been identified in *B. poitrasii*. Sequence analysis of these genes and comparison with known chitin synthase sequences showed that four of these genes (*BpCHS1-4*) belong to class I-II chitin synthases while the others belonged to class IV-V. Using the primer sets mentioned above, class III chitin synthases were not found in *B. poitrasii*.

Southern hybridization gave different patterns for the eight gene fragments. This confirmed that these eight genes were indeed distinct and that the class IV and V

genes were not just the products of downstream amplification of class I-III genes. Multiple bands were observed for the case of *BpCHS1*, *BpCHS5, BpCHS6*, *BpCHS7* and *BpCHS8*. Multiple bands on southern blots can be explained to a certain extent as the products of cross hybridization reactions of probes. It also suggests a possibility of gene duplication for CHS genes in *B. poitrasii*. Similar observations have been made by Motoyama *et al.* (1994) and Thomsen and Beauvais (1995) for class II CHS in another zygomycete, *R. oligosporus*

An unrooted phylogenetic tree constructed using class II *CHS* genes from different fungi showed that the class II *CHS* genes clearly separated into three clusters belonging to ascomycete, zygomycete and deuteromycete. This is consistent with the traditional taxonomy methods based on morphological features. The phylogenetic tree showed that the chitin synthase genes of *B. poitrasii* are similar to other chitin synthases from zygomycetes viz., *RoCHS*, *PbCHS*, *McCHS* and *RrCHS*. A similar phylogenetic tree to study the taxonomic relatedness of fungi has also been prepared using chitin synthase genes from *Metarhizium anisiopliae var anisopliae* (Nam *et al.*, 1998).

RT-PCR was used to study the differential expression of *BpCHS* genes during different stages of growth under different nutritional and temperature conditions. Fungal growth was monitored through visual observations as well as by measuring the dry weight of cells and glucose consumption. It was observed that more genes were expressed when the cells were grown in mycelium favouring conditions compared to yeast-favouring conditions. This can be correlated to the fact that the mycelium form cells of this fungus contains 1.5 times more chitin as compared to that of the yeast form cells (Khale and Deshpande, 1992) and also that the chitin synthase activities are higher for the mycelium-form cells as compared to that for the yeast-form

cells(Chapter 4; Table 5.2). *BpCHS3* and *BpCHS4* genes were found to express exclusively in the mycelium-from cells. *BpCHS1* and *BpCHS5* expressed prominently in the young yeast cells. *BpCHS8* was expressed constitutively. Apart from showing form-specific expression of genes, CHS genes also showed differential expression depending upon growth phase. *BpCHS1* is expressed in high levels in young yeast cells while *BpCHS5* is expressed in high levels in older yeast cells. This difference in expression may be due to the requirement of the cell for different types of chitin synthases at different stages of growth. In *S. cerevisiae* and *C. albicans* specific chitin synthases have been shown to be involved in septum formation (Bulawa, 1993). *CHS* genes also expressed differently depending upon the nutritional and environmental conditions. *BpCHS2* and *BpCHS6* showed high levels of expression in mycelium form cells in the absence of glucose but not in the presence of glucose. It is interesting that more number of genes were expressed during Y→ M in the absence of glucose than in the presence of glucose even at 24 h after all the glucose has been consumed and the cells had reached stationary growth phase under both conditions. This indicated that chitin synthase gene expression at any given time was not only a function of the current growth conditions but was also influenced by the chitin deposition pattern followed till then.

The results relating to gene expression during Y → M transition in the absence of glucose are compiled and summarized in Table 5.2. During Y→ M transition, *BpCHS6* and *BpCHS8* were expressed in moderate to high levels at all stages of the transition including the yeast innoculum. *BpCHS5* is expressed in high levels only in the yeast innoculum. Therefore, it is possible that *BpCHS5* codes for chitin synthases that are involved in yeast-form specific growth processes such as budding. *BpCHS2* shows high levels of expression in the exponential phase but the levels of expression

decreased in the stationary phase. Therefore, it is likely that *BpCHS2* codes for chitin synthases that are involved in germ tube formation and hyphal tip growth.

Table 5.2: *CHS* gene expression during yeast to mycelium transition in the absence of glucose at 28°C.

	0 h	6-8 h	24 h
Morphology	Yeast	60% cells show germ tube formation	Mycelium
Chitin synthase activity	Low	Maximum	Maximum
CHS genes: High level of expression (Figure 5.4)	*BpCHS5* *BpCHS6*	*BpCHS2* *BpCHS4* *BpCHS6* *BpCHS8*	*BpCHS4* *BpCHS6* *BpCHS8*
CHS genes: Moderate level of expression (Figure 5.4)	*BpCHS8*	*BpCHS3* *BpCHS7*	*BpCHS2* *BpCHS3* *BpCHS7*
CHS genes: Low level of expression (Figure 5.4)	*BpCHS1* *BpCHS7*	*BpCHS1* *BpCHS5*	*BpCHS5*

The expression of multiple chitin synthase genes in *B. poitrasii* at different stages of growth and under different growth conditions has been studied. These observations will be useful to understand the role of cell wall metabolism, particularly chitin synthesis, in the dimorphic behaviour of *B. poitrasii*.

Chapter 6: Conclusion

In this thesis, the role of the cell wall on dimorphism in the zygomycetous fungus, *Benjaminiella poitrasii*, was studied using three different approaches. In the first approach, the regeneration of protoplasts of *B. poitrasii* under different dimorphism triggering conditions was studied to ascertain the role of cell wall components and metabolic processes in the determination of morphological outcome. The second approach involved biochemical studies on chitin synthases from yeast- and mycelium form cells. The effect of various inhibitors of regulatory mechanisms involved in chitin synthesis was also studied. In the third approach, the chitin synthase genes of *B. poitrasii* were identified and their differential regulation during dimorphic transition was studied.

Our studies on the protoplast regeneration showed that the protoplasts from both yeast and mycelial cells formed irregular masses during the initial 10-12 h possibly due to the non-regulated cell wall synthesis. The morphological distinction (yeast or mycelium) was seen after 36h. This highlighted the importance of initial cell wall synthesis in determining the cell wall deposition pattern. Protoplast regeneration studies were carried out in the presence of specific inhibitors for different processes involved in cell wall metabolism such as chitin synthesis/ degradation, mannan synthesis, protein synthesis and regulatory events for different biochemical pathways in the cell. In the presence of cycloheximide (protein synthesis inhibitor, 3-20 μM), no differentiation either in the yeast-or mycelial-form up to 36 h was noted. The presence of nikkomycin (chitin synthase inhibitor, 5-20 μM) led to the formation of deformed cells under mycelium favoring conditions while that of phenyl methyl sulfonyl fluoride (serine protease inhibitor, 0.5-5.5 mM) led to the formation of deformed cells under yeast- favoring conditions. In the presence of glucono-δ- lactone (glycosidase inhibitor, 2-30 mM) only yeast- form cells were observed under all

regeneration conditions. The glucan and mannan synthase, Ca-calmodulin, cAMP-dependent protein kinase inhibitors studied did not affect the morphological outcome. Thus, among all the tested cell wall synthesis inhibitors, chitin metabolism inhibitors showed a distinctive effect on the regeneration of protoplasts suggesting that the respective enzymes significantly contribute in determining the morphological outcome of a dimorphic fungus *B. poitrasii.*

The protoplast regeneration studies indicated that chitin metabolism contributed significantly in the morphological outcome. Therefore, further studies on the biochemical properties of membrane bound chitin synthases from *B. poitrasii* were undertaken. The mixed membrane fractions (MMF) isolated from yeast and mycelium-form cells of *B. poitrasii* were used to study the properties of membrane bound chitin synthases. Chitin synthase activity increased during yeast to mycelium transition, reaching the maximum level at 6h and remained practically unchanged thereafter. This observation correlated well with the earlier report that the cell walls of mycelium-form cells of *B. poitrasii* have much higher levels of chitin and chitosan compared to yeast-form cells (Khale and Deshpande, 1992).

Different mechanisms are suggested to be regulatory for chitin synthesis in fungi. These include: activation of zymogenic chitin synthases by trypsinisation, phosphorylation- dephosphorylation, membrane stress, involvement of chitinases and chitin synthase inhibitors in controlling chitin synthesis (Gooday, 1995; Adams *et al.*, 1993; Wenke *et al.*, 1993; Deshpande *et al.*, 1997). Earlier studies have shown that chitin synthase from yeast-form cells of *B. poitrasii* was more zymogenic as compared to that from mycelium-form cells. In this study, metal ions, like Mg^{2+}, Co^{2+} and Mn^{2+} were found to be effective as activators for chitin synthases from both yeast and mycelium-form cells. The addition of H-7, an inhibitor of cAMP-dependant

protein kinases, did not have any significant effect on the chitin synthase activity of mycelium-form cells but decreased chitin synthase activity of yeast-cells slightly. On the other hand, TFP (a calcium calmodulin inhibitor) completely inhibited chitin synthase activity in both yeast- and mycelium-form cells thus indicating that primary signal transduction mechanism associated with chitin synthase activity involves calcium calmodulin dependant kinases. However, further experimentation with the purified enzymes is necessary to ascertain the role of phosphorylation in the activation of chitin synthases in *B. poitrasii*. Membrane solubilisation of mixed membrane fractions of yeast- and mycelium- form cells using digitonin, Triton X-100 and Tween 80 resulted in very high levels of activation of the chitin synthases. The isoelectric focussing experiment with digitonin solubilized mixed membrane fractions (MMF) of yeast- and mycelium-form cells resolved chitin synthase activities into 3 peaks for yeast MMF and 4 peaks for mycelium MMF.

The differences in activity, properties and regulation of chitin synthases in yeast- and mycelium-form cells and the importance of chitin metabolism in the determination of morphological outcome immediately suggested the possibility of using chitin synthase as a target for the development of antifungal drugs. With this in mind, three different tests were used for the initial screening of inhibitor preparations from various sources for the presence of chitin synthase inhibitors. These tests were hyphal tip-bursting, effect on germ tube formation during yeast-mycelium transition in *B. poitrasii* and direct measurements of chitin synthase activity of yeast- and mycelium-form cells. Cell free extracellular broths of various microbial cultures grown in an inhibitor-production medium were screened. Hyphal tip bursting (HTB) was the test used for the screening of the cell wall metabolism inhibitors. The addition of these culture-filtrates inhibited growth and/or cell wall metabolism. Cell free

extracellular broth of *Chaetomium* sp., *Volutella* sp. and *Sclerotium* sp. inhibited chitin synthase activity from mycelium-form cells of *B. poitrasii.* In the presence of cell free extracellular broth of *Chaetomium* sp., *Volutella* sp. and *Sclerotium* sp, reduction in the germ tube formation as compared to the control was observed. Hyphal tip bursting in *B.poitrasii* was observed only in the presence of the cell free extracellular broth of *Chaetomium* sp.

The extracellular culture broths of *Chaetomium* sp and *Volutella* sp were taken up for evaluation as potential inhibitors of chitin synthase activity in selected pathogenic fungi - *Candida albicans*, *Sclerotium rolfsii* and *Aspergillus niger*. The chitin synthase activity from *C. albicans* was inhibited by the extracellular broth of *Chaetomium* sp while the chitin synthase activity in *A. niger* was inhibited by the extracellular broth of *Volutella* sp.

Differences in the properties of chitin synthases of yeast- and mycelium-form cells observed in the biochemical studies indicated the possibility of multiple chitin synthase types and hence, multiple chitin synthase genes in *B. poitrasii.* Differential expression of chitin synthase genes offers yet another opportunity to influence dimorphic transition. The chitin synthase (CHS) genes of a variety of fungi have been well studied and are found to be present in the multiple forms (Bowen *et al.,* 1992; Mellado *et al.,* 1995; Bulawa, 1993; Cid, 1995). The differential expression of the CHS genes during morphogenesis has also been studied for the case of *C. albicans*, *Paracoccidiodis brasiliensis* and *Phycomyces blackesleeanus* (Munro *et al.,* 1998; Nino-Vega *et al.,* 1999; Miyazaki *et al.*, 1997). Molecular studies on chitin synthase genes performed as part of this work showed the presence of at least eight chitin synthase genes in the genome of *B. poitrasii.* These genes were labeled as *BpCHS1-8.* Sequence determination and analysis of these genes showed that these genes belonged

to chitin synthase classes I, II, IV and V. The phylogenetic tree constructed (using the CHS gene sequences) to study the evolutionary relationship between *B. poitrasii* and other fungi revealed that *B. poitrasii* is closely related to *Rhizopus oligosporus* and *Phycomyces blakesleeanus.* Expression of these genes was studied through RT-PCR. Different chitin synthase genes were found to be differently expressed during various stages of dimorphic transition and growth. *BpCHS3* and *BpCHS4* expressed exclusively in the mycelium-from cells. *BpCHS1* and *BpCHS5* expressed prominently in the young yeast cells. *BpCHS8* was the only gene that was expressed constitutively.

Thus, during the course of this work, cell wall metabolism in general and chitin metabolism in particular was shown to be important in determining the morphological outcome during dimorphic transition in *B. poitrasii*. Further biochemical and molecular studies of chitin synthases showed their multiplicity and different biochemical characteristics that significantly contributed in the dimorphic behavior of *B. poitrasii*. The use of chitin synthesis as a potential target for the antifungal drugs was also evaluated.

Chapter 7: References

Literature Cited

- Adams DJ, Causier BE, Mellor KJ, Keer V, Milling R, Dada J (1993) In Chitin enzymology, (Muzzarelli RAA eds) Eur Chitin Soc, Ancona, pp.15-25.
- Adams DJ, Gooday GW (1983) In Systemische fungizide und antifungale verbindungen, Academie -Verlag, Berlin, pp 39-45.
- Ainsworth and Bisby (1995) Dictionary of Fungi, CABI publishing, New York.
- Aronson JM (1965) In: The fungi- An advanced treatise Vol IV. (Ainsworth GC, Sussman AS eds) Academic press, New York, pp 49-76.
- Arroyo-Begovich A, Ruiz-Herrera J (1979) J Gen Microbiol 113:339-345.
- Aufauvre-Brown A, Mellado E, Gow NAR, and Holden DW (1997) Fungal Genet Biol 21:141-152.
- Au-Young J, Robbins PW (1990) Mol Microbiol 4:197-207.
- Bailey DA, Feldmann PJ, Bovey M, Gow NA, Brown AJ (1996) J Bacteriol 178:5353-60.
- Bartnicki- Garcia S (1968). Ann Rev Microbiol 22:99
- Bartnicki-Garcia S (1980) In: Advances in protoplast research. (Ferenczy L, Farkas GL eds) Akademiai kiado, Budapest, Pergamon Press, Oxford, pp. 475-484.
- Bartnicki-Garcia S (1973) Symposium of Society for General Microbiology 23:245-67.
- Beth Din A, Specht CA, Robbins PW, Yarden O (1996) Mol Gen Genet 250:214-222.
- Beth-Din A, Yarden O (1994) Microbiology 140:2189-2197
- Binding H, Weber HJ (1974) Mol Gen Genet 135: 273-276

- Binks PR, Robson GD, Goosey MW, Humphreys A, Trinci APJ (1990) J Gen Microbiol 137: 615-620.
- Bolker M, Urban M, Kahmann R (1992) Cell 68:441-450.
- Borgia PT, Iartchouk N, Riggle PJ, Winter KR, Koltin y, Bulawa CE (1996) Fungal Genet Biol 20:193-203.
- Boucher I , Dupuy A, Vidal P, Neugebauer WA, Brzezinski R (1992) Appl Microbiol Biotechnol 138:188-193
- Bowen, A. R., Chen- Wu, J.L., Momany, M., Young, R., Szanizlo, P.J., Robbins, P.W. (1992) Proc. Natl. Acad. Sci. USA 89: 519-523.
- Braun PC, Calderone RA (1978) J Bacteriol 133:1472-7.
- Braun PC, Calderone RA (1979) J Bacteriol 140:666-670.
- Brillinger GU (1979) Arch Microbiol 121:71-74.
- Briza P, Ellinger A, Winkler G, Breitenbach M (1990) J Biol Chem 265:15118-23.
- Brown AJP, Gow NAR (1999) Trends in Microbiol 7:333-338.
- Brown AJP (1994) In: Molecular biology of the pathogenic fungi: a laboratory manual (Maresca B, Kobayashi GS eds.) pp. 127-134. Telos Press, New York.
- Bulawa CE (1992) Mol Cell Biol 12:1764-1776.
- Bulawa CE, Miller DW, Henry LK, Becker JM (1995) Proc Natl Acad Sci USA 92:10570-10574.
- Bulawa CE, Slater M, Cabib E, Au-Young J, Sburlati A, Adair WL, Robbins PW (1986) Cell 46:213-225.
- Bulawa CE.(1993) Annu Rev Microbiol 47:505- 534.
- Cabib E (1987) Adv Enzymol Relat Areas Mol Biol 59:59-101.

- Cabib E (1991) Antimirob Agents Chemother 35:170-173.
- Cabib E, Drgonova J, Drgon T (1998) Ann Rev Biochem 67:307-333.
- Cabib E, Farkas V (1971) Proc Natl Acad Sci USA 68:2052-2056.
- Cabib E, Sburlati A, Bowers B, Silverman SJ (1989) J Cell Biol 108:1665-1672
- Camargo EP, Dietrich CP, Sonneborn D, Strominger JL (1967) J Biol Chem 242:3121-8.
- Cano-Canchola C, Sosa L, Fonzi W, Syphard P, Ruiz-Herrera J (1992) J Bacteriol 174:362-6.
- Carbonell LM (1969) J Bacteriol 100:1076-1082.
- Cassone A, Simonetti N, Strippoli V (1973) J Gen Microbiol 77:417-427.
- Chattaway FC, Wheeler PR, O'Reilly J (1981) J Gen Microbiol 123:233-40.
- Chattaway FW, Bishop R, Holmes MR, Odds FC, Barlow AJE (1973) J Gen Microbiol 75:97-109.
- Chen-Wu JL, Zwicker J, Bowen AR, Robbins PW (1992) Mol Microbiol 6:497-502.
- Chitnis MV, Deshpande MV (2001) A manuscript communicated to Microbiol Res.
- Choi W-J, Santos B, Duran A, Cabib E (1994) Mol Cell Biol 14:7685-7694.
- Cid VJ, Duran A, Rey F, Snyder MP, Nombela C, Sanches M (1995) Microbiol Rev 59:345-386.
- Cole GT,Sun SH (1985) In: Fungal Dimorphism (Szaniszlo PJ Eds) pp. 281-333 Plenum Press, New York.
- Craig GD, Campbell JM, Peberdy JF (1981) Trans Br Mycol Soc 77:579-85.
- Da Silva SP, Borges Walmsley MI, Pereira IS, Soares CM, walmsley AR, Felipe

MS (1999) Mol Microbiol 31:1039-50.

- Dahn U, Hagenmaier HJ, Hohne HI, Konig WA, Wolf G, Zahner H (1976) Arch Microbiol 107:143-60.
- Davila T, San-Blas G, San-Blas F (1986).J Med Vet Mycol 24:193-202.
- Davis B, (1985) In: Fungal protoplasts: Applications in Biochemistry and Genetics (Peberdy JF, Ferenczy L eds.) Marcel Dekker , New York, Basel, pp.45-71
- Debona M, GordeeRS (1994) Annu Rev Microbiol 48:471-97.
- Dermastia M, Komel R (1993) Period Biol 94: 241-243.
- Deshpande MV (1996) Indian J Med Microbiol 14:
- Deshpande MV (1998) Recent trends in Mycoses 55-63.
- Deshpande MV, O'Donnell R, Gooday GW (1997) FEMS Microbiol Lett 152:327-332
- Din AB, Specht CA, Robbins PW, Yarden O (1996) Mol Gen Genet 250:214-222.
- Dlugonski J, Sedlaczek L, Jaworski A (1984) Can J Microbiol 30: 57-62
- Drouhet E, Dupont B, Improvisi L, Lesourd M, Prevost MC (1990) J Med Vet Mycol 28:425-36.
- Eck RV, Dayhoff MO (1996) Atlas of protein sequence and structure 1996. National Biomedical Research Foundation, Silver Spring, Maryland.
- Eddy AA, Williamson DH (1959) Nature 183: 1101-1104
- Edwards JE (1991) New Engl J Med 324:1060-2.
- Egidy G, Paveto MC, Passeron S, Galvagno MA (1989) Exp Mycol 13:428-32.
- Elorza MV, Margui A, Rico H, Miragall F, Sentandreu R (1987) J Gen Microbiol

133:2315-2325

- Elorza MV, Rico H, Gozalbo D, Sentandreu R (1983) Ant van Leeuwenhoeck 49:457-469
- Elorza V, Marcilla A, Sanjuan R, Mormeneo S, Sentandreu R (1994) Arch Microbiol 161:145-151.
- Endo A, Kakiki K, Misato T (1970) J Bacteriol 104:189-196.
- Eveleigh DE, Sietsma JH, Haskins RH (1968) J Gen Microbiol 52: 89-97
- Eyssen H (1977) Agricultura 25: 21-44.
- Farkas V (1979) Microbiol Rev 43:117-144
- Farkas V (1985) In: Fungal protoplasts: applications in genetics and biochemistry(Peberdy JF, Ferenczy L, Eds) Dekker, New York Basel, pp 3-29.
- Farkas V (1990) Acta Biotechnol 10:225-238.
- Farkas V, Svoboda A (1980) Curr Microbiol 4:99-104.
- Felsenstein J (1985) Evolution 39:783-791.
- Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) version 3.5 p. Distributed by the author. Department of Genetics, University of Washington, Seattle.
- Ferve M (1979) In: Fungal walls and hyphal growth (Burnett JH, Trinci APJ eds) Cambridge University Press, Cambridge. pp. 225-63.
- Fiedler HP, Kurth R, Langharig J, Delzer J, Zahner H (1982). J Chem Tech Biotechnol 32:271-280.
- Finkelman MAJ, Zajic JE, Vardanis A (1980) Appl Environ Microbiol 39:923-925.
- Fitch WM (1971) Systematic Zool 20:406-416.

- Frost DJ, Brandt KD, Cugier D, Goldman R (1995) J Antibiot 48:306-10.
- Furter R, Rast DM (1985) FEMS Microbiol Lett 28:205.
- Gabriel M (1968) Folia Microbiol 13: 231-234
- Gabriel M, Kopecka M (1995) Microbiology (Reading, UK) 141:891-9
- Garrison RG (1978) Cytological and ultrastructural aspects of dimorphism. In: Fungal dimorphism (Szaniszlo PJ and Harris JL eds.), Plenum press, New York,pp.15-46.
- Ghormade (2000) A thesis submitted to University of Pune.
- Ghormade V, Deshpande MV (2000) Naturwissenschaften 87:236-40.
- Ghormade VS, Lachke SA, Deshpande MV (2000) Folia Microbiol 45:231-238.
- Gibson RK, Buckey CE, Peberdy JF (1976) Protoplasma 89:381-387.
- Glaser L, Brown DH (1957) J Biol Chem 228:729-42.
- Gooday GW (1977) J Gen Microbiol 99:1-11.
- Gooday GW (1979) In: Fungal walls and hyphal growth. (Burnett JH, Trinci APJ eds) Cambridge University Press, Cambridge, UK. pp. 203-223.
- Gooday GW (1989) In: Bioactive metabolites from microorganisms. (Burshell ME, Grafe U Eds) Elsevier, Amsterdam. pp. 139-150.
- Gooday GW (1990) In: Biochemistry of cell walls and membranes in fungi (Kuhn PJ, Trinci APJ, Jung MJ, Goosey MW, Copping LG Eds) Springer-Verlag, Berlin. Pp. 61-79.
- Gooday GW (1995) In: The growing fungus (Gow NAR, Gadd GM Eds.) Chapmann & Hall, London, pp. 43-62
- Gooday GW, de Rousset-Hall A (1975) J Gen Microbiol 89:137-45.
- Gooday GW, Brydon LJ, Chappell LH (1988) Mol Biochem Parasitol 29:223-

225.

- Gooday GW, de Rousset-Hall A, Hunsley D (1976) Trans Br Mycol Soc 67: 193-200.
- Gooday GW, Schofield DA (1995) Can J Bot 73(Suppl.1): S114-S121
- Gow LA, Selitrennikoff CP (1984) Curr Microbiol 11: 211-216.
- Gow NA (1993) Trends Microbiol 1:45-50.
- Gow NR (1995) In: The growing fungus (Gow NAR, Gadd GM eds.), Chapmann & Hall, London, pp. 403-422.
- Gow NAR, Hube B, Bailey D and 9 other authors (1995) Can J Bot 73(Suppl 1) S335-S342.
- Gow NAR, Robbins PW, Lester JW, Brown AJP, Fonzi WA, Chapman T, Kinsman OS (1994) Proc Natl Acad Sci USA 91:6216-6220.
- Gozalbo D, Dubon F, Sentandreu R (1985) FEMS Microbiol Lett 26:59-63.
- Graybill GR (1989) Eur J Clin Microbiol Infect Dis 8:402-12.
- Harold FM (1995) Microbiol 141:2765-2778
- Hector RF, Braun PC (1987) In: Recent trends in the discovery, development and evaluation of antifungal agents. (Fromtling RA ed) Prous. Science publishers, Barcelona. pp. 369-382.
- Herrera-Estrella L, Ruiz-Herrera J (1983) Exp Mycol 7:362-369.
- Horsch M, Rast DM (1993) In: Chitin enzymology (Muzzarelli RAA Ed) European Chitin Society, Ancona, Italy. pp. 47-56.
- Horsch M, Sowdhamini R (1996) In: Chitin Enzymology vol 2. Muzzarelli RAA (ed) Italy: Atec Edizioni pp. 447-458.
- Howard DH, Herndon RL (1961) J Bacteriol 81:522-527.

- Hube B, Monod M, Schofield DA, Brown AJ, Gow NA (1994) Mol Microbiol 14:87-99.
- Hunsley D, Gooday GW (1974) Protoplasma 82:125-146.
- Inderlied CB, Sypherd PS (1978) J Bacteriol 133:1282-1286.
- Isaac S, Ryder NS, Peberdy JF (1978) J Gen Microbiol 105: 45-50.
- Isono K, Nagatsu J, Kobinata K, Sasaki K, Susuki S (1967) Agric Biol Chem 31:190-199.
- Jan YN (1974) J Biol Chem 249:1973-1979.
- Kamada T, Bracker CE, Bartinicki-Garcia S (1991) J Gen Microbiol 137: 1241-1252.
- Kapteyn JC, Van Den Ende H, Klis FM (1999) Biochem Biophy cta 1426:373-383.
- Katz D, Rosenberger RF (1971) J Bacteriol 108:184-190.
- Kelkar HS, Shankar V, Deshpande MV (1990) Enzyme Microb Technol 12: 510-514.
- Keller FA, Cabib E (1971) J Biol Chem 246:160-6.
- Kerkenaar A, Barug D (1984) Pestic Sci 15:199-205.
- Khadori N (1989) Eur J Clin Microbiol Infect Dis 8:33-51.
- Khale A (1990) A PhD thesis submitted to University of Pune.
- Khale A, Srinivasan MC, Deshmukh SS, Deshpande MV (1990) Ant van Leeuwenhoek 57:37-41.
- Khale A, Deshpande MV (1992) Ant van Leewenhoek. 62, 299-307.
- Khale-Kumar A and Deshpande MV (1993) J Bacteriol 175:6052-6055.
- Kobinata K, Uramoto M, Mizuno T, Isono K (1980) J Antibiot 33:244-246.

- Kollar R, Petrakova E, Ashwell G, Robbins PW, Cabib E (1995) J Biol Chem 270:1170-1178.
- Konomi M, Webster D, Osumi M (1999) Nippon Joshi Daigaku Kiyo, Rigakubu 7: 33-43 (Japanese, Chem. Abstr. 132:134480, 2000)
- Kovac l, Subik J (1970) Acta Fac Med Univ Brun 37:91-94.
- Kreger DR, Kopecka M (1975) J Gen Microbiol 92:207-220.
- Kulkarni RK, Nickerson KW (1981) Exp Mycol 5:148-54.
- Lane JW, Garrison RG (1970) Can J Microbiol 16:747-749.
- Laska BA, Borgia PT (1980) J Bacteriol 141:565-569
- Liu H, Styles CA, Fink GR (1994) Science 262:1741-4.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) J Biol Chem 193:265-275
- Machida S, Saito M (1993) J Biol Chem 268:1702-1707.
- Madzak C, Blanchin-Ronald S, Cordero-Otero RR, Gaillardin C (1999) Microbiol 145:75-87.
- Magee PT (1997) Science 277:5322-5223.
- Maresca B, Carratu L, Kobayashi GS (1994) Trends Microbiol 2:110-114
- Maresca B, Kobayashi, GS (1989) Microbiol Rev 53:186-209.
- Maresca B and Kobayashi GS (2000) Contrib Microbiol 5:201-16.
- Martinez-Cadena G, Ruiz-Herrera J (1987) Arch Microbiol 148:280-285.
- Matsunobu T, Hiruta O, Nakagawa K, Murakami H, Miyadoh S, Uotani K, Takabe H, Satoh A (1996) Meiji Seika Kenkyu Nenpo 35:28-50 (Japanese, Chem. Abstr: 126, 235083, 1997).
- McCarthy PJ, Troke PF, Gull K (1985) J Gen Microbiol 131:495-503.

- McCleary BV (1978) Carbohyd Res 67:213-21.
- McMurrough I, Bartnicki-Garcia S (1971) J Biol Chem 246:4008-16.
- Mellado E, Aufauvre-Brown A, Specht C, Robbins P, Holden D (1995) Mol Gen Genet 246:353-359.
- Mellado E, Aufauvre-Brown A, Gow NAR, Holden DW (1996) Mol Microbiol 20:667-679.
- Mio T, Yabe T, Sudoh M, Satoh Y, Nakajima T, Arisawa M, Yamada-Okabe H (1996) J Bacteriol 178:2416-2429.
- Miyaji M, Nishimura K (1977) Mycopathol 60:73-78.
- Miyazaki A, Ootaki T (1997) J Gen Appl Microbiol 43:333-340.
- Miyazaki A, Momany M, Szanizlo PJ, Jayaram M, Ootaki T (1993) Gene 134:129-134.
- Mizoguchi J, Saito T, Mizuno K, Hayano K (1977) J Antibiot 30:308-13.
- Montgomery GWG, Gooday GW (1985) FEMS Microbiol Lett 27:29-33.
- Mormeneo S, Rico H, Iranzo M, Aquado C, Sentandreu R (1996) Arch Microbiol 166:327-35.
- Motoyama T, Sudoh M, Horiuchi H, Ohta A, Takagi M (1994) Biosci Biotech Biochem 58:1685-1693.
- Muller H, Furter R, Zahner H, Rast DM (1981) Arch Microbiol 130: 195-197.
- Munro CA, Gow NAR (1995) In: Antifungal agents discovery and mode of action (Dixon GK, Copping LG, Hollomon DW, eds) Oxford: BIOS Scietific Publisher Ltd. pp 161-171.
- Munro CA, Schofield DA, Gooday GW, Gow NAR (1998) Microbiol 144:391-401.

- Munro C.A, Winter K, Buchan A, Henry K., Becker JM, Brown AJP, Bulawa CE, Gow NAR (2001) Molec. Microbiol. 39: 1414 - 1426.
- Musilkova M, Fencl Z (1966) Folia Microbiol 11:472-474.
- Musilkova M, Fencl Z (1968) Folia Microbiol 13:235-239.
- Nagahashi S, Sudoh M, Ono N, Sawada R, Yamaguchi E, Uchida Y, Mio T, Tagagi M, Arisawa M, Yamada-Okabe H (1995) J Biol Chem 270:13961-13967.
- Nam J-S, Lee D-H, Lee K-H, Park H-M, Bae K-S (1998) FEMS Microbiol Lett 159:77-84.
- Necas O (1971) Bacteriol Rev 35:149-170.
- Necas O (1980) In: Advances in protoplast research (Ferenczy L, Farkas GL eds). Akademiai Kiado, Budapest: Pergammon Press, Oxford. pp 151-161.
- Necas O, Svoboda A (1981) In: Yeast cell envelopes: Biochemistry, Biophysics and Ultrastructure. vol II (Arnold WN, Ed) CRC Press, Boca Raton, Fla. pp 105-128.
- Necas O, Svoboda A (1985) In: Fungal protoplasts: Applications in biochemistry and genetics (Peberdy JF, Ferenczy L,Eds), Marcel Dekker, New York, Basel, pp. 115-133
- Nelson N (1944) J Biol Chem 153:375-380
- Niimi M, Niimi K, Tokunaga J, Nakayama H (1980) J Bacteriol 142:1010-14.
- Nino-Vega GA (1997) A PhD thesis submitted to the University of Aberdeen.
- Nino-Vega GA, Buurman ET, Gooday GW, San-Blas G, Gow NAR (1998) Yeast, 14: 181-187.
- Nino-Vega GA, Munro CA, San-Blas G, Gooday GW, Gow NA (2000) Med Mycol 38:31-39.

- Odds FC (1988) Candida and candidosis, Balliere Tindall, London.
- Oki T (1992) Boston Birkhauser pp:64-87.
- Oki T, Konishi M, Tomatsu T, Tomita K, Saitoh K (1988) J Antibiot 41:1701-4.
- Oki T, Tenmyo O, Hirano M, Tomatsu K, Kamei H (1990) J Antibiot 43:763-70
- Orlean P (1987) J Biol Chem 262:5732-39.
- Orlowki M (1991) Microbiol Rev 234-258.
- Ortega JKE, Gamow RI, Ahlquist CN (1975) Plant Physiol 55:333-337.
- Pancaldi S, Poli F, Dall'Olio G, Vannini GL (1984) Arch Microbiol 137:185-187.
- Pancaldi S, Poli F, Dall'Olio G, Vannini GL (1988) Microbios 54:81-86.
- Pardo M, Monteoliva L, Pla J, Sanchez M, Gil C, Nombela C (1999) Yeast 15:459-472.
- Pearson WR (1990) Meth Enzymol 183:63-98.
- Peberdy JF (1976) In: Microbial and plant protoplasts. (Peberdy JF, Rose AH, Rogers HJ, Cocking EC Eds) Academic Press, New York pp 39-50.
- Peberdy JF (1979a) Ann. Rev. Microbiol. 33:31-39
- Peberdy JF (1979b) In: Fungal walls and hyphal growth (Burnett JH, Trinci APJ, Eds) Cambridge University Press, Cambridge. pp.49-69.
- Peberdy JF (1989) Mycol Res 93:1-20
- Peberdy JF (1990) In: Biochemistry of cell walls and membranes in fungi. (Kuhn PJ, Trinci APJ, Jung MJ, Goosey MW eds) Berlin/New York: Springer-Verlag. pp5-24
- Peberdy JF, Moore PM (1975) J Gen Microbiol 90:228-36.
- Pine L, Webster RE (1962) J Bacteriol 83:149-157.
- Poli F, Pancaldi S, Vannini GL (1986) Eur J Cell Biol 42:79-83.

- Popov B, Reuter G, Meyer HW (1980) Z Alleg Mikrobiol 20:47-62.
- Porter CA, Jaworski EG (1966) Biochemistry 5:1149-54.
- Ramirez-Leon IF, Ruiz-Herrera J (1972) J Gen Microbiol 72: 281-290
- Rast DM, Horsch M, Furter R, Gooday GW (1991) J Gen Microbiol 137:2797-2810.
- Reyes E, Novaes - Ledieu M, Garcia - Mendoza C (1983) Curr Microbiol 9:301-304
- Reyes F, Perez-Leblic MI, Martinez MJ, Lahoz R (1984) FEMS Microbiol Lett 24:281-283
- Rico H, Miragall F, Sentandreu R (1985) Exp Mycol 9:241-253.
- Rodriguez Aguirre MJ, Garcia Acha I, Villanueva JR (1964) J Microbiol Serol 30:33-44.
- Roncero C, Duran A (1985) J Bacteriol 163:1180-1185.
- Ruiz-Herrera J, Bartnicki-Garcia S (1976) J Gen Microbiol 97:241-249.
- Ruiz-Herrera J (1992) Fungal cell wall: Structure, Synthesis and assembly. CRC press, Boca Raton, pp. 5-22.
- Sabie FT, Gadd GM (1992) Mycopathol 119:147-56.
- Sabie FT, Gadd GM (1989) Mycopathol 108:47-54.
- Sakuda S (1996) In: Chitin Enzymology (Muzzarelli RAA, Ed), Atec Edizioni, Italy, pp.203-12.
- Sakuda S, Isogai A, Matsumoto S, Susuki S (1986) Tetrahedron Lett 27:2475-2478.
- Sakuda S, Isogai A, Matsumoto S, Susuki S (1987) J Antibiot 40:296-300.
- Sakurai T, Cheeptham N, Mikawa T, Yokota A, Tomita F (1999) J. Antibio 52:

508-11.

- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Press, USA.
- San-Blas G (1979) Exp Mycol 3:249.
- San-Blas and San-blas (1983) CRC Crit Rev Microbiol 11: 101-127.
- Sburlati A, Cabib E (1986) J Biol Chem 261:15147-15152.
- Schofield DA (1994) A PhD thesis submitted to Dept of Molecular and Cell Biology, University of Aberdeen.
- Schwencke J, Magana-Schwencke N, Laporte J (1977) Ann Microbiol 128A:3-18.
- Selitrennikoff CP, Lilley BL, Zucker R (1981) Exp Mycol 5:155-161.
- Shaw JA, Mol PC, Bowers B, Silverman SJ, Valdivieso MH, Duran A, Cabib E (1991) J Cell Biol 114:111-123.
- Schulz BE, Kraepaelin G, Hinkelmann W (1972) J Gen Microbiol 82:1-13.
- Sietsma JH, Wessels JGH (1990) J Gen Microbiol 136:2261-65.
- Sietsma JH, Wessels JGH (1991) NATO ASI ser, SerH, 53(Fungal Cell Wall Immune Response): 81-95 (CA 116:147550)
- Silverman SJ (1989) Yeast 5:459-467.
- Silverman SJ, Sburlati A, Slater ML, Cabib E (1988) Proc Natl Acad Sci USA. 85:4735-4739.
- Smith TA, Barker JHA, Jung M (1990). J. Gen Microbiol 136: 985-992.
- Soll DR (1992) Clinical Microbiol Rev 5:183-203.
- Somashekar D, Joseph R (1996) Bioresource Technol 55:35-45.
- Somogyi M (1952) J Biol Chem 195:19.

- Spect CA, Liu Y, Robbins PW, Bulawa CE, Iartechouk N, Winter KR, Riggle PJ, Rhodes JC, Dodge CL, Culp DW, Borgia PT (1996) Fungal Genet. Biol. 20:153-167.
- Staneck JL, Roberts GD (1974) Appl Microbiol 28: 226-231
- Sternlicht E, Katz D, Rosenberger RF (1973) J Bacteriol 114: 111-123.
- Stewart PR, Rogers PJ (1978) In: The filamentous fungi. Vol 3-Developmental Mycology (Smith JE, Berry DR eds.) Edward Arnold pp. 165-196.
- Stoldt VR, Sonneborn A, Leuker CE, Ernst JF (1997) 16:1982-91.
- Sudoh M, Nagashaki S, Doi M, Ohta A, Takagi M, Arisawa M (1993) Mol Gen Genet 241: 351-358.
- Sullivan PA, McHugh NJ, Romana LK, Shepherd MG (1984) J Gen Microbiol 130:2213-2218
- Suresh K, Subramanyam C (1997) FEMS Microbiol Lett 150:95-100.
- Sutar *et al.* (1986)
- Suzuki S, Isono K, Nagutsa J, Mizutani T, Kawashima Y, Mizuno T (1965) J Antibiot 18:131-132.
- Svoboda A and Necas O (1974) Proceedings of the Fourth International Symposium on Yeasts, Vienna, pp. D13.
- Sypherd PS, Borgia PT, Paznokas JL (1978) In: Adv Microb Physiol (Rose AH and Morris JG, Eds) Academic press, New York 18, pp.67-104
- Szaniszlo PJ (1985) In: Fungal dimorphism (Szaniszlo PJ, Harris JL, Eds.), Plenum press, New York, pp. 3-11.
- Taft CS, Stark T, Selitrennikoff CP (1988) Antimicrob Agents Chemother 32:1901-3

- Takeuchi T, Hara T, Naganawa H, Okada M, Hamada M et al (1988) J antibiot 41:808-11.
- Tariq VN, Devlin PL (1996) Fungal Genetics Biol 20: 4-11.
- Thomas KR,Davis B (1980) Microbios 28:69-80.
- Thompson JD, Higgins DG, Gibson TJ (1994) Nucl Acid Res 22: 4673-4680.
- Thomsen L, Beauvais A (1995) FEMS Microbiology Lett 129:115-120.
- Toyama H, Yokoyama T, Shinmyo A, Okada H. (1984a) J Biotechnol 1:25-35.
- Toyama N, Ohara H, Ogawa K (1984b) Bull Faculty Agric Miyazaki Univ 31: 101-108.
- Trilla JA, Cos T, Duran A, Roncero C (1997) Yeast 13: 795-807.
- Tully M, Gilbert HA (1985) Gene 36: 235-240.
- Tyrrell D, MacLeod DM (1972) J Invertebr Pathol 19:354-360.
- Ukei T, Numata K, Sawada Y, Nishio M, Ohkuma H (1993) J Antibiot 46:455-64.
- Valdivieso MH, Mol PC, Shaw JA, Cabib E, Duran A (1991) J Cell Biol 114:101-109.
- van den Bossche H (1990) In: Biochemistry of cell walls and membranes in fungi.(Kuhn PJ, Trinci APJ, Jung MJ, Goosey MW, Copping LG eds) Spriger Verlag, Berlin. pp. 135-157.
- van der Valk P, Wessels JGH (1976) Protoplasma 90:65-87.
- Vermeulen CA, Raeven MBJM, Wessels JGH (1979) J Gen Microbiol 114:87-97.
- Vyas P, Deshpande MV (1989) J Gen Appl Microbiol 35:343-350
- Walsh TJ, Hathorn JW, Sobel JD, Merz WG, Sanchez V et al (1991) New Engl J

Med 324:1026-31.

- Watanabe M, Tohyama H, Hiratani T, Watabe H, Inoue S, Kondo S-I (1997) J Antibiot 50:1042-1051
- Waterfield WF, Sisler HD (1988). BioTechniques 6: 832-834
- Wenke J, Anke H, Sterner O (1993) Biosci Biotechnol Biochem 57:961-4.
- Wessels JGH (1986) International Rev Cytol 104:37-79.
- Wessels JGH (1990) In: Tip growth in plant (eds) Academic press pp 1-29.
- Wessels JGH, Mol PC, Sietsma JH, Vermeulen CA (1990) In: Biochemistry of cell walls and membranes in fungi. (Kuhn PJ, Trinci APJ, Jung MJ, Goosey MW, Copping LG, Eds) New York, Heidelberg, Berlin: Springer-Verlag pp.81-95.
- Wessels JGH, Sietsma JH (1979) In: Fungal walls and hyphal growth. (Burnett JH, Trinci APJ Eds) Cambridge University Press, Cambridge, pp 27-48.
- Wessels JGH, Sietsma JH (1981) In: Encyclopedia of Plant Physiology , vol 13B, Plant Carbohydrates II. (Tanner W, Loewus FA, Eds) Springer-Verlag, Berlin, pp 352-415.
- Woestemeyer A, Woestemeyer J (1998) Microbiol Res 153: 97-104
- Yanai K, Kojima N, Takaya N, Horiuchi H, Ohta A, Takagi M (1994) Biosci Biotech Biochem 58:1828-1835.
- Yarden J-C, Gonneau M, Sarthou P, Le Goffic F (1984) J Becteriol 160:884-888.
- Yarden O, Yanofsky C (1991) Genes Develop 5:2420-2430.
- Youatt J, Gow NAR, Gooday GW (1988) Protoplasma 146:118-126.
- Zinjarde SS, Pant A, Deshpande M (1998) Mycol.Res. 102:553.
- Zhu W-Y and Gooday GW (1992) Mycol. Res. 96: 371-377.

LIST OF PUBLICATIONS, PATENTS AND PRESENTATIONS

Publications

1. **M.V. Chitnis** and M. V. Deshpande. Isolation and regeneration of protoplasts from the yeast and mycelial forms of a dimorphic fungus *Benjaminiella poitrasii*: Possible role of chitin metabolism in determining morphological outcome during regeneration. (Manuscript communicated to *Microbiological Research*).
2. R.S. Patil, A. Deshpande, A. Natu, P. Nahar, **M.V. Chitnis**, V. Ghormade, R.S. Laxman, S. Rokade and M.V. Deshpande. Biocontrol of root infecting plant pathogenic fungus, *Sclerotium rolfsii* using mycolytic enzymes and chitin metabolism inhibitors singly and in combination. (In press, *Journal of Biological Control*)
3. **M.V. Chitnis**, C. A. Munro, A. J. P. Brown, N. A. R. Gow, G. W. Gooday and M. V. Deshpande. Chitin synthase genes of *Benjaminiella poitrasii* and their differential expression under dimorphism triggering conditions. (In preparation for *Fungal Genetics and Biology*).

Patents

1. M.V. Deshpande, R. Patil, **M.V. Chitnis** and R. Seeta Laxman. A process for the preparation of mycolytic enzymes containing mainly chitosanase. Submitted to CSIR to file Indian patent (NF 224/99).

2. M.V. Deshpande, M. Desai, **M.V. Chitnis** and R. Patil. A process for the preparation of mycolytic enzyme complex used for the isolation of fungal protoplasts. Submitted to CSIR to file Indian patent (NF 116/99).

Presentations

1. **M.V. Chitnis** and M.V. Deshpande. Use of Mycolytic Enzyme Complex of *Myrothecium verrucaria* in isolation of protoplasts from different fungal genera. Presented in the poster session of the Annual meeting of SBC(I), held at Vishakapatnam, India, December 1997.
2. V. Ghormade, **M.V. Chitnis** and M.V. Deshpande. Comparison of temperature and glucose as a morphogenetic trigger in yeast – mycelium transition of *B. poitrasii*. Presented at the Sixth International Mycological Congress – IMC6 at Jerusalem, Israel, August 1998.
3. **M.V. Chitnis**, V. Ghormade and M.V. Deshpande. Regulation of chitin metabolism in the dimorphic fungus *Benjaminiella poitrasii*. Presented at the Third International Symposium on Chitin Enzymology and Fourth Conference of the European Chitin Society at Senigallia, Ancona, Italy, May 2001.