BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF PROGRAMMED CELL DEATH IN DIMORPHIC FUNGUS *BENJAMINIELLA POITRASII*

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Contents

List of Tables

List of Figures

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CERTIFICATE

Certified that the work incorporated in the thesis entitled "**Biochemical and molecular characterization of programmed cell death in dimorphic fungus *Benjaminiella poitrasii***" submitted by Mr. Fazal Shirazi was carried out under our supervision. Such material as has been obtained from other sources has been acknowledged in the thesis.

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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled, **"Biochemical and molecular characterization of programmed cell death in dimorphic fungus *Benjaminiella poitrasii*"** submitted by me for the degree of Doctor of Philosophy to the University of Pune, is the record of work carried by me at Biochemical Sciences Division, National Chemical Laboratory, Pune - 411008, Maharashtra, India, under the supervision of Dr. M. V. Deshpande (research guide) and Prof. W. N. Gade (co-guide). The work is original and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other university or other institute of higher learning. I further declare that the material obtained from other resources has been duly acknowledged in the thesis.

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List of Abbreviations

Abbreviation	Full Form
BSA	Bovine serum Albumin
CHA	Cyclohexylamine
d	Day
DAB	Diaminobutanone
dNTP	Deoxynucleoside triphosphate
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
h	Hour(s)
IC_{50}	50 % inhibitory concentration
µM	Micromolar
µg	Microgram
µl	Microliter
mM	Milimolar
min	Minute(s)
PMSF	Phenyl methyl sulphonyl fluoride
sec	Seconds
TCA	Trichloro acetic acid
TNBS	2,4,6-trinitrobenzenesulfonic acid
Tris	Tris (hydroxymethyl) aminomethane

ABSTRACT

Programmed cell death (PCD) is a regulated intracellular process that functions during normal cellular development, in response to stress and as defense against pathogen attack in plants and animals (Biella *et al*., 2002). In fungi it serves to eliminate cells that are incompetent for mating/ susceptible due to mutations/ aging or viral infections (Herker *et al*., 2004; Ivanovska and Hardwick, 2005; Reiter *et al*., 2005). Hamann *et al.* (2008) described the role of apoptotic processes in fungal pathogenicity, competitiveness, propagation, ageing and lifespan control. PCD is classified mainly into two categories: apoptosis and necrosis pathways.

Several antifungal agents *viz* acetic acid, viscosinamide, amphotericin B, farnesol etc. are known to induce PCD in *Candida albicans*, *Rhizoctonia solani*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, (Madeo *et al*., 2002; Phillips *et al*., 2003; Semighini *et al*., 2006a). The apoptotic inducers manifest there effects *via* the biochemical correlates of apoptosis such as caspases, superoxide dismutase (SOD, E.C 1.15.1.1) etc (Ramsdale, 2006).

Dimorphic fungi are useful model systems to understand the eukaryotic developmental processes as they differentiate from unicellular yeast to filamentous form for survival and proliferation (Gow, 1995). In case of *C. albicans*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidiodes brasilensis*, *Ustilago maydis* and *Uromyces* sp. and other human and plant pathogens dimorphism is important in pathogenesis. Number of enzymes of C and N pathways, cell wall metabolism and sulphur metabolism are known biochemical correlates of these morphological transitions. A nonpathogenic, zygomycetous dimorphic fungus *Benjaminiella poitrasii*, was studied earlier to understand the mechanism of Y-H transition in response to temperature, pH and glucose. Furthermore the stable morphological (monomorphic yeast form) mutants can be used to understand the mechanism of differentiation (Khale *et al*., 1990).

In *B. poitrasii* it was already reported that different enzymes involved in ammonia assimilation and chitin synthesis such as NAD- (E.C 1.4.1.2)/NADP-glutamate dehydrogenase (E.C 1.4.1.4), chitin synthase (E.C 2.4.1.16), chitinase (E.C 3.2.1.14), *N*-acetylglucosaminidase (E.C 3.2.1.52) and polyamine synthesizing enzyme ODC (ODC, E.C 4.1.1.17) were biochemically correlated with Y-H transition (Khale *et al*., 1992; Ghormade *et al*., 2000; Chitnis *et al*., 2002; Ghormade *et al*., 2005a). Among these, the relative proportion of NADP-/NAD- dependent GDH regulated by phosphorylation or dephosphorylation was found to have a significant correlation with Y-H transition (Khale and Deshpande, 1993; Khale *et al*., 1992). Furthermore, *B. poitrasii* possessed three

GDHs, one requiring NAD while the other two were form specific which use NADP as a coenzyme (Amin *et al.*, 2004a). The quantitative relationship between these two enzymes was expressed as the GDH ratio (NADP-/NAD-GDH). Increase in the GDH ratio led to a change in morphology from H to the Y form. GDH ratio (≥0.15) was associated with Y form. Transition of the parent strain from the H to the Y form was preceded by an increase in the GDH ratio. Reverse trends were observed for Y-H transition, for which a lowering of the ratio occurred. Morphological shift from Y-H form was accompanied with the change in GDH and ODC activities. The NAD-GDH and ODC enzyme levels were lower in the *B. poitrasii* yeast form as compared to the hyphal form cells whereas the NADP-GDH enzyme levels were higher in yeast form cells (Khale *et al*., 1993; Ghormade *et al*., 2005a).

In fungi as differentiation is one of the mechanisms for survival and a number of biochemical correlates of differentiation and apoptosis are common, there is a possibility of a link between differentiation and apoptosis. If so, then understanding of differentiation, per se could be useful to design apoptosis inducers for the control of fungal pathogenesis.

An extensive literature survey on fungal and yeast apoptosis, their signaling pathways, genes and inducers of apoptosis is presented in the **Introduction (Chapter I)**. Dimorphism and enzymes involved in morphogenesis especially NAD-/NADP-GDH, GS, GOGAT and ODC were also presented to understand their correlation with apoptosis, if any.

Materials and methods (Chapter II) used during investigation were described. This chapter describes materials and methods used in study. The details of growth and maintenance of *B. poitrasii* and wine spoilage yeasts (*Dekkera bruxellensis*, *Metschnikowia pulcherrima*, *Zygosaccharomyces rouxii*, *Candida krusei*, *Debaryomyces hansenii* and *Pichia anamola*) are described. The microbiological and microscopic techniques used for growth and differentiation studies are discussed. Antifungal bioassays such as hyphal tip bursting (HTB), Y-H transition and disc diffusion method are discussed. Extraction, purification and identification of antifungal metabolite using biochemical and biophysical technique such as NMR, thin layer chromatography is accounted. Enzyme assays for estimation of GDH, ODC, SOD (E.C 1.15.1.1) and caspase-like activities -1 (E.C 3.4.22.36), -3 (E.C 3.4.22.56), -8 (E.C 3.4.22.61) are mentioned. Apoptosis detection assays as analyzed by exposure of phosphatidylserine by annexin V-FITC staining, DNA fragmentation by terminal deoxynucleotidyl transferase–

mediated dUTP nick end labeling (TUNEL), chromatin condensation by 4'6-diamino-2-phenylindole (DAPI), reactive oxygen species (ROS) production by dihydrorhodamine (DHR123) were described. Molecular techniques including isolation of genomic DNA, RNA, cDNA synthesis, PCR, cloning and DNA sequencing and analysis have been elaborated.

Biochemical studies of differentiation as pre-programmed cell death process in *B. poitrasii* was carried out (Chapter III). In the present investigation different effectors of the Y-H transition and its biochemical correlates *viz.* GDH and ODC were tested in *B. poitrasii*. The additives assessed were commercially available compounds like diaminobutanone (DAB, ODC inhibitor), cyclohexylamine (CHA, spermidine synthase inhibitor), isophthalic acid (NAD-GDH inhibitor), amphotericin B (ergosterol synthesis inhibitor), fluconazole (14α-demethylase inhibitor); phenazine-1-carboxamide (PCN, isolated and purified from *Pseudomonas* sp. B18); chemically synthesized compounds triazole linked β-lactam–bile acid conjugates (18B, 20B and 24 B) and tetrapeptide linked-cholic acid derivative (DS16), strobilurin derivative (PC229); acetic acid and H_2O_2.

During Y-H transition NAD-GDH activity increased by 11 fold whereas NADP-GDH activity decreased by 6 fold. In case of ODC, the activity increased by 4.5 fold. NADP-GDH ($r = -0.862$, $p = 0.0001$) and NAD-GDH ($r = 0.862$, $p = 0.0001$) activities correlated significantly with ODC activity. Y-H transition in *B. poitrasii* was associated with increase in polyamine levels (>1.5 fold of putrescine and spermidine and 4 fold of spermine levels higher in H cells than Y form cells). Similarly increase in polyamine levels during Y-H transition was also reported in case of other fungi such as *C. ulmi*, *Fusarium moniliforme*, *M. rouxii*, *Y. lipolytica* (Marshall *et al*., 1979; Calvo-Mendez *et al*., 1987; Guevara-Olvera *et al*., 1993).

The additives analyzed in this study inhibited Y-H transition (80-94%). At their inhibitory concentration the *in vitro* NAD-GDH activity decreased in case of isophthalic acid (16 mM), and acetic acid (20 mM), while NADP-GDH activity was unaltered. In presence of H_2O_2 (50 mM) and amphotericin B (4 µM) NADP-GDH activity was induced in Y form of *B. poitrasii*, while there was no change in NAD-GDH activity. Above observations were evident with increase in NADP-/NAD-GDH ration (≥0.41). Chemically synthesized additives 18-24B inhibited Y-H transition by 16-95% at their respective concentration, while DS16 inhibited germ tube formation by 51.6-95%. However no change in GDH activities was observed in the presence of chemically

synthesized additives. In case of microbial extracts *Bacillus* culture inhibited Y-H transition by 24-68% while *Pseudomonas* sp. inhibited by 36%. The maximum germ tube inhibition was observed for *Bacillus* sp B15 (68%). Yeast form NAD-GDH and NADP-GDH were inhibited by *Bacillus* sp. B1 (42.9%) and B21 (61.5%) respectively. Phenazine-1-carboxamide (PCN), isolated and purified from *Pseudomonas* sp. B18 was also studied for its effect on Y-H transition inhibition (22-224 μM). PCN led to inhibition of germ tube formation from 62.6-82.3% with increasing concentration (22-67 μM) as compared to control.

ODC activities were inhibited with increase in concentration of additives. Maximum inhibition (≥85%) was observed in presence of DAB (12 mM), CHA (3 mM), 18B (51 μM), 20B (26 μM), 24B (39 μM), DS16 (87 μM) and PCN (89 μM). In presence of microbial culture filtrates ODC activities were inhibited from 14.7 to 87.9 %, whereas B14 showed no inhibition. *Bacillus* B1, B15 and *Pseudomonas* B18 showed 87.9, 85.7 and 93.5 % inhibition of ODC activity respectively.

Biochemical and molecular studies of programmed cell death in *B. poitrasii* and validation studies using yeasts species involved in wine fermentation for PCD was discussed (Chapter IV). All the above additives which affected Y-H transition and biochemical correlates (GDH and ODC) were found to induce apoptosis with characteristics of apoptotic phenotype. The possible correlation of differentiation and apoptosis in *B. poitrasii* was studied. Varied concentrations of above mentioned additives were analyzed for apoptosis by annexin V-FITC, TUNEL, DAPI, DHR123, propidium iodide, SOD and caspase-like activities (-1,-3 and -8) in *B. poitrasii*.

Increase in annexin, TUNEL, DAPI and DHR123 positive cells were observed with increasing concentration of additives like isophthalic acid (16 mM), acetic acid (20 mM), H_2O_2 (50 mM), amphotericin B (2 μM), DAB (15 mM), CHA (4 mM), 18B (110 μM), 20B (105 μM), 24B (92 μM), DS16 (115 μM) and PCN (89 μM). Fluconazole and strobilurin (PC229) failed to induce apoptosis, only displayed necrotic phenotype. SOD and caspase-like activities also increased with increasing inhibitor concentration, maximum activity was observed at apoptotic phase, whereas decrease in activity was seen at necrotic phase. Chronologically aged cells showed increase in apoptotic phenotype as they approached the stationary phase, with increase in SOD and caspase-like activity. Similar trend was observed during sporulation in *B. poitrasii*. These results showed that differentiation or Y-H transition may precede cell death and can be used as a checkpoint to study linkage between morphogenesis and apoptosis.

Molecular studies of ODC and metacaspases from *B. poitrasii* was carried out to understand their role in differentiation and apoptosis. A PCR based approach to fish out the genomic and c-DNA clones of ODC gene from *B. poitrasii* was followed. The full length ODC gene (1349 bp) was obtained with primers designed from flanking ends of known sequences. *B. poitrasii* ODC gene was homologous to *Aspergillus niger* (53%), *Chaetomium globosum* (58%), *Gibberella zeae* (59%), *Magnaporthe oryzae* (60%), *Metarhizium anisopliae* (55%), *Mucor circinelloides* (51%), *Podospora anserina* (56%), *Tuber melanosporum* (55%), *U. maydis* (47%), *Yarrowia lipolytica* (95%). The phylogenetic tree with ODC data showed close relatedness between *B. poitrasii* and *Y. lipolytica*.

Similar approach was followed to fish out metacaspase gene from *B. poitrasii*. Partial gene fragment (467 bp) was amplified using primers AF1 (ATCAACGATGTCAMSAACATGTC) and AR1 (TCTACTCRACWCAGGGTAT). The *B. poitrasii* metacaspase partial fragment was homologous to metacaspase genes of *Aspergillus clavatus* (93%), *Aspergillus fumigatus* (93%), *Aspergillus nidulans* (93%), *Neosartorya fischeri* (93%), *Neurospora crassa* (82%), *P. brasiliensis* (89%), *Penicillium marneffei* (93%), *Talaromyces stipitatus* (91%), *S. cerevisiae* (67%) and *Schizosaccharomyces pombe* (67%). In addition the cause effect relationship can be studied in detail by the development of systems for reverse genetics in *B. poitrasii* and the analysis of *BpODC* and metacaspase disruptant strains will further clarify the role of these genes in the differentiation and apoptotic process.

Screening of chemically synthesized compounds and microbial metabolites using saprophytes, human and plant pathogenic fungi. Chemically synthesized triazole-linked β-lactam–bile acid conjugates (B), bile acid dimers linked with triazole and bis- β-lactam (D) and tetrapeptide linked-cholic acid derivatives (S) were tested *in vitro* for antifungal activity. The compounds 17 -24 B, SB09, SB32-39, 15, 17, 19, 20D and DS16 were active against all the strains tested, whereas SB51-52 and DS 14 were found to be inactive. Additionally among the 22 compounds tested, 5 triazole-linked β-lactam–bile acid conjugates (B compounds) were found to affect germ-tube formation, while 2 bile acid dimers linked with triazole and bis- β-lactam (D17, D19) and 1 cholic acid derivative (DS16), were effective in inhibiting Y-H transition.

Among the microbial metabolites screened 5 bacterial cultures showed 60-70% hyphal tip bursting. These cultures showed ≥ 5 mm zone of inhibition and were used for screening of potential glutamate dehydrogenase and ornithine decarboxylase inhibitors.

Effect of PCN on yeasts species involved in wine fermentation was sided for PCD. Wine spoilage yeasts underwent apoptosis in response to purified additive PCN as shown by % apoptotic cells, *D. bruxellensis* - 40-60% at 89.6 µM (MIC, 358 µM), *M. pulcherrima*- 40-60%, 89.6 µM (MIC, 201 µM), *Z. rouxii*- 25-70%, 112 µM (MIC, 573 µM), *C. krusei*- 10-70%, 89.6 µM (MIC, 143 µM), *D. hansenii*- 25-75%, 224 µM (MIC, 571 µM) and *P. anamola*- 15-70%, 71.7 µM, (MIC, 287 µM). PCN also inhibited ODC activities in all wine spoilage yeasts at the above mentioned concentration. Inhibition of ODC and induction of apoptosis showed connection between the commitment of yeasts cells to undergo morphogenesis and to die.

Extraction, purification and identification of phenazine-1-carboxamide from *Pseudomonas* sp. B-18 was done (Annexure I). A promising antifungal metabolite from *Pseudomonas* sp. B-18 was isolated and purified. The acetone extract of cell biomass was fractionated using methanol and the residue was purified using silica gel column chromatography with methanol: chloroform (5:95) mixture for elution. Antifungal activity in fraction D was identified using plate assay against plant pathogens *Fusarium oxysporum*, *Magnaporthe grisea*, *Drechslera oryzae* and saphrophyte *B. poitrasii*. Growth inhibition of 20-30 mm was observed at concentration (50 µg).The purified compound was isolated as pale yellow needles in methanol, which showed formula weight of 223 using ESI-MS spectrum with molecular formula $C_{13}H_9N_3O$ (phenazine-1-carboxamide, PCN). Amide nature of compound was detected by FT-IR.

In the **conclusions (Chapter V)**, Y-H transition and the biochemical correlates of *B. poitrasii* served to screen antifungal agents/effectors of differentiation and apoptosis inducers. Studies using effectors of morphogenesis showed that Y-H transition and its biochemical correlates GDH and ODC could serve as a check point to study apoptosis. PCN, 18, 20 and 24B was identified as potential ODC inhibitors and PCD inducers, which can be used as potential agent for control of human pathogens and food spoilage yeasts. The isolation of ODC and metacaspase gene was done which will be helpful to investigate its possible role in differentiation and apoptosis. Purification and isolation of PCN was done from *Pseudomonas* sp B18 because of its ability to inhibit morphogenesis and to induce apoptosis. PCN was also found to inhibit ODC activity and induced apoptosis in yeasts making it useful to control wine spoilage yeasts.

The references were given in **Chapter VI**.

CHAPTER I

INTRODUCTION

1. INTRODUCTION

Programmed cell death (PCD) is a regulated intracellular process that functions during normal cellular development, in response to stress and defence against pathogen attack in plants and animals (Biella *et al*., 2002). Lockshin and Williams (1965) first described PCD as the developmental process in which silkmoth muscle cells were systematically destroyed. Kerr *et al.* (1972) first described apoptosis as controlled cell deletion resulting in PCD. This form of cell death was distinguished from necrosis by its controlled pathway of self destruction. Etymologically 'apoptosis' in Greek language means falling of petals from flowers or leaves from trees. Thompson (1995) proposed that apoptosis or PCD was controlled by a genetic pathway distinct from necrosis. Apoptosis was studied extensively in a nematode *Caenorhabditis elegans*. The characterization of cell death genes (*ced-3*, *ced-4* and *ced-9*) responsible for apoptosis of specific neuronal cells in *C. elegans,* aided the identification of homologous genes in vertebrates (Reed, 1997). *Ced-3*, that encoded an aspartic specific cysteine protease or caspase, was homologous to interleukin-1 converting enzyme that has a caspase like function in mammalian cells (Yuan *et al*., 1993). The human homolog of *ced-9* belonged to the B-cell lymphatic-leukemia protooncoprotein-2 (Bcl-2) family of proteins (Reed *et al*., 1998). Zou *et al*. (1997) reported the involvement of mitochondria in apoptosis through the release of cytochrome *c*. Upon apoptotic signals, cytochrome *c* was released from mitochondria that, in turn, interacted with apoptosis protease activator factor (Apaf-1) and dATP for further activation of caspase (Li *et al.*, 1997; Vaux, 1997).

In humans, PCD provides certain advantages such as regulation of cell number and defence against potentially dangerous self replicative lymphocytes, virus infected and tumour cells (Stellar, 1995). In fungi it served to eliminate cells that were incompetent for mating or susceptible due to mutations, ageing or viral infections (Severin and Hyman, 2002; Fabrizio *et al*., 2004b; Herker *et al*., 2004; Ivanovska and Hardwick, 2005; Reiter *et al*., 2005). Recently, Hamann *et al.* (2008) described the role of apoptotic processes in fungal pathogenicity, competitiveness, propagation, ageing and lifespan control.

Denmeade and Issacs (1996) suggested that transformation of mammalian cells (normal to cancerous) was an intermediate stage between growth and apoptosis. The growth of any cell was determined by the quantitative relationship between the rate of cell proliferation and cell death. The disruption of this relationship resulted in continuous cell proliferation. Adjusting quantitative cell kinetics relation may help cell death to

exceed proliferation. So an understanding of the regulators of cell proliferation and apoptosis is important. Denmeade and Issacs (1996) demonstrated that proliferating cells could be induced to undergo apoptosis at any stage of the proliferative cell cycle (G1, S, G2 and M).

The connection of apoptosis with growth and morphogenetic adaptation throughout the life cycle of the fungal plant pathogen *Colletotrichum gloeosporioides* was studied by expression of mammalian Bcl-2 proteins (Barhoom and Sharon, 2007). Expression of the anti-apoptotic Bcl-2 protein led to prolonged longevity and enhanced stress resistance. These isolates had enhanced mycelium and conidia production (Barhoom and Sharon, 2007). In *Aspergillus nidulans* conidia formation was linked to apoptosis (Thrane *et al*., 2004). It was suggested that the conidia and conidiophore formation was supported by material from other parts of the hypha.

Apoptosis was implicated in stipe cavitation and gill formation in number of basidiomycetes (mushrooms) (Lu, 1991; Ramsdale, 2006). Studies on basidiomycetous fungi *Coprinus domesticus*, *Otidea onotica*, *Psathyrella candolleana* and *Tremella mesenterica* demonstrated that apoptosis played a key role in different stages of fungal fruiting-body development (Ramsdale, 2006).

In pathogenic fungi the understanding of PCD responses may offer a chance of exploiting the fungal death machinery to control fungal infections. Ramsdale (2008) discussed the role of death related signalling pathways in various pathogens such as *Aspergillus fumigatus*, *Candida albicans*, *Colletotrichum trifolii* and *Magnaporthe grisea*. The comparative knowledge of similarities and differences between death machineries of pathogens and their hosts and its role in their biology will identify apoptosis as a feasible target for development of antifungal agent.

1.1 Differentiation in fungi

The series of events leading to development of a distinct form and function in fungi is defined as differentiation. Denmeade and Issacs (1996) mentioned that transformation of cells or differentiation is an important event between growth and apoptosis. Fungi display several patterns of differentiation during their life span. Differentiation relies mainly on pattern of cell wall which ultimately determines the cell shape of the fungus. In yeast cells in general, non-polar regulated cell wall growth occurs. The yeast cells of *Mucor* sp. and *Paracoccidioides brasiliensis* exhibit multipolar budding. In haploid *Saccharomyces cerevisiae* the pattern is axial, whereas diploid *C.*

albicans and *S. cerevisiae* exhibited polar budding during pseudohyphae formation (Gow, 1995).

Fungal hyphae extend by synthesizing a new wall at their apex and, as they increase in length, additional sites are formed in the sub-apical region for wall synthesis. These give rise to lateral branches, with wall synthesis confined to the tip. Although wall extension is limited to the tip wall, thickening may occur on the lateral sides behind the apex (Wessels *et al.*, 1990). The hyphal tip receives a continuous supply of membrane vesicles which provide enzymes and precursors for the apical intussusception (Cabib *et al*., 1988). Hyphae of *C. albicans* and other dimorphic fungi extend apically. Mycelium of *C. albicans* is like small cell compartment and like yeast cells are joined from end to end. In contrast hyphae of *Mucor rouxii* and *Histoplasma capsulatum* resemble to hyphae of true mycelium (Gow, 1995).

Septa formation is another differentiation mechanism that leads to compartmentalization and addition of rigidity to fungal hyphae. Septa formation among zygomycetes occurs late to compartmentalize autolysing older hyphae from younger growing hyphae. *S. cerevisiae* cell division cycle and septa formation for separation of older cells represents one of the simple morphogenetic pathways extensively studied from the biochemical and genetic perspectives (Cabib *et al*., 1989). According to Gull (1978), the main function of the septa is to give hyphae the ability to undergo differentiation such as sporulation.

Fungal differentiation lead to variations in pattern of asexual and sexual spore formation, adapted for dispersal as well as for survival (Cole, 1986). Asexual or sexual spores are resistant structures formed from vegetative hypha in response to nutritional deprivation. A variety of morphologically distinct asexual spores are produced by fungi. Sporangiospores are produced by zygomycetous fungi such as *Rhizopus* and *Benjaminiella poitrasii*. However zygomycetous entomopthorales produce conidia in *Conidiobolus* sp., *Ballocephala verrucospora* (Deshpande, 1992; Latge *et al*., 1989). The fungal sexual spores show remarkable diversity e.g. zygospores (*Phycomyces*, *Mucor* and *Benjaminiella*), ascospores (*S. cerevisiae*, *Neurospora crassa*) and basidiospores (*Schizophyllum commune, Ustilago maydis*) to name a few (Bolker *et al*., 1995; Staben, 1995; Ghormade *et al*., 2005b).

In some fungi hyphae are interwoven to form small aggregates called sclerotia that are able to resist adverse conditions (Willets, 1972). *Sclerotium rolfsii* was used as a

model to elucidate the biochemical changes occurring during sclerotium formation (Chet and Henis, 1989).

Among the different morphological transitions being studied to unravel the mechanism of eukaryotic differentiation, the study of fungal dimorphism stands out distinctly due to its reversible nature (Gow, 1995). The study of dimorphism is important as it played important role in pathogenesis of most plant and human fungal pathogens.

1.2 Dimorphism: a form of fungal differentiation

Dimorphic fungi grow either in yeast (Y) or hypha (H) forms reversibly in response to environmental conditions for survival and proliferation (Gow, 1995; Deacon, 2006). Differentiation or cellular transformation in dimorphic fungi follows two basic patterns of cell wall formation, polarized regulated for hypha and non-polarized regulated for yeast (Chitnis and Deshpande, 2002). Hyphal forms of dimorphic fungi differentiate into Y form by lateral and/or by terminal budding. The Y-H transition occurs by germ tube formation and further extension by hyphal tip elongation.

Dimorphism is displayed by fungi across different taxonomic groups *viz*. zygomycetes like *Mucor*, *Mycotypha*, *Benjaminiella*, ascomycetes such as *Candida*, *Yarrowia*, basidiomycetes like *Ustilago*, *Ophiostoma* (Bartnicki-Garcia and Nickerson, 1962a, b; Schulz, 1974; Khale, 1990; Brunton and Gadd, 1991; Gow, 1995; Zinjarde *et al*., 1998). Some of these fungi are important human or plant pathogens while others are known so far as saprophytes. The main cause of pathogenesis was the ability of the fungi to grow in two alternative forms, Y or H.

Among the zygomycetes *Mucor racemosus*, *M. rouxii*, *M. genevensis* and *M. bacilliformis* showed dimorphic behaviour (Orlowski, 1991). In *M. rouxii*, *M. microspora* and *M. africana* hyphal growth was observed under aerobic conditions while the yeast form was reported under anaerobiosis (Bartnicki-Garcia and Nickerson, 1962a; Schulz *et al*., 1974). In *M. rouxii* the presence of high glucose concentration in the medium favoured the yeast form, while hyphal growth was supported at low glucose concentrations (Bartnicki-Garcia, 1963; Sypherd *et al*., 1978). In the family Mycotyphaceae, *M. africana* and *M. microspora*, *Benjaminiella poitrasii*, *Benjaminiella multispora* and *Benjaminiella youngii* exhibited dimorphism (Benny *et al*., 1985; Kirk, 1989). Y or H forms of *M. africana* and *M. microspora* were obtained by change in temperature, pH, glucose (Schulz *et al*., 1974; Benny *et al*., 1985).

Candida sp. are opportunistic human pathogen that exhibit morphological change to facilitate infection in the host cell (Scully *et al*., 1994). *C. albicans* mutants defective in Y-H transitions were reported to be non-pathogenic (Lo *et al*., 1997). The environmental factors that influence the transition between yeast and hyphal growth were mainly temperature, pH and serum. *C. albicans* Y form was favoured by acidic pH while alkaline pH supported H form (Stewart *et al*., 1988). Brown and Gow (1999) reported that nitrogen deprivation stimulated filamentation in *C. albicans*. During Y-H transition the presence of zinc affected germ tube formation in *C. albicans* (Sabie and Gadd, 1990).

In *Yarrowia lipolytica* a saprophytic fungus, Y-H transition takes place in response to nutritional factors like *N*-acetylglucosamine, ammonium sulphate, glutamine and glutamate (Rodriguez and Dominguez, 1984; Novotny *et al*., 1994; Ruiz-Herrera and Sentandreu, 2002). *Y. lipolytica* grew in the Y form at acidic pH whereas H form grew at neutral pH (Szabo, 1999; Ruiz-Herrera and Sentandreu, 2002). In NCL, a marine isolate of *Y. lipolytica* exhibited Y-H transition under anaerobic conditions (Zinjarde *et al*., 1998).

S. cerevisiae, an ascomycete and one of the most studied model organisms to understand eukaryotic systems, also exhibited differential levels of certain enzymes in its yeast and pseudo- or true hyphal form. The co-regulation of starch hydrolysis and dimorphism in *S. cerevisiae* was extensively reviewed by Melane *et al.* (1997). *S. cerevisiae* pseudohyphae formation was observed when grown on solid media in response to starvation for nitrogen and polarized growth was enhanced in the presence of Mn^{2+} salt in the medium (Gimeno *et al*., 1992; Asleson *et al*., 2000).

In the human pathogenic fungus *H. capsulatum* the temperature shift from 25-37°C triggered H-Y while addition of zinc to growth media inhibited Y-H transition (Pine and Peacock, 1958; Maresca and Kobayashi, 1989). The dimorphic fungi *Sporothrix schenckii*, *Blastomyces dermatitidis*, *Penicillium marneffei*, *P. brasilensis* and *Coccidioides immitis* exhibited yeast form at 37°C (Cole and Sun, 1985; Domer, 1985; Travassos, 1985; Kudeken *et al*., 1996; Nachman *et al*., 1996).

Dimorphism in the plant pathogen *U. maydis* was shown to be influenced by pH (Ruiz-Herrera *et al*., 1995; Ruiz-Herrera and Martinez-Espinoza, 1998). Ruiz-Herrera *et al*. (1995) reported that nitrogen source was an important factor for hyphal growth in *U. maydis*.

Wangiella dermatitidis, a dimorphic human pathogen, displayed polymorphic forms such as thin walled and thick walled yeast, multicellular form, moniliform hyphae and true hyphae during the course of transition (Kester and Garret, 1995).

Benjaminiella poitrasii, a NCL isolate was used extensively as a model for dimorphic studies and antifungal screening. *B*. *poitrasii*, homothallic fungus produced yeast and mycelium during its vegetative phase, sporangiospores in its asexual phase and the sexual phase comprised of zygospores. The vegetative, yeast phase cells produced uni-, bi- and multipolar budding under yeast forming conditions. In Y-H transition, cells exhibited emergence of bud like structures that eventually produced germ tubes. The reverse transition M-Y was initiated by producing terminal and lateral budding. Asexual spore formation started with the swelling of the hyphal tip followed by emergence of stub like outgrowths on the surface of vesicle that later formed sporangiospores apically. The zygospore formation took place in a zig-zag manner by fusion of two opposing and equal size gametangia (Ghormade *et al*., 2005b). Dimorphism in *B*. *poitrasii* was investigated using morphological, biochemical and genetic approaches (Khale *et al*., 1990; Ghormade and Deshpande, 2000; Chitnis and Deshpande, 2002). The studies in *B*. *poitrasii* on sporangiospores and zygospore germination under different conditions suggested that dimorphism was an intrinsic ability of the organism, expressed throughout the life cycle (Ghormade and Deshpande, 2000). *B*. *poitrasii* exhibited rapid reversible Y-H transition that was used for screening of antifungal compounds. In *B*. *poitrasii*, the yeast form was favoured by glucose, high temperature (37°C) and acidic pH. The biochemical correlates of differentiation in *B*. *poitrasii* such as glutamate dehydrogenase, chitin synthase, chitinase, *N*-acetyl glucosaminidase and ornithine decarboxylase are well characterized (Khale and Deshpande, 1993; Deshpande, 1998b; Ghormade *et al*., 2000; Chitnis *et al*., 2002; Amin *et al*., 2004a; Ghormade *et al*., 2005a). Dimorphic fungi could provide a good model for study of apoptosis and for screening apoptosis inducing agents. Dimorphic fungus *B*. *poitrasii* can be used to study the role of differentiation in apoptosis.

1.2.1 Biochemical correlates of dimorphism

The change in the cell wall architecture facilitates the morphological change in fungi, accompanied by increased or decreased levels of the carbon and nitrogen metabolizing enzymes involved in the cell wall synthesis and degradation. These enzymes were studied in various fungi as biochemical correlates of the morphological transition (Deshpande, 1996).

Nitrogen metabolism

Ammonia assimilating enzymes

In fungi ammonia assimilation pathway include; NAD- (E.C 1.4.1.2) and NADP-GDH (E.C 1.4.1.4) and glutamine synthetase (GS) (E.C 6.3.1.2)/ glutamate synthase (GOGAT) (E.C 1.4.1.13) enzymes. The GS and GOGAT enzymes are more effecient in plants (Stewart *et al*., 1995). It was reported that NAD- and NADP-GDH enzymes are more effiient in fungi like *Candida utilis*, *S. cerevisiae* and *M. racemosus* for the ammonia assimilation (Fergusson and Sims, 1974; Peters and Sypherd, 1979; Avendano *et al*., 1997).

Glutamate dehydrogenase

GDH catalyze either reductive amination of 2-ketoglutarate to glutamate or oxidative deamination through the reverse reaction using NAD (P) H as a cofactor.

$$NH_4^+ + \text{2-Ketoglutarate} + NAD(P)H \xleftrightarrow{GDH} \text{Glutamate} + NAD(P)^+ + H_2O$$

GDHs were shown to have a biochemical correlation with Y-H transition in the *Mucor* (Bartnicki-Garcia and Nickerson, 1962 a, b). Peters and Sypherd (1979) reported two GDHs, NAD-GDH and NADP-GDH in *M. racemosus*. The H-Y transition in *Mucor* sp. correlated with the decreased NAD-GDH activity. In *B. poitrasii*, the relative proportion of NADP- to NAD-GDH was important in deciding the morphological outcome (Khale *et al*., 1992). Two isoenzymes of NADP-GDH were expressed differentially in yeast and hyphal forms while NAD-GDH was common for both the forms (Amin *et al*., 2004a).

Glutamine synthetase (GS) and GOGAT

Glutamine synthetase catalyzes the synthesis of glutamine from ammonium, ATP and glutamate. Glutamine produced by GS is essential for protein synthesis and its amide nitrogen is used to synthesize many essential metabolites such as nucleic acids, aminosugars, histidine, tryptophan, asparagine and various cofactors (Barrs *et al*., 1995). It also plays a key role in the chitin synthesis pathway in fungi. With fructose 6-phosphate it forms glucosamine 6-phosphate, which in turn gives UDP-, *N*-acetyl glucosamine, monomer of chitin.

$$\text{Glutamate} + \text{Ammonium} + ATP \xrightarrow{GS} \text{Glutamine} + ADP + Pi$$

GOGAT catalyzes the transfer of L-glutamine amide group to the carbon of 2-ketoglutarate to give L-glutamate. It plays a key role in the ammonia assimilation in microorganisms and plants.

$$\text{Glutamine} + \text{2-Ketoglutarate} + \text{NAD(P)H} \xrightarrow{\text{NAD(P)-GOGAT}} \text{2-Glutamate} + \text{NAD(P)}^{+}$$

Polyamine metabolism

Ornithine decarboxylase (ODC, E.C 4.1.1.17), spermidine synthase (E.C 2.5.1.16) and spermine synthase (E.C 2.5.1.22)

Putrescine, spermidine and spermine are low-molecular weight polyamines synthesized in the cells from their immediate precursor, ornithine. These ubiquitous aliphatic polycations bind to phospholipids, proteins, DNA and are essential for cell growth, differentiation and transformation (Garcia *et al*., 1980; Ruiz-Herrera *et al*., 1983; Tabor and Tabor, 1985; Calvo-Mendez *et al*., 1987; Pignattin *et al*., 2004).

Ornithine decarboxylase (ODC) is an important enzyme in the biosynthetic route of the polyamines (Ruiz- Herrera and Martinez-Espinoza, 1998). In *M. circinelloides* and *M. racemosus* aerobiosis triggered Y-H transition, accompanied by increased levels of ODC transcription and enzyme activity (Blasco *et al*., 2002). Similarly in plant pathogenic fungi *U. maydis* and *Ceratocystis ulmi* high levels of polyamines were associated with hyphal form as compared to the yeast form (Marshall *et al*., 1979; Reyna-Lopez and Ruiz-Herrera, 2004). In human pathogenic fungi *Histoplasma capsulatum* and *P. brasiliensis*, H-Y transition was accompanied with the increase in ODC activity (Marshall *et al*., 1979; San-Blas *et al*., 1996b). In *B. poitrasii*, the Y-H transition was related to elevated levels of polyamine (Ghormade *et al*., 2005a). Similar observations were made in case of *Y. lipolytica* (Jimenez-Bremont *et al*., 2001).

Polyamines play role in expression and activation of mitogen activated protein kinase (MAPK) signal transduction pathways. They enhance MAPK and tyrosine kinase activities (Auvinen *et al*., 1995; Flamigni *et al*., 1999). Spermidine stimulated the phosphorylation of p42 and p44 protein kinases that play central role in the Ras/MAPK cascade (Bachrach *et al*., 2001). Polyamines stabilize cellular nucleic acids and membranes and activation of protein kinases and transcription factors (Bachrach *et al*., 2001).

Sulphur metabolism

The sulphur metabolism includes enzymes such as cysteine reductase, cysteine oxidase and sulfite reductase. In dimorphic fungi *C. albicans* and *A. pullulans* glutathione metabolism was found to alter with yeast to hypha transition (Gunasekaran *et al*., 1995; Jürgensen *et al*., 2001). In case of *H. capsulatum*, temperature affected the redox state of the -SH groups or the general redox potential in cells that determined the form of the organism.

Cell wall metabolism

Cell wall composition of dimorphic fungi belonging to different taxonomic groups differs from species to species. Significant differences in the chemical composition of cell wall between yeast and hypha were observed for *B. poitrasii*, *M. rouxii*, *C. albicans*, *S. schenckii*, *B. dermatitidis*, *H. capsulatum* and *P. brasiliensis* (San-Blas and San-Blas, 1983; Orlowski, 1991; Khale *et al*., 1992). The enzymes involved in cell wall synthesis and degradation, like chitin synthase, glucan synthetase, chitinase and *N*-acetylglucosaminidase play important role in the dimorphism. It was reported in *B. poitrasii* that chitin synthase activity and yeast-hyphal morphogenesis were both subjected to regulation by osmotic pressure, phosphorylation and calcium (Deshpande *et al*., 1997). In *B. poitrasii*, Chitnis *et al*. (2002) reported that eight chitin synthase genes were expressed differentially during Y-H transition. Glucan (α-1, 3-glucan) is another common polysaccharide present in the cell walls of fungal pathogens like *H. capsulatum*, *B. dermatitidis* and *P. brasiliensis* (Deshpande, 1996). Other cell wall associated enzymes endochitinase and *N*-acetylglucosaminidase were also reported to be involved in the yeast to hypha transition of *B. poitrasii* (Ghormade *et al*., 2000). Most of the studies of fungal morphogenesis were dealt with biochemical changes, which occur prior to the dimorphic transition.

1.3 Genes involved in the regulation of morphogenesis

In various dimorphic fungi, genes related to various functions in the cell have been extensively studied. At NCL, Chitnis *et al*, (2002) reported the presence of eight distinct chitin synthase (*CHS*) genes in *B. poitrasii*. Two of these genes viz. *BpCHS2* and *BpCHS3* were hyphal form specific (Chitnis *et al*., 2002). In *C. albicans* microarray analysis was carried under different growth conditions to investigate differential expression of genes during yeast to hypha transition. Transcriptional factors *EFG1* and *CPH1* were found to be important for germ tube formation in *C. albicans* (Nantel *et al*., 2002). Genes encoding for cellular regulators like protein kinases (*STE12, STE20,* and

PKC1), transcriptional activators (*TUP1, EFG1*) and heat shock proteins (*HSP70*) were observed to play an important role in the morphological transition of *S. cerevisiae*, *C. albicans* and *H. capsulatum* respectively (Maresca and Kobayashi, 1989; Liu *et al*., 1994; Magee, 1997). In *C. albicans*, the expression of some genes coding for cellular building blocks changed during yeast to hypha transition (Gow, 1995). For instance, the genes *ECE1* (gene that is expressed in relation to the extent of cell elongation) and *CHS2* were expressed in the hyphal form. The genes that were expressed in the cells of only one form, such as *HYR1* (hyphal regulated), a hypha specific gene in *C. albicans* and *YPS3* (yeast phase specific), a yeast specific gene and *MS8*, mold specific gene in *H. capsulatum* were known but the information of their products is still unknown (Bailey *et al*., 1996; Maresca and Kobayashi, 1989; Tian and Shearer, 2002).

1.4 Programmed cell death (PCD) in yeasts and fungi

1.4.1 Types of PCD

PCD may be classified into three main categories: apoptosis, autophagy and necrosis. The first two are programmed and genetically regulated, whereas the third one is environmentally induced by physical or chemical injuries. The onset of apoptosis was identified with a set of the morphological and biochemical hallmarks. They are as follows: rapid loss of mitochondrial membrane potential, externalization of the phospholipid phosphatidylserine on the plasma membrane, DNA fragmentation and cell shrinkage (Madeo *et al*., 2002). Apoptosis and its related pathways are described in nearly all major life forms (Ramsdale, 2006). Although the mechanisms of apoptosis may differ in detail, some of the proteins and regulatory factors involved in the apoptotic pathways are conserved among diverse organisms (Table 1.1) (Biella *et al*., 2002). During the past decade, apoptosis was reported in unicellular yeasts and in some filamentous fungi (Madeo *et al*., 1997; Lu, 2006). In fungi the involvement of apoptosis was demonstrated in different biological processes, development and ageing (Table 1.2).

Autophagy, a form of cell death, involves disintegration/dissolution of cell components. It is characterized by the targeting of organelles (Golgi, polyribosomes and endoplasmic reticulum) by the lysosome prior to the onset of nuclear pyknosis (irreversible condensation of chromatin in the nucleus). In *S. cerevisiae* autophagic vesicles were associated with apoptotic cell death suggesting a crosstalk between two pathways (Yamaki *et al*., 2001).

Necrosis or accidental cell death results when cells die from severe or sudden injury (Ueda and Shah, 1994). Morphological changes leading to necrosis include

swelling of mitochondria and endoplasmic reticulum, loss of membrane integrity and extensive inflammatory response (Kerr *et al*., 1972).

1.4.2 Detection of morphological and cytological features of fungal apoptosis

The morphological and cytological features of fungal cell death were studied using different filamentous fungi and yeasts (Table 1.2). Various techniques for detection of apoptosis were developed with the aid of fluorescent stains. They are externalization of the phospholipid phosphatidylserine (PS) on the plasma membrane as detected by fluorescein isothiocynate labelled annexin V, identification of DNA fragmentation by terminal deoxynucleotidyl transferase–mediated dUTP nick end labelling (TUNEL) and loss of membrane integrity by propidium iodide (PI). Chromatin condensation was detected by 4,6-diamino-2-phenylindole (DAPI) staining while generation of reactive oxygen species (ROS) was recognized by oxidation of dihydrorhodamine (DHR123) to the fluorescent rhodamine (Fig. 1.1). Other techniques involved detection of mitochondrial membrane potential by selective uptake of fluorescent cationic dye JC1 (5,5′,6,6′tetrachloro-1,1′,3,3′-tetramethylbenzimidazoylcarbocyanine iodide) and cell shrinkage by microscopy as reported in *S. cerevisiae* and different fungi (Fig.1.1, Table 1.2) (Madeo *et al*., 2002). The cytological phenotypes of apoptosis observed in fungi in response to external stimuli or genetic defects or induction of heterologous pro-apoptotic genes, exhibited hallmarks of apoptosis similar to those exhibited by well studied nematodes. The biochemical markers involved release of cytochrome *c* from mitochondria, increase in enzymatic activities of superoxide dismutase (SOD) and caspases in response to reactive oxygen species (ROS) generation and apoptotic stimuli. In case of apoptosis in mammalian cells, later stages included blebbing of plasma membrane and enclosure of organelles in membrane-bound sacs. DNA was cleaved into smaller fragments (~200 bp) by the caspase-activated DNase (CAD) (Wyllie, 1980; Enari *et al*., 1998; Ribeiro *et al*., 2006). However, DNA ladders were generally absent in fungi with the exception of *M. racemosus* and *S. cerevisiae* (Roze and Linz, 1998; Madeo *et al*., 1999; Ribeiro *et al*., 2006).

Table. 1.1 Apoptosis related pathways in different life forms

Organism	Proteins and regulatory factors involved in apoptosis										
	Caspase	Para-Caspase	Meta-caspase	HtrA-Family	Serine/threonine protein kinase	BCL-2	BIR	p53	NFkB	TNF	TNFR
Prokaryotes	-	+	+	+	+	-	-	-	-	-	-
Fungi (Yeast)	-	-	+	+	+	-	+	-	-	-	-
Nematodes	+	+	-	+	+	+	+	-	-	+	+
Arthropods	+	-	-	+	+	+	+	+	+	+	+
Vertebrates	+	+	-	+	+	+	+	+	+	+	+
Plants	-	-	+	+	+	-	-	-	-	-	+

HtrA, High temperature resistance A; Bcl-2, B-cell lymphatic-leukemia protooncoprotein-2; BIR, Baculovirus Inhibitor of apoptosis protein; NFkB, nuclear factor-kB; TNF, tumour necrosis factor; TNFR, TNF receptor. Modified from Koonin *et al*., 2002

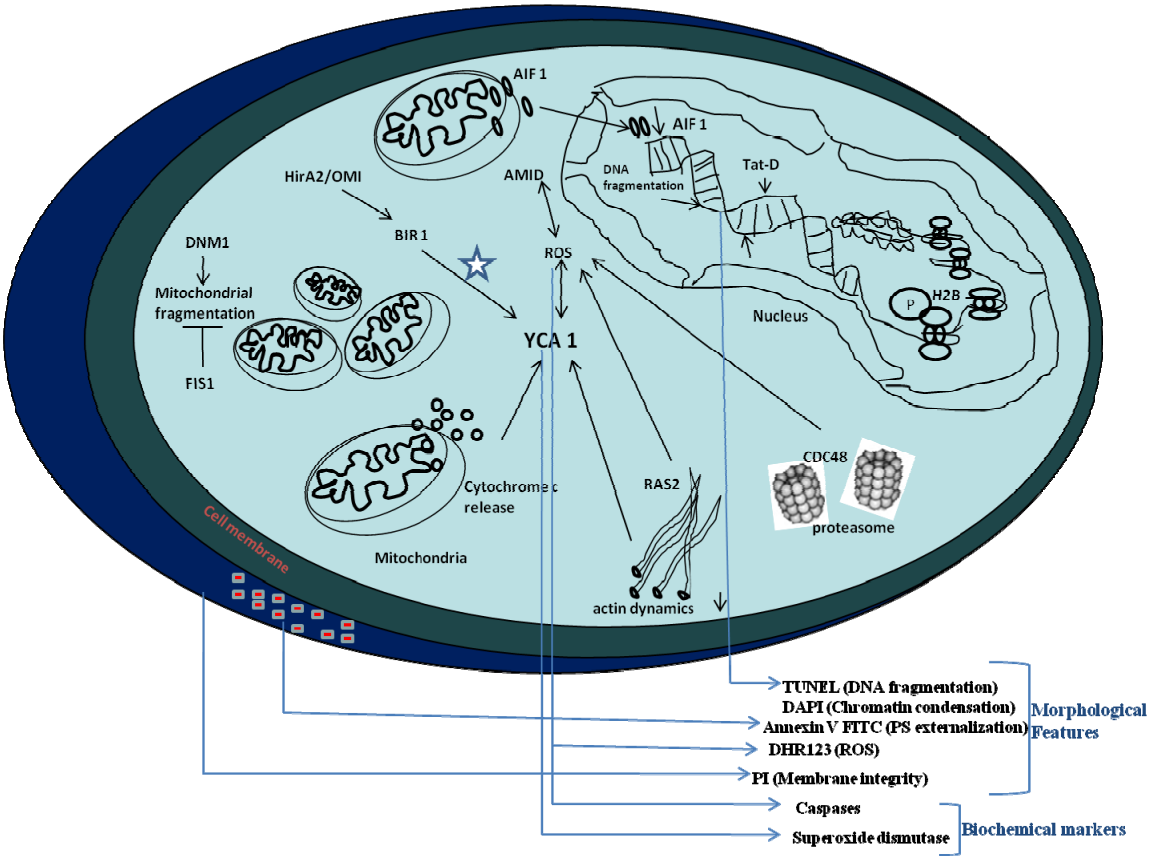

Fig. 1.1 Possible mechanism involved in apoptosis and their detection.

☆ indicates pathways that are known in mammals but not in yeast.

Table. 1.2 Apoptotic markers in fungi

Organisms	Biological Process/Life cycle/Treatment	Apoptotic Marker						
		DNA cleavage	PS	Membrane integrity	Mitochondria involvement	Protein synthesis dependent	Metacaspase	Inhibition by caspase inhibitor
Filamentous fungi								
Aspergillus fumigatus	Stationary Phase	+	+	+	ND	+	+	+
	H_2O_2	+	+	+	ND	+	_	_
	Amphotericin B	+	+	+	ND	+	_	_
Aspergillus nidulans	Asexual sporulation	ND	ND	ND	ND	ND	+	ND
	Sphingoid long chain	+	+	+	+	+	_	ND
	Antifungal protein	+	+	+	+	+	_	ND
	Farnesol	+	+	+	+	ND	ND	ND
Colletotrichum trifolli	Ras overexpression	+	+	+	+	ND	ND	ND
Coprinus cinereus	Meiosis defect	+	ND	ND	ND	ND	ND	ND
Magnaporthe grisea	Asexual sporulation	ND	ND	ND	ND	ND	+	ND
Neurospora crassa	Vegetative incompatibility	+	ND	ND	ND	ND	ND	ND
Yeast								
Candida albicans	Stationary Phase	+	+	+	ND	+	+	+
Saccharomyces cerevisiae	H_2O_2	+	+	+	ND	+	_	_
	Amphotericin B	+	+	+	ND	+	_	_
Schizosaccharomyces pombe	Merocyanine 540 (Phototoxic agent)	+	+	+	+	+	_	_

PS, Phosphatidylserine; Amphotericin B, ergosterol inhibitor; Farnesol, antitumour agent; Ras, RAt sarcoma ; ND, Not detected; Phillips *et al*., 2003; Robson, 2006; Veneault-Fourrey *et al*., 2006.

1.5 Yeast and the study of apoptosis

The *S. cerevisiae* was used to understand the role of apoptosis and its advantages in unicellular organisms (Longo *et al*., 2005). It was suggested that apoptosis in unicellular yeasts served to eliminate cells that are incompetent for mating, susceptible to viruses, mutations or ageing (Severin and Hyman, 2002; Fabrizio *et al*., 2004a, b; Herker *et al*., 2004; Ivanovska and Hardwick, 2005; Reiter *et al*., 2005). *S. cerevisiae* apoptosis served as a functional model to study the role of conserved genes and pathways involved in apoptosis that are potentially relevant to human (Fig. 1.2). The discovery of an apoptotic *S. cerevisiae* strain carrying a *CDC48* or *CDC13* mutation shed light on its mammalian orthologue valosin containing protein (VCP), which was involved in polyglutamine triggered neurodegenerative disorders (Madeo *et al*., 1997; Shirogane *et al*., 1999). *S. cerevisiae* cells harbouring a mutation in the cell division cycle gene, *CDC48*, exhibited characteristic markers of mammalian apoptosis (Madeo *et al*., 1997; Temple *et al*., 2005; Braun *et al*., 2006; Zischka *et al*., 2006; Mroczek and Kufel, 2008). Formic acid, an inducer of yeast apoptosis, caused cell death in mammalian ocular cells. Though the molecular mechanisms in mammalian cells are unidentified, it was demonstrated in yeasts that formic acid induced loss of mitochondrial membrane potential, early ROS burst and mitochondrial destruction (Du *et al*., 2008). Apoptosis in *S. cerevisiae* and *Kluyveromyces lactis* was induced by low doses of the killer toxin zygocin that triggered ROS production (Reiter *et al*., 2005).

The *S. cerevisiae* was used to characterize apoptosis morphologically, biochemically and genetically. Acetic acid, an apoptotic agent induced cell death in yeast cells of *S. cerevisiae*. The accumulation of acetic acid was associated with acidification of the intracellular environment that resulted in apoptosis (Ludovico *et al*., 2001). Stress conditions such as presence of high glucose or sorbitol concentration in culture medium induced cell death in *S. cerevisiae*. The death process was accompanied by morphological and biochemical indicators of apoptosis (Silva *et al*., 2005). Ammonia, a stress sensing molecule induced apoptosis in *S. cerevisiae* by oxidative stress (Hamann *et al*., 2008). The metal ions Cu^{2+} and Mn^{2+} (>6 mM) induced apoptosis in *S. cerevisiae* (Liang and Zhou, 2007; Carmona-Gutierrez *et al*., 2010a).

S. cerevisiae mutants lacking oxidative stress *sod* genes established their role for maintenance of cellular redox homeostasis (Drakulic *et al*., 2005). Phillips *et al*. (2003) reported that apoptosis was induced in yeast form of *C. albicans* by environmental stress and amphotericin B while hypha served as an escape route to evade apoptosis.

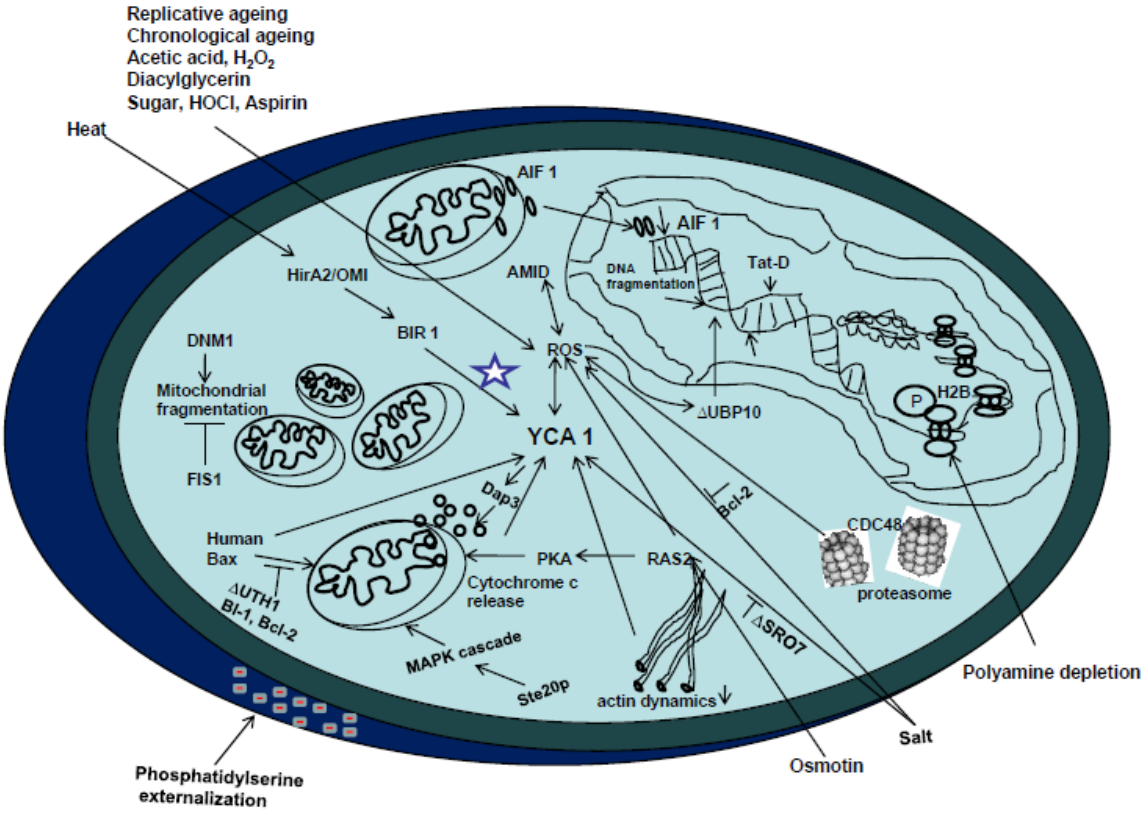

Fig. 1.2 The apoptotic yeast cell

☆ indicates pathways that are known in mammals but not in yeast.

Although apoptosis in fungi and mammals share characteristic features, several details of their underlying mechanisms differ. Fungi lack biochemical homologues of mammalian aspartate specific cysteine proteases or caspases that are important for apoptosis. Instead, they possess functional homologues of caspases known as metacaspases, which are cysteine proteases (Uren *et al*., 2000). Plant, protozoan and fungal metacaspases displayed arginine and lysine specific cysteine proteases (Watanabe and Lam, 2005; Gonzalez *et al*., 2007; Hamann *et al*., 2007). *S. cerevisiae* metacaspases revealed its role in apoptosis (Table 1.3) (Madeo *et al*., 2002; Bettiga *et al*., 2004; Khan *et al*., 2005; Weinberger *et al*., 2005). Both caspase-dependent and caspase-independent cell death executors were responsible for cell death in *S. cerevisiae* (Guaragnella *et al*., 2006; Hauptmann *et al*., 2006) (Table 1.3). *S. cerevisiae*, *C. albicans* and *Schizosaccharomyces pombe* had a single metacaspase encoding gene, while *A. fumigatus*, *A. nidulans* and *N. crassa* had two each (Mousavi and Robson, 2004; Cheng *et al*., 2003; Thrane *et al*., 2004; Fedorova *et al*., 2005). Among pathogenic fungi studies of the role of metacaspases in the cell death response were carried out with *A. fumigatus* (Mousavi and Robson, 2003; Richie *et al*., 2007) (Table 1.1).

Table. 1.3 Involvement of metacaspase in yeast apoptosis

Biological Process/Treatment	Proapoptotic markers	Mca1p activity detected		Mca1p-independent	Mca1 deletion on cell viability
		FLICA Substrate			
Chronological ageing	PSE, ROS,CA, CHrC, DB	FITC-VADFMK	ND	Yes	Yes
Bax expression	ND	ND	ND	ND	No
Defect in deubiquitination	PSE,ROS, CA, DB	FITC-VADFMK	ND	ND	ND
Glycosylation defect	PSE, ROS,CA, CHrC, DB	No	VEID, IETD	VEID, IETD, FITC	No
G2/M arrest (*cdc13-1*)	ND	No	ND	ND	No
H_2O_2	CA, CHrC, DB,	FITC-VADFMK	VEID, IETD	ND	Yes (10%: 60%)*
K1, K28 zygocin	PSE, ROS, CHrC, DB	ND	ND	ND	Yes
Mitochondrial fission (*fis1*/ H_2O_2)	PSE	ND	ND	Yes	Yes (50%: 90%)*
NaCl stress (*sro7*/NaCl)	ROS, CHrC, DB, CA	FITC-VADFMK	ND	ND	Yes (70%: 95%)*

Proapoptotic markers: CA, caspase activity; CHrC, chromatin condensation by 4',6-diamidino-2-phenylindole (DAPI); DB, DNA breaks by terminal deoxynucleotidyl transferase-mediated UTP nick end labelling (TUNEL) assay; PSE, phosphatidyl serine externalization; ROS, reactive oxygen species. *Proportion of surviving cells containing functional *Mca1* gene to those containing mutant *mca1*gene.

FITC-VAD-FMK, fluorescein-5-isothiocyanate-VAD-fluoromethylketone; FLICA, fluorochrome-labelled cell-permeable inhibitors of caspases; ND, not detected. VEID, *N*-acetyl-Val-Glu-Ile-Asp -7-amino-4-methylcoumarin; IETD, *N*-acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin.

Modified from Vachova and Palkova, 2007

1.5.1 Mitochondrial cell death pathway in yeast

Cytochrome *c* was released from mitochondria which promoted cell death. Apoptosis inducing factor (*Aif)*, a mammalian, apoptogenic mitochondrial intermembrane protein, acted independently of Bcl-2 and caspase pathways (Lu, 2006). Wissing *et al.* (2004) reported that the *S. cerevisiae* mitochondrial ortholog of the *Aif* caused DNA damage. Like the mammalian *Aif*, the *S. cerevisiae Aif1p* was localized within the mitochondrial membranes and was translocated to the nucleus upon induction of apoptosis. It induced apoptosis and had DNase activity requiring cofactors, Mg^{2+} and Ca^{2+} (Wissing *et al*., 2004). Its apoptogenic function required cyclophilin (*Cyp1*) like mammalian *Aif* (Lu, 2006). Furthermore, apoptosis induced by verexpression of *Aif* in *S. cerevisiae* cells was inhibited by deletion of the metacaspase gene *Yca1*.

Treatment with the antifungal agents acetic acid, amiodarone, H_2O_2, ethanol, hyperosmotic stress, pheromone, ageing and expression of mammalian proapoptotic proteins also induced mitochondrial fragmentation in *S. cerevisiae* (Ludovico *et al*., 2001; Skulachev, 2002; Madeo *et al*., 2004; Mazzoni *et al*., 2005; Sokolov *et al*., 2006; Gourlay *et al*., 2006). Fannjiang *et al*. (2004) identified the mitochondrial death pathway shared between yeast and mammals (Fig.1.3A). In addition to *S. cerevisiae*, mitochondrial fragmentation was also reported in other model systems like *Drosophila melanogaster* and *C. elegans* (Jagasia *et al*., 2005; Goyal *et al*., 2007). Mitochondrial fragmentation is an early feature of apoptosis in flies and mammals. Regulators of mitochondrial fragmentation, such as dynamin related protein1 (Drp1) and mitochondrial fission 1 protein (Fis1) were shown to regulate apoptosis and cytochrome *c* release (Karbowski and Youle, 2003; Fannjiang *et al*., 2004).

The protein machinery involved in yeast mitochondrial fission were Dnm1 (homologue of mammalian DRP1), Mdv1(yeasts homologue of mitochondrial division), CCR4 associated factor (Caf4) and Fis1 (fission) (Cerveny and Jensen, 2003; Griffin *et al*., 2005; Yu *et al*., 2005; Hoppins *et al*., 2007; Merz *et al*., 2007). Deletion of these fission proteins, except Caf4, caused mitochondrial degradation (Griffin *et al*., 2005). *Dnm1*-knockout in *S. cerevisiae* strains were resistant to several different death stimuli and displayed delayed mitochondrial fragmentation during cell death (Fannjiang *et al*., 2004; Ivanovska and Hardwick, 2005).

Overexpression of *Drp1* and *Fis1* genes in *S. cerevisiae* protected Ca^{2+} induced cell death due to blockage of Ca^{2+} signals (Frieden *et al*., 2004). However, like *Drp1*, *Fis1* also had a pro-survival function, as knockdown of mammalian *Fis1* resulted in cell senescence (Lee *et al*., 2007). *S. cerevisiae* mitochondrial fragmentation occurred shortly following addition of a death stimulus (Fannjiang *et al*., 2004). Mitochondrial fragmentation was recently suggested to contribute to mitochondrial degradation by triggering autophagic cell death (Twig *et al*., 2008).

The Bcl-2 family proteins are regulators of apoptosis in mammalian cells. The proapoptotic Bcl-2 associated X protein (Bax) contained BH1 (Bcl-2 homology), BH2 and BH3 conserved domains. The radiation sensitive protein of *S. pombe* (*SpRad9*), a DNA-damage checkpoint protein contained BH-3 domain and considered as critical domain for death (Harris and Thompson, 2000). It was suggested that *SpRad9* represented the first member of the Bcl-2 protein family in *S. pombe*.

Earlier studies revealed that cytosolic and mitochondrial superoxide dismutase (*sod1* and *sod2* respectively) were required for long term survival of *S. cerevisiae* cells (Fabrizio and Longo, 2003) (Fig. 1.3B). *UTH1*, was possibly involved in autophagic death and needed for efficient Bax mediated killing in *S. cerevisiae* (Camougrand *et al*., 2003). While deletion of *UTH1*does not prevents the insertion of Bax into the mitochondrial outer membrane or cytochrome *c* release, it inhibited the appearance of ROS production (Camougrand *et al*., 2003). This suggested that Bax triggered cell death by a cytochrome *c* independent but autophagic cell death (Camougrand *et al*., 2003; Kissova *et al*., 2006). The dying cells employed an apoptotic pathway activated by mitochondrial ROS and the debris from dying cells provided nutrients for the surviving (better adapted) genetic variants (Hardwick and Cheng, 2004).

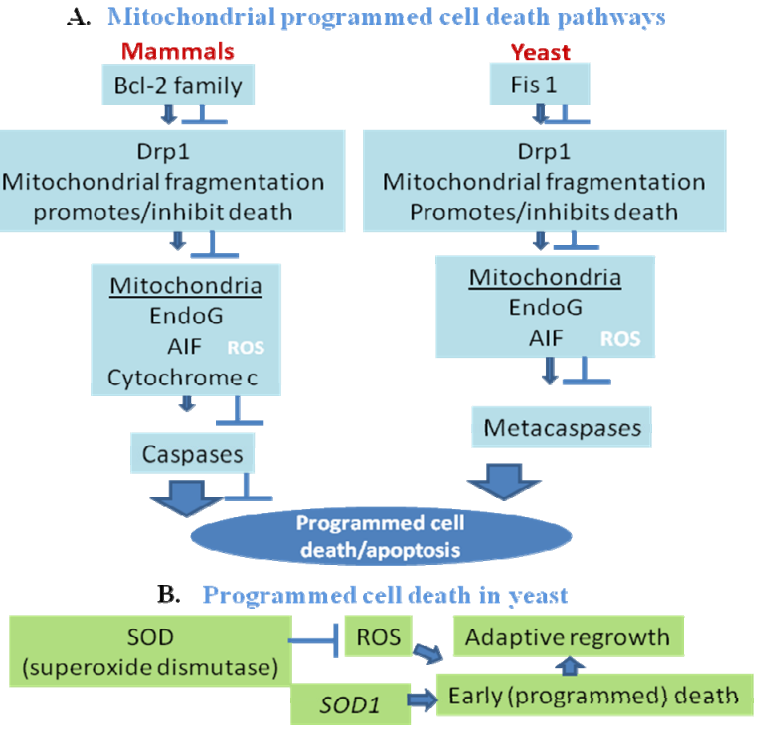

Fig. 1.3 Death pathways in mammals and yeast
(A)*Fis1* of the yeast *S. cerevisiae* and mammalian *Bcl2* inhibit mitochondria-dependent cell death. Orthologs of Mammals and *S. cerevisiae* encode dynamin-like GTPase *Drp1/Dnm1*, *Fis1*, *Aif*, caspase-like factors, and EndoG (endonuclease G), cytochrome *c*. **(B)** The induction of early (programmed) death by yeast correlates with the ability of yeast cultures to ultimately survive following environmental stresses that lead to elevated ROS levels.

Homologues of core regulators of mammalian apoptosis are conserved in yeasts and filamentous fungi (Table 1.4) (Madeo *et al*., 2002; Fahrenkrog *et al*., 2004; Wissing *et al*., 2004; Fedorova *et al*., 2005; Li *et al*., 2006; Walter *et al*., 2006; Buttner *et al*., 2007; Frohlich *et al*,. 2007; Madeo *et al*., 2009). However, several apoptotic factors are missing in yeasts, including the Bcl-2/Bax family and Apaf-1 (Zha and Reed, 1997). In

both human and *S. cerevisiae* cells, histone modifications (histone H2B phosphorylation at serine 14 in human and at serine 10 in yeast and H2B deacetylation at lysine 11 in yeast) play an important role in apoptotic chromatin condensation and apoptosis (Cheung *et al*., 2003; Ahn *et al*., 2005, 2006).

Table. 1.4 Fungal protein homologue of putative apoptotic mediators

Protein	*S. c*	*S. p*	*A. f*	*N. c*	*U. m*	Function
Aif1p	+	-	-	-	-	Caspase independent apoptosis
Asf1p	+	+	+	+	+	Cell cycle, chromatin assembly, mating
Cdc6p	+	+	+	+	+	Cell cycle control, DNA replication
Cdc13p	+	-	-	-	-	Cell cycle control, telomere-binding
Cdc48p	+	+	+	+	+	Ubiquitin-proteosome system
CDC48	+	+	+	+	+	Cell division cycle, AAA ATPase
DAP3	+	+	+	+	+	Mitochondrial fragmentation
DNM1/ Drp1	+	+	+	+	+	Apoptosis
FadA/GpaA	+	+	+	+	+	Regulation of sexual differentiation
FIS1p	+	+	+	+	+	Ethanol-induced apoptosis
Hel10p	+	-	+	-	-	Apoptosis
HtrA2	+	+	+	+	-	Mitochondrial homeostasis
Oxa1	+	+	+	+	+	Regulation of life span
Mmi1p	+	+	+	+	+	Negative regulation of apoptosis
Ndi1p/AMID	+	+	+	+	+	Mitochondrion-associated inducer
Nma11p	+	+	+	+	+	Nuclear mediator of apoptosis
Rad50p	+	+	+	+	+	Maintenance of genome integrity
RMP1	+	+	+	+	+	Regulation of development and life span
Sod1p	+	+	+	+	+	Response to stress
Ste20p	+	+	+	-	+	Regulation of sexual differentiation
Stm1p	+	+	+	+	+	Caspase independent apoptosis
Tat-D	+	+	+	+	+	Apoptosis
Ubp10p	+	-	-	-	-	Ubiquitin-proteosome system
Uth1p	+	+	+	+	-	Response to stress
Yap1	+	+	+	+	+	Oxidative stress tolerance
Yca1p	+	+	+	+	+	Metacaspase

S.c, *S. cerevisiae*; *S.p*, *S. pombe*; *A.f*, *A. fumigatus*; *N.c*, *N. crassa* ; *U.m*, *U. maydis.* Fedorova *et al*., 2005; Frohlich *et al*., 2007.

1.6 Fungal cell death responses

In fungi, organised cellular degradation is linked to phenomena such as autophagy, autolysis, ageing/senescence, somatic and sexual incompatibility and spore discharge mechanisms (Ramsdale, 2006). Death events in fungi are triggered either by harsh physical and chemical agents, or can be developmentally regulated.

Autophagy

In filamentous fungi, autophagy was reported during nutrient starvation and also as a part of the heterokaryon incompatibility response (Pinan-Lucarre *et al*., 2003; Ramsdale, 2006). The molecular mechanism of autophagy was also demonstrated in *S. cerevisiae*. Overexpression of Ras/protein kinase A/cAMP pathways in *S. cerevisiae* led to blockage of autophagy (Budovskaya *et al*., 2004; Ramsdale, 2006). While in mammals the overexpression of Ras resulted in caspase independent autophagic cell death, emphasizing the differences between mammals and fungi (Ramsdale, 2006).

Autolysis

Autolysis, a feature displayed by growing hyphae, allowed reallocation of resources from older part of the hypha to the growing tip (Ramsdale, 2006). Autolysis was linked to nutritional deprivation in fungi. In *Penicillium chrysogenum*, carbon-limited cultures underwent autolysis associated with ROS production (Sámi *et al*., 2001). Mousavi and Robson (2003) demonstrated that autolytic cultures of *A. fumigatus* displayed characteristic features of apoptotic cells such as externalization of phosphatidylserine and membrane rupture. They demonstrated that autolysis was an active process inhibited by addition of cycloheximide to cultures and that the apoptotic phenotype was abolished by addition of caspase inhibitor *z*-VAD-fmk (benzyloxycarbonyl-VAD-fluoromethylketone).

Ageing

In fungi, few cases of ageing are reported (Osiewacz *et al*., 1990). In the well-established ageing model *Podospora anserina*, an ascomycetous fungus, linear decline in strain-specific growth period was associated with activation of the apoptotic machinery (Hamann *et al*., 2007). Further deletion of the two metacaspase genes, *PaMca1* and *PaMca2*, resulted in an increased lifespan that indicated the involvement of caspases in ageing and cell death.

Studies with unicellular yeasts highlighted the close link between apoptosis responses and ageing. Two types of ageing are recognized in yeasts – replicative ageing and chronological ageing (Bitterman *et al*., 2003; Ramsdale, 2006). Replicative ageing is defined as the number of cell divisions which can be undertaken by a mother cell before it ceased to divide. Chronological ageing is defined as the loss of viability of single cells over time in stationary phase. The death of aged cells was considered ‘useful’ as it limited the spread of deleterious mutations and other ‘acquired’ characteristics such as

damaged proteins that preferentially segregate in the mother cells (Ramsdale, 2006). Interestingly, markers of apoptosis, phosphatidylserine externalization and DNA-strand breaks, were also reported during replicative ageing of *S. cerevisiae* cells (Laun *et al*., 2001; Herker *et al*., 2004).

Somatic incompatibility

Fusion of two genetically incompatible hyphae induced a cell-death reaction termed 'heterokaryon incompatibility (HI)' (Glass and Dementhon, 2006). In vegetative or heterokaryon incompatibility apoptosis was linked to the recognition of non-self, due to allelic differences at the incompatibility loci. These processes prevented the spread of harmful genetic elements (van Diepeningen *et al*., 1997; Ramsdale, 2006). Somatic incompatibility was associated with the generation of ROS and an increased proteolytic activity in *P. anserina* (Ramsdale, 2006). Studies on the role of the suicide programme in fungal reproduction are limited. In *S. cerevisiae*, the mating cascade was linked to cell death by induction of mitochondria-dependent apoptotic cell death after exposure to pheromone (Severin and Hyman, 2002). In the filamentous ascomycete *P. anserina*, formation of ascospores and fruiting bodies were severely impaired in the mutant with two metacaspase genes deletions (Hamann *et al*., 2007). In *Neurospora crassa* and *P. anserina*, HI reaction displayed clear characteristics of apoptosis (Marek *et al*., 2003; Pinan-Lucarre *et al*., 2007).

Meiotic death and spore germination

Fungi undergo meiosis during sexual spore formation. Meiotic apoptosis in fungi was studied in *C. cinereus* mutants (Lu *et al*., 2003; Ramsdale, 2006). In the sporulation defective mutants (*spo11*), apoptosis was triggered at the tetrad stage with condensed chromatin and other apoptotic phenotypes (Lu *et al*., 2003). During pathogenesis, development of turgor pressure in appressoria of plant pathogen *M. grisea* relied upon the death of the germinating spore (Liu *et al*., 2007). In basidiomycetous fungi genomic disparity between nuclei in spores led to post-germination mortality e.g. *Heterobasidion annosum* and *Stereum hirsutum* (Ramsdale, 1998).

Apoptosis in host–pathogen and antagonistic interactions

Several fungi cause diseases in plants, animals and humans. During infection by the opportunistic human pathogen *A. fumigatus* the host cells responded by production of reactive oxygen species (ROS) directed against the germinating spores leading to apoptosis (Mousavi and Robson, 2004; Thrane *et al*., 2004).

Apoptosis was also induced during antagonistic interactions, in which fungi compete for nutrients with other species growing on the same substrate. Secretion of molecules that induce apoptotic processes in a competitor provides a major selective advantage. Farnesol, an apoptosis inducing molecule produced by *C. albicans*, prevented hypha formation of *A. nidulans* during quorum sensing (Hamann *et al*., 2008). Farnesol induced apoptosis in *A. nidulans* provides a selective advantage to *C. albicans* in antagonistic interactions (Semighini *et al*., 2006a). The induction of apoptosis in *A. nidulans* by farnesol was dependent on poly (ADP-ribose) - polymerase (PARP) an enzyme, that in higher organisms was involved in DNA-damage recognition, DNA repair and induction of apoptosis (Semighini *et al*., 2006b). *S. cerevisiae* and *K. lactis* produce killer toxins that cause apoptotic cell death in susceptible strains (Hamann *et al*., 2008).

1.7 Effectors of apoptosis

Reactive oxygen species (ROS)

ROS are the chief regulators of apoptosis. In *S. cerevisiae* exogenous oxygen stress induced apoptotic cell death in presence of H_2O_2 (Madeo *et al*., 1999). Cell death induced by acetic acid was accompanied by increased ROS levels in yeasts (Ludovico *et al*., 2002). Heterokaryon incompatibility led to increased production of ROS as detected in *P. anserina* and *C. cinerea* (Silar, 2005). The *S. cerevisiae* cell division cycle mutant (*cdc48S565G*) exhibited the apoptotic phenotype with accumulation of ROS (Madeo *et al*., 1999). In the yeast, oxygen stress was induced in chronologically aged cells and aminoacids starved cells (Laun *et al*., 2001; Eisler *et al*., 2004; Fabrizio *et al*., 2004b; Herker *et al*., 2004). Pozniakovsky *et al.* (2005) demonstrated that ROS levels were elevated in pheromone and amiodarone induced cell-death cascade in *S. cerevisiae*.

Superoxide dismutase (SOD)

The enzyme SOD acted as a defence against oxidative damage (Gessler *et al*., 2007). Metal toxicity caused by Cu^{2+}, Cd^{2+} and As^{3+} increased the ROS production causing apoptosis that was alleviated by over-expression of *sod2*, encoding the mitochondrial manganese superoxide dismutase. In fungal cells, the two types of SOD found are Cu/Zn-SOD in the cytosol, encoded by the gene *sod-1* and Mn-SOD in the mitochondria, encoded by *sod-2*. Expression of *sod-1* and *sod-2* genes increased under oxidative stress in the stationary growth phase in *S. cerevisiae* (Gessler *et al*., 2007). In *N. crassa* and *S. cerevisiae sod-1* mutants were hypersensitive to redox mediators and increased oxygen tension. However, *sod-1* mutants of *N. crassa* were less sensitive to UV, heat shock and γ-irradiation, due to increased activity of mitochondrial Mn-SOD.

Increased SOD activity was also observed during spore germination in *N. crassa* (Gessler *et al*., 2007).

Glutathione

Glutathione (GSH) is a tripeptide that contains a peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side chain. GSH plays an important role in overcoming oxidative stress. A *S. cerevisiae* strain lacking glutathione, caused by deletion of γ-glutamyl-cysteine synthetase gene, exhibited apoptosis and was sensitive to treatments with H_2O_2 (Madeo *et al*., 1999). Furthermore, the apoptotic phenotype was suppressed in *S. cerevisiae* by oxygen radical scavengers (Madeo *et al*., 1999).

Actin

In yeast, the actin dynamics play an important role in apoptosis. In aged cells decreased actin turnover was reported, due to specific mutations in actin gene or treatment with actin-stabilizing drugs such as jasplakinolide (Gourlay *et al*., 2004). Further, increased actin turnover by deletion of the *Scp1* (*S. cerevisiae* calponin) gene encoding the actin-bundling protein SCP1, led to lifespan extension of the dividing and non-dividing *S. cerevisiae* cells (Gourlay *et al*., 2004). The role of actin dynamics was linked to the Ras-cAMP signalling, an important regulatory pathway of longevity.

Caspases

Caspases are aspartate specific cysteine proteases that contain conserved pentapeptide sequences of Glu-Ala-Cys-Arg-Glu-Gly-Gly at their active site (Cohen, 1997; Thornberry and Lazebnik, 1998). Caspases or proteases of the caspase family display the following characteristic features: first, they are cysteine proteases that use cysteine as the nucleophylic group for substrate cleavage, and second, they cleave the peptide linkage carboxy-terminal to aspartic acid residues (Alnemri *et al*., 1996). There are two types of cell death inducing caspases: initiator caspases and effector caspases. Initiator caspases (2, 8, 9 and 10) cleave inactive pro-forms of effector caspases, thereby activating them. Effector caspases (3, 6 and 7) in turn cleave other protein substrates within the cell, to trigger the apoptotic process. The initiation of this reaction is regulated by caspase inhibitors.

Recently Uren *et al*. (2000) identified two new families of caspase-like proteases with significant similarity to caspases i.e. metacaspases and paracaspases. They identified metacaspases in plants and fungi and paracaspases in metazoans and

Dictyostelium discoideum. Uren *et al*. (2000) identified an *S. cerevisiae* open reading frame for a caspase like protein fitting into category of metacaspases, that was later named by Szallies *et al*. (2002) as metacaspase 1 (MCA1). Madeo *et al*. (2002) proposed its caspase like function in *S. cerevisiae* and named it initially as yeast caspase-1 (*Yca1*). Madeo *et al*. (2002) and Hauptmann *et al*. (2006) reported a high proteolytic activity of Mca1p, overproduced in *S. cerevisiae*, towards the mammalian caspase substrates Ac-VEID-AMC (*N*-acetyl-Val-Glu-Ile-Asp-7-amino-4-methylcoumarin) and Ac-IETD-AMC (*N*-acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin). This activity was completely inhibited by the caspase inhibitor *z*-VAD-FMK. According to the substrate specificity, Mca1p was proposed to function as an initiator caspase in *S. cerevisiae* (Madeo *et al*., 2002). For measuring metacaspase activity in fungi, caspase (-1,-3 and -8) like activities are determined because of structural similarity of metacaspase to caspases specifically members of both families contained the caspase-hemoglobinase fold (Bozhkov *et al*., 2010; Carmona-Gutierrez *et al*., 2010b).

Mca1p dependent caspase-like activity was demonstrated using fluorescein-5-isothiocyanate-carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (FITC-VAD-FMK) and fluorogenic substrate D_2R (Asp-Asp Rodamine) in ageing colonies (Herker *et al*., 2004; Vachova and Palkova, 2005). Mca1p independent activities with specificity similar to caspases 6 and 8 were activated during death of *S. cerevisiae* cells defective in glycosylation (Hauptmann *et al*., 2006). *S. cerevisiae* protease activity similar to effector caspases (caspases 3 and 7) were reported using the inhibitor DEVD-VAD in H_2O_2 induced death of a *fis1* mutant (Fannjiang *et al*., 2004; Madeo *et al*., 2009).

Caspase generally target three groups of protein substrates: (1) caspases themselves, (2) proteins that are inactivated for cell death and (3) proteins that are required for execution of apoptosis (Kidd, 1998). The first group comprises of pro-caspases that may be autoactivated and may require “upstream” caspases for efficient proenzyme activation. The second group of substrates are proteins whose inactivation occurs during cell death eg. PARP, the 70-kDa protein component of the ribonucleoprotein, the catalytic subunit of the DNA-dependent protein kinase, DNA topoisomerase I and actin etc. (Cohen, 1997; Kidd, 1998). The third group of proteins are required to execute apoptosis eg. cleavage of mammalian gelsolin (actin binding protein), by caspase-3 like protease constitutively activated this protein and the cleaved product degraded actin filaments (Kidd, 1998).

1.8 Signalling pathways leading to apoptosis

Understanding of fungal apoptosis responses requires the elucidation of the signal pathways which transmit information about the status of the cell and the environment to the suicide machinery. Both pharmacological approaches and functional genetic analyses have revealed some of the components of the death-related signal transduction pathways. Many extracellular stimuli induced apoptosis by affecting the signalling transduction pathways implicated in control of cell growth (Evan and Littlewood, 1998).

Ras and cAMP

Ras the protein sub-family of small GTPases and cyclic adenosine monophosphate (cAMP) are involved in cell signalling. Both pro- and anti-apoptotic functions for Ras were identified in fungi. Lovastatin, an inhibitor of Ras prenylation (hydrophobic modification), induced an apoptosis-like cell death response in hyphal form but not yeast form of *M. racemosus* (Roze and Linz, 1998). Exogenous application of dibutyryl cAMP initiated morphogenesis from hyphal to yeast-like growth and prevented lovastatin-induced cell death response. Crosstalk between cAMP and G protein signalling occurs in several fungi (Glass and Dementhon, 2006). In *S. cerevisiae*, ROS production, expression of antioxidant proteins and the apoptotic response that follows treatment with osmotin was partially dependent upon the induced suppression of *Ras2*/cAMP pathway (Ramsdale, 2006).

The Ras pathway was strongly linked to morphogenetic signals in a number of fungi. One common theme was finding of an interrelationship between cell death and fungal morphogenesis/differentiation (Ramsdale, 2006). In *C. albicans* known signalling pathways *EFG1* (enhanced filamentous growth), *RIM101* (regulator of IME2), *TEC1* (transposon enhancement control), *CPH1* (*Candida* pseudohyphal regulator) that contribute to morphogenesis were not linked to apoptotic pathways (Phillips *et al*., 2003). In *C. albicans* and *S. cerevisiae* mutations in *cdc35* (encoding adenylate cyclase), *tpk1* and *tpk2* (which encode regulatory subunits of PKA) that block Ras signalling suppressed or delayed the apoptotic response upon exposure to low levels of acetic acid, H_2O_2 and amphotericin B (Feng *et al*., 1999; Phillips *et al*., 2003, 2006). However, mutations that resulted in constitutive activation of the Ras pathway accelerated entry into the apoptotic pathway for *C. albicans* and *Colletotrichum trifolii* (Glass and Dementhon, 2006; Phillips *et al*., 2006).

In *A. nidulans* resistance to *Penicillium* antifungal protein (PAF) and farnesol was associated with mutations in α and β subunit of G protein, whereas mutations that

hyperactivated G protein signalling (*ΔflbA*) resulted in increased sensitivity to farnesol (Leiter *et al*., 2005; Glass and Dementhon, 2006).

Phosphoinositide 3-OH kinase and protein kinase B signalling

The phosphoinositide 3-OH kinase and protein kinase B signalling pathways protect variety of cell types against apoptosis (Mathieu *et al*., 2001). In *S. cerevisiae*, addition of wortmannin, protein kinase B inhibitor caused ROS-induced cell death. Since protein kinase B declined during ROS induced cell death and it was activated by phosphorylation, it was suggested that phosphoinositide 3-OH kinase/protein kinase B regulation may play role in apoptosis. In mammals, phosphoinositide 3-OH kinase inhibited caspase-3 further delaying the onset of p53 mediated apoptosis (Ramsdale, 2006).

Protein phosphatase 2A

In *S. cerevisiae*, E4 a viral regulator protein, formed a complex with protein phosphatase that interacted with the anaphase-promoting complex, leading to its inactivation and concomitant cell cycle arrest/apoptosis. It was associated with the production of ROS (Kornitzer *et al*., 2001).

Calcineurin and calcium

Ca^{2+}/calmodulin/calcineurin (Ca-CaM regulated phosphatase) signals may possibly affect fungal cell death response. In *S. cerevisiae* antifungal azole activity was altered by addition of Ca^{2+}, inhibitors of Ca-CaM (trifluoperazine and calmidazolium) and calcineurin (cilcosporin and pimecrolimus) (Ramsdale, 2006; Carmona-Gutirrez *et al*., 2010a). Apoptosis induced by mating pheromones and salts in the yeast was shown to be influenced by mutations in calmodulin (Gourlay *et al*., 2006). Although mode of the cell death has not yet been ascertained, it might be speculated that the inhibition of the Ca^{2+} – calmodulin- calcineurin signalling pathway could induce apoptosis in the face of external stresses (Ramsdale, 2006).

Sphingolipids and protein kinase C

Sphingolipids are membrane components that play a role in signal transmission and cell recognition, alteration in which affects apoptosis. A member of this family, accelerated cell death (*Acd1*) gene translocation responsible for spingosine across the plasma membrane in *Arabidopsis thaliana*, affected apoptosis during the plant hypersensitivity response (Brodersen *et al*., 2002). Screening of *N. crassa* mutants by Ferket *et al*. (2003) revealed several mutants with altered sphingolipid compositions

which were resistant to apoptotic stimuli (defensins). The addition of D-erythro-sphingosine, an inhibitor of protein kinase C to lovastatin-treated *M. racemosus* cells accelerated apoptosis (Roze and Linz, 1998).

MAP kinase cascades

MAPK cascades participate in a wide array of cellular functions, such as cell growth, cell differentiation and apoptosis (Cross *et al*., 2000). Osmotin (antifungal cytotoxic agent) activated the mating, invasive growth and pseudohyphal growth, MAP kinase cascades of *S. cerevisiae* independently of both the pheromone receptor and the associated G protein coupled alpha subunit (Ramsdale, 2006). The pathway was activated rapidly by the phosphorylation of *ste7* (sterile) and preceded the phenotypic changes associated with apoptosis.

1.9 Differentiation and apoptosis

Polyamines in apoptosis

The role of polyamines in cell proliferation are well elucidated, more recently these polyamines were also implicated in cell death process or apoptosis (Schipper *et al*., 2000; Nitta *et al*., 2002; Oredsson, 2003; Wallace and Fraser, 2003; Wallace *et al*., 2003). Depletion of polyamines resulted in cell-growth arrest, mainly at the G1 phase which played a role in the transcription of genes involved in growth arrest and apoptosis (Kramer *et al*., 2001; Li *et al*., 2001b; Ackermann *et al*., 2003).

Inhibition of polyamine biosynthesis either protected or sensitized cells exposed to death stimuli. Polyamine depletion also prevented apoptosis induced by green tea extract in bladder carcinoma cells, while it induced apoptosis in neonatal cardiomyocytes (Facchini *et al*., 2003). Treatment with an ODC inhibitor difluoromethylornithine (DFMO) caused complete depletion of polyamines in mouse fibroblasts lacking spermine synthase gene that eventually resulted in cell death (Stefanelli *et al*., 2000). DFMO diminished putrescine and spermidine concentrations in a wide variety of cells causing cell cycle arrest mostly in G1 by impairing protein synthesis and topoisomerase II (Rudkin *et al*., 1984; Berntsson and Oredsson, 1999; Ackermann *et al*., 2003). An apoptosis related example of polyamine - protein interactions was the inhibition of apoptotic endonucleases by spermine which stabilizes the inactive enzyme conformation (Brune *et al*., 1991; Ribeiro and Carson, 1993; Urbano *et al*., 1998).

Exogenous polyamines stabilised the chromatin structure in cell nuclei, while polyamine depletion caused increased nuclease dependent chromatin degradation and

impaired DNA repair mechanisms (Basu *et al*., 1992; Snyder and Bhatt, 1993; Seiler and Raul, 2005). Inhibition of ODC, AdoMetDC or the perturbation of polyamine regulation by structural analogues of spermidine activated apoptosis by chromatin destabilization (Seiler and Raul, 2005).

Most studies have dealt with action of polyamines at mitochondrial phases of apoptosis, whereas less is known about the effect of these polyamines on signal transduction pathways located upstream mitochondrial phase and caspase activation (Pignattin *et al*., 2004). The mitochondrial phases of apoptosis culminate with activation of proteolytic cascades involving the caspases (Thornberry and Lazebnik, 1998). The involvement of polyamines in apoptosis-related pathways at the mitochondrial level was investigated in different cell models *viz.* intestinal epithelial cells, mouse fibroblasts and human breast cancer cells. In whole cells and cell-free models of apoptosis, polyamines particularly spermine activated caspases and induced the release of cytochrome *c* from mitochondria to trigger the death program (Stefanelli *et al*., 1998, 1999, 2000). In ODC overproducing mouse myeloma cells, accumulation of putrescine provoked apoptotic death by release of cytochrome *c* from the mitochondria followed by activation of caspase cascades (Erez *et al*., 2002). On the other hand, in various lymphoid cell lines, complete depletion of polyamines with combined use of ODC and S-adenosylmethionine decarboxylase (SAMD) inhibitors caused disruption of mitochondrial membrane potential resulting in caspase activation and apoptotic cell death (Nitta *et al*., 2002). ODC overproducing cells showed enhanced MAPK activity as polyamines phosphorylated the MAPK, which in turn triggered the expression of nuclear oncogenes (Flamigni *et al*., 1999; Bachrach *et al*., 2001). Nuclear transcription factor-κB (NF-κB) was implicated in control of cell growth, survival and apoptosis (Karin and Ben-Neriah, 2000). In normal intestinal epithelial cells, polyamines negatively regulated NF-κB activation (Li *et al*., 2001a). A schematic illustration depicting possible relationships linking ODC/polyamines with signal transduction pathways in the regulation of cell growth or cell death is shown in Fig. 1.4.

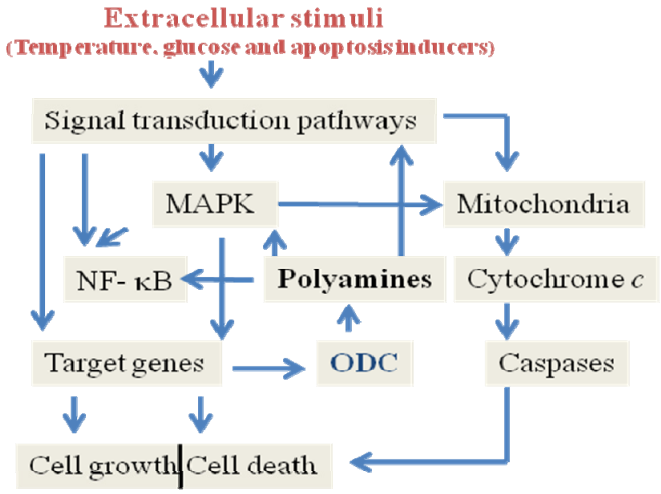

Fig. 1.4 Involvement of polyamines in controlling the balance between cell growth and apoptosis (Pignattin *et al*., 2004).

1.10 Yeast as a model for screening of antitumour and antifungal drugs

Yeasts provide an attractive tool to study the mode of action of both antitumour and antifungal drugs due to their common eukaryotic origin and similar apoptotic machinery. Genetic changes in tumour cells often include alterations in the control of cell cycle and the regulation of the cell death process (Perego *et al*., 2000). Studies in *S. cerevisiae* were the first to reveal the cellular target of rapamycin, an immunosuppressant drug broadly used in human tissue transplants (Heitman *et al*., 1991). Interestingly, most of the antitumour drugs used nowadays were first selected as antimicrobial agents (Almeida *et al*., 2008). Treatment of cancer and fungal diseases face the challenge of toxicity and resistance hence newer targets are of significance. Differences in the architecture of yeast apoptosis may allow the targeting of non-conserved genes or gene products as novel and specific antifungal drug targets to combat the increasing number of fungal infections seen in immunocompromised individuals (Almeida *et al*., 2008).

Apoptosis-inducing antitumour drugs in yeast

Paclitaxel, arsenic, bleomycin and valproate (VPA) represent well studied antitumour drugs that induce yeast apoptosis (Table 1.5). Farnesol is known to induce apoptosis in a lung carcinoma cells and fungi such as *S. cerevisiae*, *A. nidulans* and *C. albicans* (Machida *et al*., 1999; Semighini *et al*., 2006a; Joo *et al*., 2007).

Fredericamycin A (FMA), antibiotic product of *Streptomyces griseus*, camptothecin and podophyllotoxin from the plants *Camptotheca acuminate* and *Podophyllum peltatum* respectively are among the antitumour drugs known to induce apoptosis in *S. cerevisiae* and mammalian cells due to the inhibition of topoisomerases (Hartmann and Lipp, 2006). Other antitumour drugs, including 5-fluorouracil, selenium, coumarin and 1,10-phenanthroline are described as cytotoxic agents that lead to

apoptosis in *S. cerevisiae* (Coyle *et al*., 2004; Cai *et al*., 2007; Hoskins and Scott, 2007; Thati *et al*., 2007).

Antifungal drugs

S. cerevisiae was a practical and conventional system for studying the properties of antifungal compounds (Barns *et al*., 1991). Fungi affected human welfare by destroying crop plants, causing food spoilage and life-threatening diseases in immuno-compromised individuals (Talbot, 2003). Most antifungal drugs in current use belong to structural classes that affect specific fungal cellular targets, such as ergosterol biosynthesis (amphotericin B and azole drugs).

Allylamines are non-competitive inhibitors of squalene epoxidase which led to ergosterol depletion e.g.- cyclosporin A (Onyewu *et al*., 2003). Caspofungin and micafungin displayed fungicidal activity against *Candida*, *Histoplasma* and *Aspergillus* (Tawara *et al*., 2000). There are reports of resistance against these drugs and the search for newer antifungals becomes necessary.

Some drugs with known antifungal capacities are already demonstrated to act as yeast-specific apoptosis-inducers (Table 1.5). Among these, some are currently used against fungal pathogens while others are still under developed *viz.* ciclopiroxolamine (Kokjohn *et al*., 2003). Compounds such as osmotin from tree frog *Phyllomedusa sauvagii* and pradimicin, from the prokaryote *Actinomadura hibisca* displayed effective antifungal capacities through the induction of apoptosis (Narsimhan *et al*., 2001; Hiramoto *et al*., 2003; Morton *et al*., 2007). Two important drugs aspirin (pain reliever) and ricin (toxin isolated from castor plants) are known to induce apoptosis in *S. cerevisiae* (Bellosillo *et al*., 1998; Narayanan *et al.,* 2005).

Table. 1.5 Overview of the antitumour and/or antifungal drugs known to induce apoptosis in yeast and their associated apoptotic phenotypes

Apoptosis inducing antitumour/antifungal drugs in yeast		
Drugs	Apoptotic phenotype	Yeast species
Cell permeability disruptor		
Amphotericin B	Phosphatidylserine exposure DNA fragmentation Chromatin condensation Arrest in G2/M	*C. albicans*
Metal cation chelator		
Ciclopiroxlamine	Chromatin condensation Sub-G0/G1 population Arrest in G2/M Nuclear dysfunction Independent of ROS accumulation Independent of metacaspase	*S. cerevisiae*
Plasma membrane binders/disruptors		
Osmotin	DNA fragmentation ROS accumulation Suppression of transcription of stress genes via RAS2/cAMP pathway Dependent on the binding to Pho36p	*S. cerevisiae*
Histone deacetylase (HDAC) inhibitors		
Dermaseptin	DNA fragmentation ROS accumulation Metacaspase independent DNA intercalating	*S. cerevisiae,* *C. albicans*
Valproate	Dependent of metacaspase DNA fragmentation	*S. cerevisiae*
Mitochondrial membrane disruptor		
Histatin	Mitochondrial membrane depolarization Mitochondrial swelling Loss of intracellular ATP and amino acids Arrest in G1 ROS accumulation	*C. albicans*
Microtubule-directed		
Paclitaxel	ROS accumulation DNA fragmentation Sub-G0/G1 population Arrest in G2/M	*S. cerevisiae*
Arsenic	ROS accumulation DNA fragmentation Arrest in G2/M PS exposure Dependent of metacaspase	*S. cerevisiae*
Bleomycin	DNA fragmentation Chromatin condensation	*S. cerevisiae*

Adapted from Almeida *et al*., 2008

Interestingly, most drugs seem to induce apoptosis in *S. cerevisiae* revealing their potential for targeting fungal-specific cell death pathways and/or regulators. Thereby, the elucidation of unique fungal apoptosis pathways/regulators may lead to better antifungal

strategies. Fungicides used in the control of food spoilage organisms are very diverse and are targeted at both narrow and wide spectra of fungal pathogens. Sulphur containing compounds inhibits the electron transport chain, whereas strobilurins block the ubiquinol-cytochrome *c* oxidoreductase of the cytochrome *bc1* complex III (Ma and Michailides, 2005). Copper fungicides, dithiocarbamates, substituted aromatics and organophosphorous compounds inhibit the activities of a wide range of enzymes and are relatively non-selective. Benzimidazoles and phenylamides are found to inhibit DNA and RNA synthesis respectively. Apoptosis may have potential applications in biotechnology as a novel strategy for controlling food spoilage organisms. The advantages over conventional strategies are less release of toxic metabolites, higher efficiency and no development of resistance in food spoilage organisms.

1.11 *Benjaminiella poitrasii*: a model system to study the relationship of differentiation and apoptosis and for screening apoptosis inducing compounds

There is a continuous interest in search for new antibiotics due to the challenges of toxicity and resistance. Screening of antifungal compounds was generally carried out using different targets and model systems. Kneifel *et al*. (1974) reported the use of zygospore formation inhibition test using *Mucor hiemalis* to screen antifungal antibiotics. Several pathogenic fungi require 24-48h for the complete yeast-hypha transition (Maresca and Kobayashi, 1989). The transition of yeast cells into hypha in *P. brasiliensis* takes 14 d. *W. dermatitidis*, displayed polymorphic forms during transition from thin walled and thick walled yeast, multicellular form, moniliform hyphae and true hyphae (Kester and Garrett, 1995). Therefore, the use of *W. dermatitidis* as a model was not suitable.

Though the *S. cerevisiae* is the popular model for screening apoptosis inducing agents and showed pseudohyphae formation, it lacks the yeast to hypha differentiation, observed commonly in pathogenic or spoilage fungi. The human pathogens *C. albicans*, *H. capsulatum*; plant pathogens *U. maydis*, *C. ulmi*; and spoilage fungi such as *Pichia membranifaciens*, *Issatchenkia orientalis* and *I. terricola* showed yeast to hypha transition. *B. poitrasii*, a dimorphic zygomycete showed rapid reversible glucose, temperature, and pH dependent yeast to hypha transition. In *C. albicans*, Phillips *et al*. (2003) observed apoptosis and differentiation during the dimorphic transition. It was reported that apoptosis was induced in *C. albicans* yeast form by environmental stress and amphotericin B while hypha served as an escape route to evade apoptosis. In dimorphic fungi the Y-H transition presents a unique mechanism that may precede

apoptosis, therefore it was interesting to evaluate this phenomenon. Furthermore, the understanding of the differentiation and apoptotic pathway in dimorphic fungi will be useful to identify inhibitors to halt fungal pathogenesis.

In fungi, it was shown that the biochemical correlates of differentiation and apoptosis are common. In *C. albicans* and *S. cerevisiae* the Ras and MAPK signalling networks were shared by the differentiation and apoptotic pathways (Gourlay *et al*., 2006; Phillips *et al*., 2006). The *Ras* deletion mutants of *C. albicans* and *S. cerevisiae* failed to show apoptosis in presence of apoptotic stimuli. In *B. poitrasii* cAMP phosphorylation/dephosphorylation regulated the relative proportion of NADP-/NAD-GDH that determined the cell wall chitin content in the yeast or hyphal forms (Khale and Deshpande, 1993). Furthermore polyamines were shown to affect GDH levels important for the yeast to hypha transition in *B. poitrasii* (Ghormade *et al*., 2005a).

The biochemical correlates of differentiation in *B. poitrasii* such as glutamate dehydrogenase, chitin synthase, chitinase, *N*-acetyl glucosaminidase are well characterized (Khale and Deshpande, 1993; Deshpande, 1998b; Ghormade *et al*., 2000; Chitnis *et al*., 2002; Amin *et al*., 2004a). These biochemical correlates were also studied in the stable, monomorphic yeast-form (Y-2 and Y-5) mutants of *B. poitrasii*.

It was reported that NAD- and NADP-GDH pathway were operative in fungi like *S. cerevisiae*, *M. racemosus* and *Candida utilis* for ammonia assimilation (Fergusson and Sims, 1974; Peters and Sypherd, 1979; Avendano *et al*., 1997). The H-Y transition in *Mucor* sp. correlated with the decreased NAD-GDH activity.

Another biochemical correlate of differentiation in *B. poitrasii*, ornithine decarboxylase, involved in polyamine synthesis, showed increased activity in the H form as compared to the Y form (Ghormade *et al*., 2005a). Similarly, ODC was also shown to be biochemically correlated with differentiation in dimorphic fungi like *M. circinelloides*, *P. brasiliensis*, *U. maydis* and *Y. lipolytica* (Jimenez-Bremont *et al*., 2001; Blasco *et al*., 2002; Nino-Vega *et al*., 2004; Reyna-Lopez and Ruiz-Herrera, 2004).

GDHs and ODC are biochemical correlates of differentiation in *B. poitrasii* that were regulated by cAMP pathway. As differentiation and apoptosis were controlled by common pathways it was interesting to explore the role of these biochemical correlates during apoptosis in *B. poitrasii*, if any. These studies would help to understand the correlation between morphogenesis and apoptosis and to screen for novel antifungal agents. An antifungal agent that overrides the apoptosis regulation and induces cell death may prove to be potent since apoptosis is usually induced at low concentrations of the

stimulating agent. Apoptosis may have potential applications, as a novel strategy for controlling food spoilage organisms.

1.12 Objectives of the present investigation

1 To study biochemical correlation between enzymes involved in cell wall metabolism and yeast-hypha reversible transition and to evaluate their possible linkage with programmed cell death (PCD) in *Benjaminiella poitrasii*.

2. Characterization of cell death processes in *B. poitrasii* and their function in differentiation process

- Screen for c DNA in *B. poitrasii* to find ornithine decarboxylase (*ODC*) gene which is involved in the morphological (Yeast-Hypha and reverse) transition.

- The identification of yeast metacaspase (*YCA1*) gene that is induced/repressed by lethal stimulus.

3. a. To screen different microbial metabolites using dimorphic transition and PCD in *B. poitrasii* as a model.

b. To study the effect of potential PCD inducers on the growth of wine spoilage yeasts such as *Zygosaccharomyces*, *Dekkera*, *Debaryomyces, Candida, Aspergillus*, *Pichia* and others.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

The materials and chemicals used in the present study were purchased from suppliers listed in the Table 2.1.

Table. 2.1 Sources of chemicals

Chemicals	Supplier
Agarose	Sisco Research Laboratory, India
Ampicillin	Sigma Chemical Co., St. Louis, USA
Annexin V FITC Kit	Sigma Chemical Co., St. Louis, USA
Bacto-peptone	Difco Industries, Detroit, USA
Cyclohexylamine (CHA)	Sigma Chemical Co., St. Louis, USA
4'6-diamino-2-phenylindole (DAPI)	Sigma Chemical Co., St. Louis, USA
Diamino butanone (DAB)	Sigma Chemical Co., St. Louis, USA
Dihydrorhodamine123	Sigma Chemical Co., St. Louis, USA
Dimethyl sulfoxide (DMSO)	Merck, Germany
Ethanol	Merck, Germany
Ethylenediamine tetraacetic acid (EDTA)	Sigma Chemical Co., St. Louis, USA
Isophthalic acid	Sigma Chemical Co., St. Louis, USA
2-Ketoglutarate	Sigma Chemical Co., St. Louis, USA
L-Ornithine	SD-Fine Chemicals, India
Oligonucleotides	IDT, USA
pGEM-Teasy vector	Promega,USA
Phusion DNA polymerase	Finnzymes, Finland
Propidium iodide	Sigma Chemical Co., St. Louis, USA
Putrescine	Sisco Research Laboratory, India
Restriction enzymes	New England BioLabs, UK
Spermidine	Sigma Chemical Co., St. Louis, USA
Spermine	Sigma Chemical Co., St. Louis, USA
Taq polymerase	Bangalore Genei, India
TUNEL assay kit	Sigma Chemical Co., St. Louis, USA
2,4,6-trinitrobenzenesulfonic acid (TNBS)	Sigma Chemical Co., St. Louis, USA
Yeast extract	Difco Industries, Detroit, USA

All other chemicals, media, buffers etc. used were of analytical grade, procured from S.D. Fine, Hi Media, SRL, Loba Chemie India.

2.2 Organisms and culture conditions

The parent strain *Benjaminiella poitrasii*, a zygomycetous, dimorphic fungus was maintained on YPG (yeast extract, 0.3 %; peptone, 0.5 %; glucose, 1.0 %; agar, 2.0 %) slants at 28°C and subcultured weekly.

Yarrowia lipolytica NCIM 3589, a dimorphic sea water isolate was maintained on MGYP (malt extract, 0.3%; glucose, 1.0%; yeast extract, 0.3%; peptone, 0.5%; agar, 2.0%) in sea water (g l^{-1}) NaCl , 24.5; $CaCl_2$..2 H_2O, 1.54; KBr, 0.1; NaF, 0.003; KCl, 0.7; H_3BO_3, 0.03; Na_2SO_4, 4.09; $NaHCO_3$, 0.2; $SrCl_2$.6 H_2O, 0.017 and $MgCl_2$.6 H_2O, 11.1.

The dimorphic human pathogenic fungi, *Candida albicans* and *Crytococcous neoformans* were maintained on YPG agar slants.

Stock cultures of *Saccharomyces cerevisiae*, *Dekkera bruxellensis* (NCIM 3534), *Metschnikowia pulcherrima* (NCIM 3109), *Zygosaccharomyces rouxii* (NCIM IF988), *Candida krusei* (NCIM 3129), *Debaryomyces hansenii* (NCIM 3146) and *Pichia anamola* (NCIM 3341) were maintained on YPG agar slants at 28°C. The yeasts were grown in YPG medium and incubated at 28°C for 24 h to obtain actively growing cells.

Escherichia coli (JM 109 strain) was used as a host strain for the cloning experiment. Strain was maintained on Luria Bertani (LB) medium (yeast extract, 0.5%; tryptone, 1.0%; NaCl, 1.0%; agar, 2.0%) and incubated at 37°C (16-18 h). For bacteria containing plasmid, the LB medium was supplemented with ampicillin (100 µg ml^{-1}). For the plasmid preparation, a single colony was inoculated into 5 ml LB medium containing 100 µg ml^{-1} ampicillin and incubated for 16 h with shaking (180 rpm) at 37°C.

The bacterial isolates *Bacillus* sp. B1, B2, B14-15, B17, B21-22, *Pseudomonas* strain B18 and *Streptomyces* sp. were maintained on MGYP agar (malt extract, 0.3%; glucose, 1.0%; yeast extract, 0.3%; peptone, 0.5%; agar, 2.0%).

The mycolytic enzyme producing fungus, *Myrothecium verrucaria* NCIM 903 and the plant pathogenic fungi *Fusarium oxysporum*, *Magnaporthe grisea* and *Drechslera oryzae* were maintained on potato dextrose (2% PDA) slants.

2.2.1 Inoculum preparation

The sporangiospores of *B. poitrasii* required for yeast (Y) to hypha (H) transition studies were obtained from 7 d old culture grown on YPG agar slants at 28°C.

Sporangiospores from 7 d old slants were harvested, washed and resuspended in 0.85% saline and count was taken using haemocytometer grid (16×10^4 spores ml^{-1}). The sporangiospores were inoculated into 50 ml of liquid 1% YPG medium and incubated at 37°C for 24 h to obtain Y cells, which were washed with 0.85% saline and used as an inoculum (16×10^4 cells ml^{-1}), unless otherwise mentioned. To obtain hyphal cells, YP (yeast extract, 0.3%; peptone, 0.5%) medium was inoculated with above mentioned inoculum and incubated at 28°C for 24 h.

Table. 2.2 Media used for *B. poitrasii*

Media composition	Transition	Enzyme activities				Apoptosis
		NAD/NADP-GDH	ODC	SOD	Caspases	
YP 28°C	+	-	+	-	-	+
YPG 0.1% 28°C	+	+	+	-	-	+
YPG 0.5% 28°C	-	-	+	-	-	+
YPG 1% 37°C	-	+	+	+	+	+

GDH, Glutamate dehydrogenases; ODC, Ornithine decarboxylase; SOD, Superoxide dismutase; Glucose % as indicated; The inoculum was prepared in the respective medium used in the study.

2.2.2 Separation of Y and H forms

B. poitrasii cells were grown in YPG 0.5 and 0.1% glucose at 28°C for 24 h and the yeast and hyphal forms were separated using glass fibre filter G1 (Jensil, India). After filtration the H form was retained on the filter, whereas the filtrate contained Y form. The purity of the morphological form was confirmed microscopically.

2.2.3 Growth conditions for the apoptosis experiment

Apoptosis experiments were performed in YPG (1%, glucose) medium with and without additives as follows: i) **commercially available compounds**- diaminobutanone (DAB, ODC inhibitor), cyclohexylamine (CHA, spermidine synthase inhibitor), isophthalic acid (NAD-GDH inhibitor), amphotericin B (ergosterol synthesis inhibitor), fluconazole (14α-demethylase inhibitor); ii) **naturally isolated compound**- phenazine-1-carboxamide (isolated and purified from *Pseudomonas* sp); iii) **chemically synthesized compounds**- triazole linked β-lactam–bile acid conjugates (18B, 20B and 24 B) and tetrapeptide linked-cholic acid derivative (DS16), strobilurin derivative (PC229, inhibitor of cytochrome *bc1* complex of respiratory chain), acetic acid and H_2O_2. The β-lactam–bile acid conjugates and tetrapeptide linked-cholic acid derivative were synthesized as described by Vatmurge *et al*. (2008 a, b) and Bavikar *et al.* (2008).

To obtain synchronized yeast cells for apoptosis experiments, the yeasts cells (section 2.2.1) were passed through G1 filters (Jinsel, India) that gave cells of equal size. These cells were inoculated in fresh 1% YPG, passed through the G1 filter and the was process repeated seven times.

The synchronized *B. poitrasii* (16×10^4 cells ml^{-1}) and wine yeasts cells (1×10^6 cells ml^{-1}) obtained were inoculated in 1% YPG medium at 37°C and 28°C respectively for 12–14 h in order to obtain actively growing cells. 16×10^4 and 1×10^6 cells ml^{-1} actively growing *B. poitrasii* and wine yeasts cells were inoculated in 1% YPG media with and without the apoptotic inducers and incubated for 200 min at 37°C and 28°C respectively under shaking condition (180 rpm). For all the experiments in case of wine spoilage yeasts the cell concentration was adjusted to 1×10^6 cells ml^{-1}. After 200 min, the cells were, washed twice with potassium phosphate buffer (PB 200 mM, pH 5.8) containing KCl (PBK 0.6 M) and processed for detection of apoptotic markers. The apoptotic markers analyzed were externalization of phosphatidylserine, membrane integrity, DNA fragmentation, chromatin condensation and production of reactive oxygen species as described in section 2.3.3.

Chronological ageing was defined as the death of cells over a time, in stationary phase. For chronological ageing, *B. poitrasii* yeast cells were grown in media as described in Table 2.2 for 6-240 h and processed for the apoptotic markers. The cells were harvested at equal time intervals and analyzed for presence of apoptotic markers.

B. poitrasii yeast cells were streaked on YPG agar plates and the plates were incubated in order to get sporangiospores (2-15 d) and zygospores (till 30 d). The sporangiospores and zygospores were isolated by repeated washing with distilled water and centrifugation as described earlier (Ghormade and Deshpande, 2000). The slants were slightly scraped to obtain sporangiospores free from yeast, mycelium and zygospores, as determined by light microscopy. The underlying mycelia bed was scrapped off and crushed with mortar and pestle in distilled water to obtain zygospores free from other growth forms. The suspension was filtered through muslin cloth and centrifuged (500g, 15 sec). Repeated washing and centrifuging resulted in a clean suspension of zygospores, as seen under light microscope. Separated sporangiospores and zygospores were used for estimation of caspases and superoxide dismutase activities (as described in section 2.3.4.4 and 2.3.4.5).

2.2.4 Isolation of microorganisms producing potential antifungal agents

Soil samples were collected from different locations in and around Pune (latitude 18.31°N, longitude 73.55 °E) Maharashtra, India. Soil sample (1g) was suspended in 10 ml sterile distilled water. Serial dilutions were made and 1ml aliquot of the diluted sample was used for pour plate method. Nutrient agar was used for isolation of bacteria and plates were incubated at 28°C.

2.2.5 Preparation of culture filtrates for screening of antifungal agents

The microbial isolates were inoculated in the medium containing soyabean meal, 1%; yeast extract, 1%; mannitol, 1.5%; starch, 5%; and incubated on rotary shaker at 28°C for 96 h. The cell free supernatant obtained after centrifugation at 10000 g for 15 min was used for further testing.

2.2.6 Screening of inhibitors

The commercially available compounds, naturally synthesized compounds, chemically synthesized compounds (as described in section 2.2.3) and culture filtrates from different microbial isolates were screened using Y-H differentiation, disc diffusion and broth microdilution method.

2.2.6.1 Yeast (Y) – hypha (H) transition in *B. poitrasii*

For the Y-H transition in *B. poitrasii*, the yeast cells (16×10^4 ml^{-1}) were washed with 0.85% saline, inoculated in YP (3 ml) medium and incubated on rotary shaker (180 rpm) at 28°C for 6 h. While for H-Y transition, hyphal cells were inoculated in 0.1% YPG and incubated at 28°C for 12 h. For the screening of compounds, different concentrations of compounds were added in the YP medium from the stocks prepared in DMSO (2.5%). Transition was observed after 6 h. Y or H cells were counted as described by Khale *et al*. (1992). In Y-H transition studies 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 using haemocytometer.

2.2.6.2 Disc diffusion method

Antifungal agents were tested against *B. poitrasii* and plant pathogens. The spores of the test organisms were suspended in 5 ml of 0.1 % Tween-80 of which 0.1 ml was spread on 1% YPG plates. Whatman filter paper No.1 discs (5 mm) impregnated with varying concentrations of the antifungal agent was placed on the plates and the plates were incubated at 28°C for 24–48 h. The diameter of the zone of inhibition was measured.

2.2.6.3 MIC and IC_{50} determination

Minimum inhibitory concentration (MIC) of compounds was determined by broth microdilution technique in accordance with the NCCLS guidelines (M27-A, 1997; M38-P, 1998). The assay was performed in a flat bottom 96- well tissue culture plate containing YPG broth (100 µl per well). The concentration range for standard and test compounds was 0.5–128 µg ml^{-1}. The plates were incubated at 28°C for fungal strains and absorbance at 600 nm was recorded after 24 h for *B. poitrasii*, *Y. lipolytica*, 48 h for *C. albicans* and *F. oxysporum*, 72 h for *C. neoformans*. MIC was determined as 90% inhibition of growth with respect to the growth control and IC_{50} was the concentration at which 50% growth inhibition was observed.

2.2.6.4 Hyphal tip bursting test

For cell wall synthesis inhibitors actively growing hyphal tips were obtained by inoculating *B. poitrasii* sporangiospores on 1% YPG agar plates, incubated at 28°C for 16-18 h (Patil *et al*., 2001). Fields with 15-20 hyphal tips were selected and culture filtrate of *Bacillus* sp. B1, B2, B14-15, B17, B21-22, *Pseudomonas* strain B18 and *Streptomyces* sp. (50 µl each) in sorbitol (0.6 M) was added. The bursting of the hyphal tips was monitored microscopically and the numbers of tips burst in 10 fields were counted to find % hyphal tip bursting (HTB).

2.3 Biochemical methods

2.3.1 Apoptosis phenotype detection assay

2.3.1.1 Isolation of protoplasts

Isolation of protoplasts of *B. poitrasii* yeasts cells was performed as described by Chitnis and Deshpande, (2002). The yeast cells of *B. poitrasii* and wine spoilage yeasts were grown and washed with potassium phosphate buffer (PB, 200 mM, pH 5.8) containing KCl (PBK 0.6 M). The lysing enzyme mixture (1.3 U chitinase from *Myrothecium verrucaria*, 5 mg ml^{-1} Sigma lysing enzyme and 0.2 mg ml^{-1} lyticase) was filter-sterilized using a 0.45 µm filter (Millipore Intertech Corporation Inc., USA). Washed *B. poitrasii* (16×10^4 cells ml^{-1}) and wine spoilage yeasts cell (1×10^6 cells ml^{-1}) were pretreated with a 10 mg ml^{-1} protease solution in PB for 30 min at 28°C. The cells were then washed with PBE (PB containing 2 mM EDTA) and then treated with a solution containing 50 mM dithiothreitol and 10 mM β-mercaptoethanol in the PBE at 37°C for 1 h. The cells were washed 5 times with PBE and then directly suspended in 1 ml of the cell wall lysing enzyme mixture containing 0.6 M KCl and were incubated at

28°C for 5 h and 3 h for *B. poitrasii* and wine spoilage yeasts respectively. The protoplasts were separated from the lysing enzyme by low speed centrifugation, at 500×g for 15 min. The protoplasts pellet was then washed with PBK, centrifuged and resuspended in the PBK buffer. The integrity of the isolated protoplasts was checked by vital staining using 0.1% (w/v) eosin in a PBK buffer.

Annexin V-FITC, TdT-mediated dUTP nick end labelling (TUNEL), DAPI and DHR 123 staining, were performed as described by Madeo *et al*. (1997, 1999, 2002).

2.3.1.2 Annexin V-FITC staining

The annexins are a group of homologous proteins which bind to phospholipids in the presence of calcium. In living cells, the phosphatidylserine was transported to the lipid bilayer by the Mg-ATP dependent enzyme, aminophospholipid translocase. At the onset of apoptosis, phosphatidylserine was translocated to the external portion of plasma membrane. Phosphatidylserine externalization was detected by reaction with FITC-coupled annexin V (Annexin V apoptosis Kit; Sigma Chemical Co).

Pretreatment of *B. poitrasii* and wine spoilage yeasts cells were carried out at 37°C for 1 h in 50 mM DTT and 10 mM β-mercaptoethanol. Yeast cells were washed with potassium phosphate buffer (200 mM, pH 5.8) containing KCl (PBK 0.6 M), digested with lysing enzyme mixture as mentioned in section 2.3.1.1. After digestion cells were washed in binding buffer (1 mM Hepes/NaOH, pH 7.4, 14 mM NaCl, 0.25 mM $CaCl_2$ in PBK centrifuged and resuspended in binding buffer. 5 µl annexin- FITC (50 µg ml^{-1}) and 10 µl propidium iodide (100 µg ml^{-1}), were added to 1 ml of cell suspension and then incubated for 20 min at room temperature in dark. Cells were observed for fluorescence with excitation and emission settings of 488 nm and 520 nm. Excitation and emission for PI was 536 and 623 nm respectively. The fluorescence was observed by epifluorescence microscope (Leitz Labor Lux, Germany). A Cannon Power Shot S-80 camera was used to record fluorescence and light microscope photographs.

2.3.1.3 Terminal deoxynucleotidyl transferase–mediated dUTP nick end labelling (TUNEL)

The enzyme terminal deoxynucleotidyl transferase catalyzes a template independent addition of deoxyribonucleoside triphosphates to the 3′-hydroxyl ends of double or single stranded DNA with either blunt, recessed or overhanging ends. Br-dUTP incorporated in to genome of apoptotic cells gave rise to stronger fluorescein signal when Br-dUTP was identified by fluorescein labelled anti-BrdU monoclonal

antibody (Sigma Chemical Co.). Non apoptotic cells do not incorporate Br-dUTP owing to the lack of exposed 3'-hydroxyl DNA ends.

B. poitrasii and wine spoilage yeast cells were fixed with 3.7% formaldehyde, digested with lysing enzyme mixture as mentioned in section 2.3.1.1. The cells were rinsed with PBK, incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. The cells were rinsed twice with PBK and incubated with 50 µl DNA labelling solution for 60 min at 37°C. After incubation the cells were rinsed three times with PBK, incubated with 100 µl of antibody solution (Anti-BrDU-Fluorescein in rinsing buffer, Sigma) for 30 min at room temperature. Cells were observed for fluorescence with excitation at 488 nm and emission at 520 nm.

2.3.1.4 4'6-diamino-2-phenylindole (DAPI) staining

4'-6-Diamidino-2-phenylindole (DAPI) was known to form fluorescent complexes with natural double-stranded DNA, showed fluorescence specificity for AT, AU and IC clusters. Apoptotic nuclei identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies.

To assess chromatin condensation, samples were stained with DAPI (Sigma). *B. poitrasii* and wine spoilage yeast cells were harvested and fixed with 3.7% formaldehyde for 30 min and washed three times with PBK. After washing cells were incubated with 1 µg ml^{-1} DAPI in PBK for 10 min at room temperature in dark. Cells were observed for fluorescence with excitation and emission settings of 350 nm and 461 nm.

2.3.1.5 Dihydrorhodamine (DHR123) staining

Dihydrorhodamine 123 was the reduced form of rhodamine 123, commonly used as a fluorescent mitochondrial dye. Dihydrorhodamine 123 itself was nonfluorescent, but it readily entered cells and was oxidized by oxidative species to fluorescent rhodamine 123 that accumulates in mitochondrial membranes. Free intracellular radicals were detected with DHR123 (Sigma).

DHR123 was added at 5 µg ml^{-1} in yeasts cell from a 2.5 mg ml^{-1} stock solution in ethanol and cells were viewed without further processing through a rhodamine optical filter after 2 h incubation (Madeo *et al*., 1999). Cells were observed for fluorescence with excitation and emission settings of 500 nm and 550 nm.

2.3.2 Inhibitor purification

2.3.2.1 Production and isolation of antifungal metabolite

Pseudomonas strain B-18 was grown in medium containing soyabean meal, 1%; yeast extract, 1%; mannitol, 1.5%; starch, 5%; and incubated at 28°C for 96 h under shaking condition (180 rpm). The culture filtrate was centrifuged at 10000 g for 15 min to separate the cells. The culture filtrate (3 L) was evaporated under vacuum at 50°C to yield yellowish brown residue (20.5 g). Residue was extracted successively with 200 ml acetone and 200 ml ethyl acetate to yield acetone extract, 1.3 g (Fraction A), ethyl acetate extract, 200 mg (Fraction B) and residue (Fraction C). Cell biomass was washed with water and suspended in 200 ml acetone for 12 h, centrifuged at 10000 g for 15 min and the acetone soluble was decanted. Acetone was evaporated under vacuum at 50°C to yield yellow residue, 1.35g. This residue was extracted successively with 200 ml chloroform and 200 ml ethyl acetate to yield product of 0.45 g (Fraction D), ethyl acetate extract, 0.2 g (Fraction E) and residual extract of biomass, 0.7 g (Fraction F) (Fig. 2.1).

The active fraction D was separated into 10 fractions using column chromatography (20 mm O.D. X 27cm length) on silica gel 200-300 mesh (20.0g) and methanol: chloroform (5:95) as eluting system. Fractions 2 to 4 which exhibited antifungal activity were purified by preparative thin layer chromatography using methanol: chloroform (5:95) as developing system to isolate compound 1 (Phenazine-1-carboxamide) 131mg.

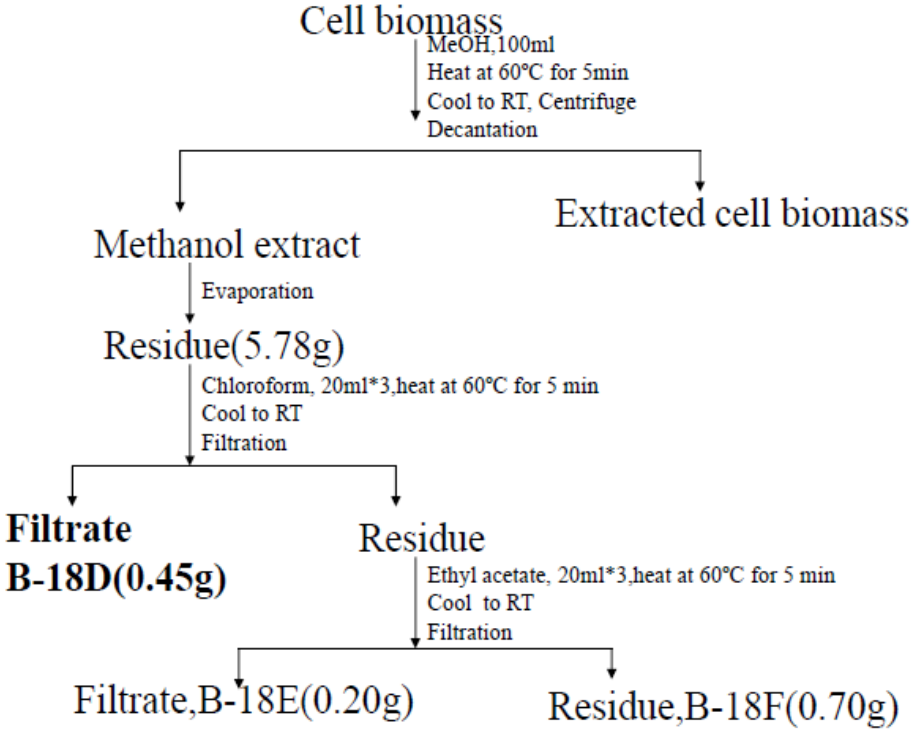

Fig. 2.1 Scheme for fractionation

2.3.2.2 Structural elucidation of phenazine-1-carboxamide (PCN)

Spectroscopic analyses were performed to determine the structure of antifungal metabolite PCN. Nuclear magnetic resonance (^{1}H NMR) spectrum was recorded on a Bruker Avance 500 MHz and 125 MHz for ^{13}C. NMR spectra were measured in deuterated chloroform ($CDCl_3$) at room temperature. The chemical shifts of the ^{1}H NMR, ^{13}C NMR and DEPT (Distortion-less Enhancement by Polarization Transfer) spectra are given in δ (ppm). Mass spectrum was recorded with electrospray ionization–mass spectrometer (ESI-MS), API-QSTAR-PULSAR in positive ion mode. Infrared spectrum was recorded using Perkin Elmer FT-IR spectrometer.

2.3.3 Cell extract preparation

Cell extracts of yeast and hyphal forms of *B. poitrasii* were obtained by the procedure described by Khale *et al*. (1992). Hyphal and the yeast form cells were collected on Whatman filter paper No.1 and washed with 0.85% saline, followed by potassium phosphate buffer (100 mM, pH 7.4) containing 1.0 mM ethylene diamine tetraacetic acid (EDTA), 1.0 mM phenyl methane sulphonyl fluoride (PMSF) and 3.0 mM dithiothreitol (DTT) for glutamate dehydrogenases (GDH).

For ornithine decarboxylase (ODC), potassium phosphate buffer (150 mM, pH 7.1) containing 0.1 mM EDTA, 1.0 mM PMSF, 2.0 mM DTT and 0.2 mM pyridoxal phosphate (PLP) was used as an extraction buffer.

For caspase , extraction buffer containing 50 mM HEPES, 0.1% CHAPS, 1.0 mM DTT and 0.1 mM EDTA, pH 7.4 was prepared according to Richie *et al*. (2007).

For superoxide dismutase (SOD), extraction buffer contained: 250 mM sucrose, 12 mM Tris-HCl and 0.1 mM DTT, pH 7.4 (Arora *et al*., 2008). Yeast and hyphal cells of *B. poitrasii* (yeast, 1g per 5ml; hyphal, 1g per 2ml of extraction buffer) were disrupted using Braun's homogenizer 5 and 4 cycles respectively of 15 s each. The samples were centrifuged at 12,000 g for 10 min at 4°C to obtain cell extracts for the estimation of enzyme activities.

2.3.4 Enzyme assays

2.3.4.1 Glutamate dehydrogenase (NAD-GDH, E.C 1.4.1.2 and NADP-GDH, E.C 1.4.1.4)

The intracellular NAD- and NADP-dependent GDH activities were assayed using the method described by Khale *et al.* (1992). The reaction mixture (1ml) contained 50-100 µl of cell extract, 200 mM NH_4Cl and 30 mM 2-ketoglutarate in 100 mM Tris-HCl

buffer (pH 8.0). Briefly the reaction was initiated by the addition of 125 µM NADH or NADPH to the sample cuvette. The control in which 2-ketoglutarate was omitted from the reaction mixture was run for each assay. The reductive amination of 2-ketoglutarate was measured by monitoring the decrease in the A_{340} of NAD(P)H. One unit of NAD- or NADP-GDH was defined as the amount of enzyme required to oxidize 1 nmol of NADH or NADPH min^{-1} mg^{-1} protein.

2.3.4.2 Ornithine decarboxylase (ODC, E.C 4.1.1.17)

2.3.4.2a Radiometric assay

In this method the release of $^{14}CO_2$ from ^{14}C-labelled ornithine was measured as described by Voige (1997) with some modifications. A 400 µl cell extract was added to L-ornithine (9.6 µmol) and 25 nCi L-[1-^{14}C] ornithine (52 mCi/mmol) prepared in 100 µl of buffer A (150 mM potassium phosphate buffer, pH 7.1; containing 5 mM DTT, 1.5 mM EDTA, 0.1 mM PLP, 0.5 mM cyclohexylamine) in a glass tube (14 x 880 mm). Reaction tubes were fitted with a rubber stopper and a glass microfibre filter (Whatman GF/C, 2.5 cm) soaked in 200 µl 2N KOH. After 30 min incubation at 37ºC, the reaction was stopped by adding 800 µl of 1M $HClO_4$ and the vials were incubated for another 30 min to trap CO_2 released. The $^{14}CO_2$ released was trapped on a filter paper disc. At the end of incubation, filter paper was transferred to a vial containing 3 ml scintillation fluid (4g PPO (2,5 diphenyloxazole) and 0.1g POPOP (1,4-bis[5-phenyl-2-oxazolyl]-benzene) in 1:1 toluene) for measuring radioactivity. The amount of radioactivity in each vial was determined by liquid scintillation spectrometry (Beckmann LS-500). The heat killed enzyme was used as a control. One unit of ODC activity was defined as one nmol of CO_2 released min^{-1} mg^{-1} protein.

2.3.4.2b Spectrophotometric assay

The cell extract (400 µl) was added to 400 µl of buffer A and the reaction was initiated by addition of 9.6 µmol L-ornithine HCl (400 µl). Reaction was carried out at 37°C for 30 min and terminated by the addition of 800 µl of 1 M $HClO_4$. The reaction mixture was centrifuged at 5000 g for 10 min and 0.5 ml of the supernatant was used for the estimation of putrescine. The heat killed enzyme was used as a control**.**

For the estimation of putrescine, 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) was used as described by Ngo *et al*. (1987). 0.5 ml of supernatant and 1 ml of 4 N NaOH was mixed by vigorous shaking. 2 ml of 1-pentanol was then added and the sample was mixed and vortexed for 20 sec. The emulsion was centrifuged at 2000 g for 5 min, and 1

ml of upper organic phase was transferred to the test tube containing 1 ml sodium borate buffer (100 mM, pH 8.0) and mixed properly. 1 ml of 10 mM TNBS dissolved in 1-pentanol was added to it and vortexed for 20 sec, then 2 ml dimethyl sulfoxide (DMSO) was added and vortexed again for 20 sec. The sample was centrifuged at 2000 g for 5 min and the absorbance of the trinitrophenyl (TNP) putrescine adduct formed in the organic phase was measured at 420 nm. Putrescine (6–250 nmol) in 100 mM potassium phosphate buffer (pH 7.0) was used as a standard.

One unit of ODC activity was defined as the amount of enzyme required to produce one nmol of putrescine min^{-1} mg^{-1} protein.

2.3.4.3 Spermidine synthase assay

The cell extract (400 µl) was added to buffer A (400 µl) and the reaction was initiated by the addition of 250 nmol putrescine (400µl). The reaction was carried out at 37°C for 30 minutes for conversion of putrescine into spermidine and terminated by the addition of 800 µl of 1 M $HClO_4$. The reaction mixture was centrifuged at 5000 x g for 10 min and the 0.5 ml supernatant was used for the estimation of the unconverted putrescine. The heat killed enzyme was used as a control**.** Spermidine synthase activity was defined as the amount of enzyme required to convert one nmol of putrescine into spermidine min^{-1} mg^{-1} protein.

2.3.4.4 Caspase (Caspase-1, **E.C 3.4.22.36**; **-3**, **E.C 3.4.22.56 and -8**, **E.C 3.4.22.61) assay**

The caspase like activities (caspase -1,-3 and -8) of *B. poitrasii* and wine spoilage yeasts cells were estimated as described by Richie *et al*. (2007). Intracellular caspase-like activities were determined using commercially available fluorogenic system that uses the peptides YVAD-AMC (*N*-acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin), DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) and IETD-AMC (*N*-acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin) as substrates. The caspase assay was performed using 0.026 mM substrate and cell extract containing 100 µg of protein made upto total reaction volume of 100 µl. After 2 h incubation in the dark at 37°C, the fluorescence at 460 nm was measured using an excitation wavelength of 360 nm. The activity is presented as relative fluorescent units (RFUs), calculated by subtracting background fluorescence in the absence of cell extract from the fluorescence obtained in the presence of cell extract.

2.3.4.5 Superoxide dismutase (SOD, E.C 1.15.1.1)

The SOD activity in *B. poitrasii* and wine spoilage yeasts was estimated as described by Arora *et al*. (2008). 20 µl of 100 mM hydroxylamine hydrochloride and 100 µg of cell extract protein was added to 30 µl of 1.6 mM nitroblue tetrazolium (NBT) and 6 µl of 10% Triton X-100 and the volume was made upto 2.1 ml with sodium carbonate buffer (50 mM, pH 10.2). The rate of NBT reduction was measured at 560 nm for 5 min using a spectrophotometer. Percentage inhibition in the rate of NBT reduction was calculated and one unit of enzyme was expressed amount of protein required to inhibit the reduction rate of NBT by 50%.

2.3.5 Estimation of protein

Protein was estimated according to Lowry *et al*. (1951) method, using crystalline bovine serum albumin as a standard.

2.4 Molecular methods

2.4.1 Isolation of genomic DNA from *B. poitrasii*

Yeast form cells of *B. poitrasii* were used to isolate genomic DNA. The cells were harvested by filtration through Whatman paper No. 1, washed with sterile distilled water, frozen immediately in liquid N_2 and stored at -80°C until use. The cells were ground using mortar and pestle under liquid N_2. The ground cells (1g) were transferred to 10 ml of extraction buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate (SDS), 50 mM DTT, 1% β-mercaptoethanol, pH 8.0) and mixed by gentle inversion 2–3 times. This was followed by phenol: chloroform (1:1) extractions. The DNA was precipitated with 0.8 volumes of isopropanol. The pellet was then washed with absolute ethanol, air-dried and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

2.4.2 Plasmid DNA isolation from *E. coli*

Plasmid isolation from *E. coli* JM109 transformants was carried out by the alkaline lysis method described by Sambrook and Russell (2001). *E. coli* cells were grown for 12 h in 5 ml LB-ampicillin (100 µg ml^{-1}) as mentioned in section 2.2**.** The 12 h grown cells were transferred to a 1.5 ml microfuge tube and centrifuged at 12,000 g for 5 min. The pellet was resuspended in 200 µl ice-cold solution containing 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0. After the addition of ice cold solution, 400 µl of freshly prepared 200 mM NaOH and 1% SDS was added, the suspension was mixed by inversion and placed on ice for 10 min. To above solution, 300 µl of 5 M potassium

acetate was added and vortexed for 10 s. The tube was then kept on ice for 10 min. The lysate was centrifuged at 10,000 g for 5 min. An equal volume of phenol-chloroform (1:1) was added and the mixture was vortexed. The suspension was then centrifuged at 12,000 g for 5 min. The aqueous phase was removed, the plasmid DNA was precipitated with 0.85 volumes of isopropanol and incubated at -20ºC for 30 min. The plasmid DNA was pelleted by centrifugation at 12,000 g for 5 min. The pellet was washed with 70% ethanol and dried at 37°C for 10 min. The plasmid DNA was redissolved in 50 µl TE buffer (pH 8.0).

2.4.3 Isolation of total RNA from *B. poitrasii*

For isolation of total RNA, *B. poitrasii* yeast cells were inoculated in 0.1 % YPG medium and incubated at 28°C for 12 h under shaking conditions (180 rpm). Yeast and hyphal cells were separated as described in section 2.2.2. Further, the yeast cells from the filtrate were harvested by filtration through Whatman filter paper No. 1, washed with sterile distilled water. The glasswares and plasticwares used for RNA isolation were treated overnight with 0.1% diethylpyrocarbonate (DEPC) and autoclaved. The separated yeast and hyphal cells were ground using mortar and pestle under liquid N_2. RNA was isolated using RNeasy mini kit (Qiagen, USA) according to the manufacturer's instructions. The RNA preparations were stored at -80°C until use. RNA concentration and purity was confirmed by measuring absorbance at 260/280. For RNA samples, absorbance at 260 nm = 1 corresponds to 40 µg ml^{-1} (Sambrook and Russell, 2001).

2.4.4 cDNA synthesis

For synthesis of cDNA, the isolated RNA samples were first treated with DNase (Promega, USA) to remove any contaminant DNA according to manufacturer's instructions. The reaction mixture (10 µl) consisted of 1 µg RNA, 1 µl of 10 x reaction buffer and 1 U μg^{-1} DNase. The reaction mixture was incubated at 37ºC for 30 min and the reaction was stopped by adding DNase stop solution (1 µl). DNase was denatured by incubating the reaction mixture at 65°C for 10 min and chilled on ice.

The cDNA was synthesized from 1 µg of RNA using sensiscript reverse transcriptase first strand synthesis kit (Qiagen, USA) according to manufacturer's instructions. The template (1 µg RNA) and primer (0.5 µg oligo dT) were mixed in a total volume of 8 µl and was incubated at 70°C for 10 min. The reaction mixture for the reverse transcription reaction consisted of 6.5 µl nuclease-free water, 2 µl of 1x reaction buffer, 1 µl of 1 U reverse transcriptase, 2 µl of 5 mM dNTPs and 0.5 µl of 5 U ribonuclease inhibitor RNasin®. The template-primer mixture (8 µl) was added to the

above reaction mixture on ice and was incubated at 37°C for 1 h. The quality of synthesized cDNA was checked by carrying out PCR reaction with ITS1 and ITS4 primers and analyzed by agarose gel electrophoresis.

2.4.5 Polymerase chain reaction (PCR) and Reverse transcriptase-PCR

To amplify ornithine decarboxylase (ODC) and metacaspase homolog of caspase gene from *B. poitrasii*, degenerate PCR primers directed towards conserved region in ODC and metacaspase were used for the PCR amplification of the genomic DNA and cDNA. The amplification was carried out using the thermal cycler (Mycycler, Biorad, USA). The PCR reactions were performed in 20 µl mixture consisting of 1x reaction buffer (with 1.5 mM $MgCl_2$), forward and reverse primers (0.5 µM of each, obtained from IDT, USA), 200 µM of each dNTPs and 0.4 U of phusion DNA polymerase (Finnzymes, Finland) and 100 to 150 ng of DNA or cDNA. Amplification conditions for ODC were: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 51°C for 45 sec and extension at 72°C for 1 min with final extension at 72°C for 7 min. For metacaspase gene PCR programme was same except annealing temperature (46°C for 45 sec). 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 size. The details of the primers used are given in Table 2.3.

Table. 2.3 List of primers (5'-3')

Forward (Metacaspase)	Reverse (Metacaspase)
AF1 ATCAACGATGTCAMSAACATGTC	AR1 ATACCCTGWGTYGAGTAGA
AF4 TGATYGGAATCAACTAYWTCGG	AR3 GGATGRCTGCAGCTCARCTGCGG
Forward (ODC)	**Reverse (ODC)**
ODC F GTACCCCCTTCTACGCCATG	ODC R TCGTCGGTGACAATTCGGAGG
ODC F1 ATGGCCCCTCACATCCTTGG	ODC R1 TTACAACTTGATGTACTGCTCAACC
ODCF4 CCTGCAAGGTGGCGTCCTAC	ODCR3 CGCTGTTGAAACCGTTGAAGG

2.4.6 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 overnight. The reaction mixture (10 µl) consisted of 50 ng template, 1x reaction buffer, 50 ng μl^{-1}pGEM-Teasy vector and 1U μl^{-1}DNA ligase. For the transformation reaction, *E. coli* JM109 competent cells were prepared by Inoue method as described by Sambrook and

Russell (2001). After overnight incubation of ligation mixture, 3 µl of product was transformed to 50 µl of competent cells. The mixture was gently flicked and the tubes were placed on ice for 20 min. The mixture was then incubated at 42ºC for 50 sec. Then 300 µl of SOC medium (tryptone, 2.0%; yeast extract, 0.5%; NaCl, 0.05%, 20 mM glucose and 250 mM KCl, pH 7.0) was added to tube and incubated at 37°C for 1 h. The transformed cells were spread on LB media containing 50 mg ml^{-1}X-gal (20 µl), 0.1 mM IPTG (100 µl) and ampicillin (100 µg ml^{-1}) and incubated at 37°C for 12 h. After incubation, the positive clones were selected by blue-white screening.

2.4.7 Colony PCR for selection of desired clone

The transformed colonies were suspended in 50 µl sterile distilled water. The suspended cells were heated at 95ºC for 5 min and centrifuged at 12,000 g for 5 min. The supernatant was used as a template for amplification. Amplification conditions for colony PCR: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 45 sec and extension at 72°C for 1 min with final extension at 72°C for 7 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 size.

2.4.8 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.

2.4.9 DNA sequencing reactions

Plasmids were isolated from the clones containing the desired fragments. Sequencing was done on an AB3730 DNA analyzer using the Big Dye terminator kit (Applied Biosystems, Inc., Foster City, CA).

2.4.10 Analysis of DNA sequence data

The sequences obtained were then subjected to FASTA3 program (www2.ebi.ac.uk/fasta3) searches and were grouped into different categories based on the maximum homology shown to known ODC and metacaspase gene from the database. Phylogenetic trees were constructed using deduced amino acid sequences and protein homologies by the maximum parsimony method included in the Clustal W program (Thompson *et al*., 1994).

2.4.11 Phylogenetic analysis

Phylogenetic analysis was performed using the deduced amino acid sequences of PCR fragments corresponding to ODC and metacaspase gene identified in the study. They were compared to the amino acid sequences of several fungal and other ODC and metacaspase sequences retrieved from Gen Bank Database. The analysis was carried out with the computer programs available in the Phylogeny Inference Package (PHYLIP) (version 3.5 p by Felsenstein, 1993).

The sequences were trimmed at the beginning and end and gaps were removed by DAMBE software. Analysis was done by the bootstrap method using maximum 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" 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 out of the 1000 phylogenies using the program "Consense" (Felsenstein, 1993) which finds the majority rule for consensus tree.

2.5 Analytical methods

2.5.1 Estimation of polyamines

The yeast and mycelium cells of *B. poitrasii* grown in YPG (0.1%, glucose), were separated by G1 filter, homogenized and used for polyamine analysis. The standard polyamines and polyamines in cell extracts were benzoylated by a modified method of Flores and Galston (1982). One ml of 2N NaOH and 10 µl of benzoyl chloride were added to 200 µl of the polyamine aliquots and vortexed for 30 sec. After incubation at 25°C for 20 min, 2 ml saturated NaCl was added to the samples to stop the reaction. The benzoyl polyamines were extracted in 3 ml of diethyl ether. The samples were centrifuged at 1500 g for 5 min and 1.5 ml of ether phase was collected and evaporated over a water bath (60°C) to dryness. The benzoyl polyamines were redissolved in 200 µl of 64% (v/v) methanol (HPLC grade; Merck, Germany). The benzoylated samples were stored at –20°C.

The benzoylated polyamines were analyzed with Waters 2690 separations module HPLC equipped with 2487 Dual λ absorbance detector (Waters). A C-18 column (4.6 X 250 mm, 5 µm particle size: Waters) was used for the separation of polyamines. The benzoylated polyamines (50 µl) were injected automatically and chromatographed at 28°C. The solvent system consisted of methanol: water, run isocratically at 64% methanol (v/v), with a flow rate of 0.5 ml min^{-1}. The benzoylated polyamines were detected spectrophotometrically at 254 nm.

2.5.2 Microscopy

2.5.2a Fluorescence microscopy

The cells stained with annexin V FITC and TUNEL were observed under the I3 filter with an excitation range of 450-490 nm and emission settings 520 nm. A-filter was used to observe cells stained with DAPI with an excitation range of 340-380 nm with emission settings 461 nm. N2.1 filter was used to observe cells stained with DHR 123 with an excitation range of 515–560 and emission settings 550 nm. The fluorescence was observed by epifluorescence microscope (Leitz Labor Lux, Germany). A Cannon Power Shot S-80 camera was used to record fluorescence and light microscope photographs

2.5.3 Agarose gel electrophoresis

Agarose gel was prepared by melting agarose in 1x TAE buffer (40 mM Tris-acetate and 1mM EDTA, pH 8.0). To cast and run the gel, Bio-Rad Mini, Midi or Maxi electrophoresis cells were used (Biorad, USA). The concentration of agarose varied according to the size of DNA molecules being separated. 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 6x gel loading buffer (0.25 % bromophenol blue, 0.25% xylene cyanol and 40% glycerol). A molecular weight marker, 200 ng of a Gene ruler ladder (Fermentas, USA) was used. DNA samples were electrophoresed through the gel at 5 Vcm^{-1}. The DNA was visualized under ultraviolet light on a Gel Doc XR system (Biorad, USA).

CHAPTER III

Biochemical studies of differentiation as a pre-programmed cell death process in *B. poitrasii*

3. INTRODUCTION

Dimorphism, a form of fungal differentiation is a phenomenon displayed by plant, human and insect pathogens during pathogenesis. The reversible morphological change from the unicellular yeast-form to the filamentous hyphal-form responds to perturbations in incubation conditions such as temperature, pH of medium, C: N ratio or metal ions (Gow, 1995; Deacon, 2006). Morphological change occurs due to change in the cell wall architecture that was influenced by levels of carbon and nitrogen metabolizing enzymes involved in cell wall synthesis and degradation. These enzymes were studied in various fungi such as *C. albicans*, *M. racemosus* and *S. cerevisiae* as biochemical correlates of the morphological transition (Doiphode *et al*., 2009). In *M. racemosus*, anerobiosis (CO_2/N_2) induced yeast-mycelium transition that correlated with the increase in NAD-GDH activity. Similar results were obtained with *M. bacilliformis* where mycelium showed two fold more NAD-GDH activity than yeast form (Peters and Sypherd, 1979). In the dimorphic fungi *M. bacilliformis*, *M. circinelloides*, *M. racemosus*, *P. brasiliensis*, *U. maydis* and *Y. lipolytica,* ODC, a polyamine synthesizing enzyme was reported to play a significant role in the differentiation (Garcia *et al.*, 1980; Ruiz-Herrera *et al.*, 1983; Guevara-Olvera *et al*., 1997; Jimenez-Bremont *et al*., 2001; Blasco *et al*., 2002; Nino-Vega *et al*., 2004; Reyna-Lopez and Ruiz-Herrera, 2004). ODC converted ornithine into putrescine, which was subsequently converted into spermidine and spermine. In *U. maydis* and *Y. lipolytica* the Y form was associated with low ODC activity and polyamine levels, while it was *vice a versa* in the hyphal form. These polyamines play important roles in nucleic acid stabilization, protein synthesis, membrane stabilization, modulation of ion channels, protection against oxygen radicals and were essential for cell homeostasis and cell growth in mammalian cells (Schipper *et al*., 2000). Chitin synthase, glucan synthetase, chitinase and *N*-acetylglucosaminidase also played an important role in Y-H morphogenesis in various fungi like *M. rouxii*, *P. brasiliensis* (San-Blas, 1979; Zou *et al*., 1990).

B. poitrasii, a non-pathogenic dimorphic fungus, was used as a model for the study of Y-H differentiation. The yeast-form was favoured by increase in glucose concentration, higher temperature (37°C) and acidic pH, whereas hyphal form favoured by low glucose concentration, low temperature (28°C) and alkaline pH (Khale *et al*., 1990; Ghormade *et al*., 2000; Ghormade and Deshpande, 2000; Chitnis and Deshpande, 2002). It was already reported that different enzymes involved in ammonia assimilation and chitin synthesis such as NADP- and NAD-dependent glutamate dehydrogenases

(GDHs), chitin synthase, chitinase, *N*-acetylglucosaminidase and polyamine synthesizing enzyme ODC showed biochemical correlation with Y-H transition in *B. poitrasii* (Khale *et al*., 1992; Ghormade *et al*., 2000; Chitnis *et al*., 2002; Ghormade *et al*., 2005a). Among these, the relative proportion of NADP- and NAD- dependent GDHs regulated by phosphorylation or dephosphorylation was found to have a significant correlation with Y-H transition (Khale and Deshpande, 1993; Khale *et al*., 1992). Furthermore, *B. poitrasii* possessed three GDHs, one requiring NAD while the other two were form specific which use NADP as a cofactor (Amin *et al.*, 2004a). It was interesting to study the biochemical role of these enzymes in connection with apoptosis, morphological differentiation and different stages of the fungal life cycle.

In the present study, effects of different additives in *B. poitrasii* were studied on Y-H transition and biochemical enzyme correlates of morphogenesis *viz.* NAD-/NADP-GDH and ODC. The additives assessed were, commercially available compounds- diaminobutanone (DAB, ODC inhibitor), cyclohexylamine (CHA, spermidine synthase inhibitor), isophthalic acid (NAD-GDH inhibitor) and amphotericin B (ergosterol synthesis inhibitor); naturally isolated compound- phenazine-1-carboxamide (isolated and purified from *Pseudomonas* sp. B18); chemically synthesised compounds- triazole linked β-lactam–bile acid conjugates (18B, 20B and 24 B) and a tetrapeptide linked-cholic acid derivative (DS16); acetic acid and H_2O_2. The possible correlation of differentiation and apoptosis in *B. poitrasii* is discussed in chapter IV.

3.1 RESULTS

3.1.1 NAD-GDH, NADP-GDH, ODC and polyamine levels in yeast and hyphal forms of *B. poitrasii*

The Y and H form cells of *B. poitrasii* were grown in 0.1% YPG, separated after 24 h and cell extracts were assessed for NAD-/NADP-GDH, ODC activities and polyamine levels as described in section 2.3.3, 2.3.4.1, 2.3.4.2 and 2.5.1 (Table 3.1).

3.1.2 GDH and ODC activities during Y-H transition in *B. poitrasii*

The intracellular GDH and ODC activities were estimated as mentioned in the section 2.3.4.1 and 2.3.4.2. It was observed that >85 % of the yeast cells germinated into hyphae after incubation at 28ºC for 24 h in 0.1% YPG. The initial NAD-GDH activity increased by 11 fold whereas NADP-GDH activity decreased by 6 fold (Table 3.2). In case of ODC, the activity increased by 4.5 fold (Table 3.2). NADP-GDH and NAD-GDH activities correlated significantly with ODC activity. Correlation coefficient value (*r*) of

NAD-GDH to ODC was 0.862 (p= 0.0001) and for NADP-GDH to ODC it was−0.862 (p= 0.0001).

Table. 3.1 NAD-GDH, NADP-GDH, ODC and polyamine levels in yeast and hyphal forms of *B. poitrasii*

Polyamine	Yeast (U mg^{-1})	Hypha (U mg^{-1})
NAD-GDH	93.0±0.30	724.0±22.0
NADP-GDH	39.0±0.20	1.4±0.20
ODC	0.891±0.11	4.1±0.22
Putrescine	51.7	83.2
Spermidine	149.0	273.9
Spermine	9.1	37.2

Cells were grown in 0.1% YPG at 28°C for 24h.

Table. 3.2 NAD-GDH, NADP-GDH and ODC activities during yeast to hypha transition in *B. poitrasii*

Time interval (h)	Germ-tube (%)	NAD-GDH (U mg^{-1})	NADP-GDH (U mg^{-1})	ODC (U mg^{-1})
0	0	83.0±7.0	20.0±2.0	0.891±0.01
6	32.1±2.0	500.0±77.0	16.0±1.0	1.10±0.09
12	56.2±3.0	780.0±82.0	12.0±2.0	2.08 ±0.30
24	85.0±6.0	979.0±100	3.0±0.5	4.08±0.30

B. poitrasii yeast cells were grown in 0.1% YPG at 28°C.

3.1.3 Effect of different additives on Y-H transition and their biochemical enzyme correlates

3.1.3.1 Effect of different additives (apoptosis inducers) on Y-H transition of *B. poitrasii*

Morphological differentiation plays a vital role in pathogenesis of fungal infection. The effect of various additives was measured by counting the germ-tube formation during Y-H transition. Y-H transition was carried out as described in materials and methods (section: 2.2.6.1). Studies on the influence of specific NAD-GDH showed that in the presence of 15 mM isophthalic acid germ tube formation was inhibited by 86.9% (Table 3.3). The reverse was true in H-Y transition. For instance, during H-Y transition percent budding cells (31% in 0.1% YPG at 28ºC after 12 h) increased to 56% in presence of 16 mM isophthalic acid. Cells exposed to acetic acid (5 and 10 mM) exhibited increased germ tube inhibition (25.6 and 81.0% respectively), whereas complete inhibition was observed with ≥20 mM concentration of acetic acid (Table 3.3).

The H_2O_2 exposed cells exhibited germ tube inhibition (10-30 mM) (Table 3.3). Amphotericin B, an ergosterol synthesis inhibitor affected Y-H transition at different concentrations (1-4 μM) (Table 3.3).

Further, to assess the effect of diaminobutanone (DAB, an ODC inhibitor) on Y-H transition, varied concentrations of DAB (8-15 mM) were used. DAB inhibited germ tube formation by 77.1-85.1% with increase in concentration (8-12 mM) as compared to control (Table 3.3). The effect of different concentrations of cyclohexylamine (CHA, a spermidine synthase inhibitor) on the Y-H transition was also studied (Table 3.3). Decrease in germ tube formation was observed with increasing CHA concentration, while no germ tubes were observed with ≥ 4mM concentration of CHA.

Table. 3.3 Effect of different additives (apoptosis inducers) on Y-H transition of *B. poitrasii*

Additives	Germ tube %
Control	53.8±2.3
Isophthalic acid (mM)	
12	26.9±2.4
15	7.0±0.4
16	ND
Acetic acid (mM)	
5	40.0±5.5
10	10.2±1.5
20	ND
H_2O_2 (mM)	
10	25.0±2.4
20	20.0±0.8
30	10.8±1.4
50	ND
Amphotericin B (μM)	
1	16.3±1.5
2	2.9±0.8
4	ND
DAB (mM)	
8.0	12.3±0.8
10	11.3±1.0
12	8.0±0.4
15	ND
CHA (mM)	
1.0	31.2±1.5
2.0	26.1±1.0
3.0	4.1±0.9
4.0	ND

Transition was observed in YP media for 6h at 28ºC. ND, Not detected.

3.1.3.2 Effect of different additives (chemically synthesized compounds) on Y-H transition of *B. poitrasii*

The effect of compounds 1, 2, 3-triazole linked β-lactam-bile acid conjugate (18B, 20B and 24 B) was tested on Y-H transition. Germ tube formation was inhibited

with increase in concentration of these compounds, while it was completely inhibited at their highest concentration used (Table 3.4). DS16 (tetrapeptide-linked cholic acid derivative) exposed cells exhibited germ tube formation inhibition (51.7-94.4%) whereas complete inhibition of germ tube formation was observed with ≥115 µM concentration of DS16 (Table 3.4).

Table. 3.4 Effect of different additives (chemically synthesized compounds) on Y-H transition of *B. poitrasii*

Additives	Germ tube %
Control	53.8±2.3
18B (µM)	
27	45.0±2.5
41	25.0±1.2
51	21.0±0.9
82	12.5±0.5
96	9.0±0.50
110	2.7±0.05
20B (µM)	
26	42.0±3.0
39	31.0±1.4
52	17.0±0.4
79	6.0±0.3
92	5.0±0.2
105	ND
24B (µM)	
26	44.0±3.5
39	25.0±1.8
52	11.0±0.5
79	6.0±0.3
92	ND
DS16 (µM)	
29	26.0±1.4
43	15.0±0.4
58	10.0±0.8
87	3.7±0.5
101	3.0±0.4
115	ND

Transition was observed in YP media for 6h at 28ºC. ND, Not detected.

3.1.3.3 Effect of different additives (microbial metabolites) on Y-H transition of *B. poitrasii*

Among the different bacterial culture filtrates (50 µl) tested, the maximum germ tube formation inhibition was observed for *Bacillus* sp B15 (68.2%), while others

exhibited inhibition in the range 23.8-60.5% (Table 3.5). Phenazine-1-carboxamide (PCN), isolated and purified from *Pseudomonas* sp. B18 was also studied for its effect on Y-H transition (22-224 µM). PCN inhibited germ tube formation by 62.6-82.4% with increasing concentration (22-67 µM) as compared to control, while no transition was observed at ≥ 89 µM concentration of PCN (Table 3.5).

Table. 3.5 Effect of different additives (microbial metabolites) on Y-H transition of *B. poitrasii*

Additives	Germ tube %
Control	53.8±2.3
Bacillus sp (B1)	29.6±2.5
Bacillus sp (B2)	40.9±3.5
Bacillus sp (B14)	30.9±1.0
Bacillus sp (B15)	17.1±2.5
Bacillus sp (B17)	21.2±1.4
Bacillus sp (B21)	33.2±3.0
Bacillus sp (B22)	31.0±2.1
Pseudomonas sp (B18)	34.6±3.1
Streptomyces sp	46.7±4.5
PCN (µM)	
22	20.1±1.9
44	11.5±0.8
67	9.5±0.5
89	ND

Transition was observed in YP media at 28ºC for 6h. The culture filtrates were 10 times concentrated of which 50 µl were added in test samples. ND, not detected

3.1.3.4 Effect of different additives (apoptosis inducers) on *in vitro* NAD-GDH, NADP-GDH and ODC activities of *B. poitrasii*

In vitro NAD-GDH, NADP-GDH and ODC activities were measured as described in materials and methods (section 2.3.4.1 and 2.3.4.2). GDH catalyzes both the NADP-dependent anabolic reaction and the NAD-dependent catabolic step. A high GDH ratio (NADP-/NAD-GDH, >0.15) seems to be associated with the Y form, whereas a lower one (<0.15) is associated with the M form. *In vitro* addition of isophthalic acid and acetic acid inhibited NAD-GDH activity (43.4-73.6 and 22.3-48.2% respectively). Thus increase in concentration of isophthalic acid and acetic acid was associated with high

GDH-ratio ≥0.41 (Table 3.6). *In vitro* addition of H_2O_2 and amphotericin B showed increase in NADP-/NAD-GDH ratio (≥4.1) with increase in concentration (10-50 mM and 1-4 µM respectively) as compared to control (Table 3.6).

Table. 3.6 Effect of different additives (apoptosis inducers) on *in vitro* NAD-GDH, NADP-GDH and ODC activities of *B. poitrasii*

Additives	NAD-GDH (U mg^{-1})	NADP-GDH (U mg^{-1})	NADP-/NAD-GDH (Ratio)	ODC (U mg^{-1})
Control	93.0 ±0.30	39 ±0.20	0.41	0.89±0.11
Isophthalic acid (mM)				
12	52.5±0.60 (43.4)	39.0±0.20	0.74	-
15	39.9±0.19 (57.0)	39.0±0.20	0.97	-
16	24.5±0.63 (73.6)	39.0±0.20	1.59	-
Acetic acid				
5	72.2±1.25 (22.3)	41.0 ±0.10	0.56	0.89±0.11
10	62.3±0.60 (33.0)	41.0 ±0.15	0.65	0.99±0.50
20	48.1±0.46 (48.2)	42.0 ±0.50	0.87	0.91±0.30
H_2O_2 (mM)				
10	91.5±3.50	36.0 ±0.80	0.39	0.89±0.11
20	94.9±3.60	44.9 ±1.00	0.47	1.01±0.51
30	96.3±0.50	51.0 ±0.50	0.53	1.02±0.35
50	96.3±0.60	65.9 ±0.30	0.68	1.15±0.30
Amphotericin B (µM)				
1	91.5±3.00	53.9 ±0.50	0.58	0.86±0.31
2	95.4±3.66	80.4±0.50	0.84	0.90±0.25
4	96.6±3.63	88.6±0.13	0.91	1.01±0.54
DAB (mM)				
8.0	-	-	-	0.21±0.03 (76.0)
10	-	-	-	0.17±0.05 (80.9)
12	-	-	-	0.14±0.04 (83.2)
15	-	-	-	ND (100)
CHA (mM)				
1.0	-	-	-	0.54±0.05 (42.8)
2.0	-	-	-	0.46±0.01 (52.0)
3.0	-	-	-	0.04±0.001(95.5)
4.0	-	-	-	ND (100)

B. poitrasii yeast cells were grown in 1% YPG at 37ºC for 24 h.
The % inhibition of enzyme activities is indicated in the parentheses.
ND, Not detected; -, Not tested.

In vitro addition of DAB and CHA led to increased inhibition of ODC activity with increase in concentration (Table 3.6). Spermidine synthase activity without CHA was 2.52 U mg^{-1} and with CHA (0.5 mM) was 0.458 U mg^{-1}. There was no change in ODC levels in presence of acetic acid, H_2O_2 and amphotericin B.

3.1.3.5 Effect of different additives (chemically synthesized compounds) on *in vitro* ODC activities of *B. poitrasii*

In vitro addition of 18B, 20B and 24B inhibited ODC activity (46.2-97.7%) of *B. poitrasii* yeast cells with increase in concentration for each compound whereas activity was completely inhibited at ≥82, 52 and 79 µM concentrations respectively (Table 3.7). *In vitro* addition of DS16 led to inhibition of ODC activity (57-87.6%) with increase in concentration (29-87 µM), whereas no activity was observed with ≥101 µM concentration of DS16 (Table 3.7).

Table. 3.7 Effect of different additives (chemically synthesized compounds) on *in vitro* ODC activities of *B. poitrasii*

Additives	ODC (U mg^{-1})
Control	0.891 ± 0.11
18B (µM)	
27	0.479±0.040 (46.2)
41	0.351±0.058 (60.6)
51	0.028±0.003 (96.8)
82	ND (100)
20B (µM)	
26	0.050±0.004 (94.3)
39	0.020±0.001 (97.7)
52	ND (100)
79	ND (100)
24B (µM)	
26	0.175±0.010 (80.3)
39	0.101±0.005 (88.7)
52	0.030±0.001 (96.6)
79	ND (100)
DS16 (µM)	
29	0.383±0.019 (57.0)
43	0.351±0.050 (60.6)
58	0.319±0.010 (64.1)
87	0.110±0.010 (87.6)
101	ND (100)

B. poitrasii yeast cells were grown in 1% YPG at 37ºC for 24 h.
The % inhibition of enzyme activities is indicated in the parentheses.
ND, Not detected

3.1.3.6 Effect of different additives (microbial metabolites) on *in vitro* NAD-GDH, NADP-GDH and ODC activities of *B. poitrasii*

The NAD-GDH, NADP- GDH and ODC activities of the crude enzyme extract obtained from yeast-form cells of *B. poitrasii* were determined in presence and absence (control) of the bacterial culture filtrates (50 µl). The NAD-GDH activity from the yeast

form was maximum inhibited (42.90%) by *Bacillus* sp B1, while *Bacillus* sp B21 inhibited NADP-GDH from the yeast form of *B. poitrasii* by 61.54% (Table 3.8).

The culture filtrate of *Bacillus* sp. B1 and B15 showed >80% inhibition, whereas B14 showed no inhibition of ODC activity. *Pseudomonas* sp. B18 showed maximum (93.5%) ODC inhibition (Table 3.8). *In vitro* inhibition of ODC activity was increased with increasing concentrations (22-224 μM) of PCN, while GDH levels were unchanged (Table 3.8).

Table. 3.8 Effect of different additives (microbial metabolites) on *in vitro* NAD-GDH, NADP-GDH and ODC activities of *B. poitrasii*

Additives	NAD-GDH ($U\ mg^{-1}$)	NADP-GDH ($U\ mg^{-1}$)	NADP-/NAD-GDH (Ratio)	ODC ($U\ mg^{-1}$)
Control	93.0±0.30	39.0±0.20	0.41	0.891 ±0.11
Bacillus sp (B1)	52.5±0.54 (42.90)	16.2±0.55 (58.39)	0.31	0.107±0.01 (87.9)
Bacillus sp (B2)	84.0±0.9 (8.54)	15.7±0.42 (60.73)	0.18	0.687±0.05 (22.9)
Bacillus sp (B14)	56.9±0.62 (38.10)	28.8±0.44 (27.28)	0.59	0.970± 0.05 (ND)
Bacillus sp (B15)	78.0±0.8 (14.64)	22.6±0.61 (42.9)	0.29	0.127±0.02 (85.7)
Bacillus sp (B17)	62.6±0.65 (31.94)	29.1±0.41 (25.45)	0.45	0.720±0.50 (19.9)
Bacillus sp (B21)	93.0±1.5 (ND)	15.0±0.44 (61.54)	0.15	0.526±0.03 (40.9)
Pseudomonas sp (B18)	58.7±0.54 (36.14)	39.3±2.1 (ND)	0.66	0.058±0.005 (93.5)
Streptomyces sp	58.7±0.54 (36.14.90)	39.2±2.1 (ND)	0.66	0.760±0.060 (14.7)
PCN (μM)				
22	90.5±3.50	37.0±0.80	0.40	0.323±0.03 (63.7)
44	92.9±3.60	40.5±1.00	0.44	0.244±0.02 (72.6)
67	94.3±0.50	41.0±0.50	0.43	0.162±0.01 (81.8)
89	93.0±0.60	45.9±0.30	0.50	0.078±0.007 (91.2)
224	93.0±0.60	35.1±0.30	0.37	0.008±0.00 (99.0)

B. poitrasii yeast cells were grown in 1% YPG at 37ºC for 24 h.
The % inhibition of enzyme activities is indicated in the parentheses. ND, Not detected

3.2 DISCUSSION

Arrest in the dimorphic transition in the pathogenic fungi was suggested to be a useful strategy for the development of antifungal drugs (Georgopapadakou and Walsh, 1994). The yeast-hypha and reverse morphological transition was targeted for screening

of antifungal agents. However, it is not always possible to use pathogens for the initial screening. Several pathogenic fungi require 24-48 h for the complete yeast-hypha transition (Maresca and Kobayashi, 1989). The transition of yeast cells into hypha in *P. brasiliensis* required more than 14 days (Deshpande, 1996). *W. dermatitidis*, a causative agent of phaeohyphomycosis in humans, in addition to the long term incubation, displayed polymorphic forms during transition from thin walled and thick walled yeast, multicellular form, moniliform hyphae and true hyphae (Kester and Garrett, 1995). Therefore, the use of *W. dermatitidis* as a model was not suitable. Saprophytic non-pathogenic fungi were also used for antifungal screening.

Frost *et al.* (1998) used whole cells of *C. albicans* to identify inhibitors towards cell wall synthesis and assembly. Inhibitors of ornithine reduced the growth of the fungus *Botrytis cinerea* (Smith *et al*., 1990). Zhu and Gooday (1992) studied the effect of nikkomycin and echinocandin on differentiated and undifferentiated hypha of *Coprinus cinereus* and on spore germination and hyphal growth of *M. rouxii*. Wenke *et al*. (1993) observed that chitin synthase inhibitor pseurotin A and 8-O-dimethyl pseurotin A isolated from submerged cultures of *A. fumigatus* inhibited the membrane bound and solubilised forms of chitin synthase. Alternately the saprophytic dimorphic fungus *B. poitrasii* was developed as a useful model for screening of antifungal agents (Salunke *et al*., 2004). The biochemical correlates of the morphological transition such as GDH, ODC, chitin synthase and chitinase were identified as effective targets.

In the present study, *B. poitrasii* was used as a model to study the effect of low fungicidal doses of different additives on Y-H transition and biochemical correlates of morphogenesis *viz.* NAD-GDH, NADP-GDH and ODC. Additives used in the study were commercially available compounds (DAB, CHA, isophthalic acid and amphotericin B), phenazine-1-carboxamide (PCN), chemically synthesised compounds (18B, 20B, 24 B and DS16), acetic acid and H_2O_2.

The NAD-GDH and ODC enzyme levels were low in the *B. poitrasii* yeast form as compared to the hyphal form cells whereas the NADP-GDH enzyme levels were higher in yeast form cells (Khale and Deshpande, 1993; Ghormade *et al*., 2005a) (Table 3.1 and 3.2). NAD and NADP-GDHs played an important role in nitrogen metabolism as they catalyze the reductive amination of α-ketoglutarate to yield glutamate, as well as the oxidative deamination of glutamate. The quantitative relationship between these two enzymes was expressed as the GDH ratio (NADP-/NAD-GDH). Khale *et al*. (1992) suggested that correlation between the GDH ratio and dimorphic behaviour in *B.*

poitrasii may exist. Increase in the GDH ratio led to a change in morphology from the H to the Y form. GDH ratio (≥0.15) was associated with Y form. Transition of the parent strain from the H to the Y form was preceded by an increase in the GDH ratio. Reverse trends were observed for Y-H transition, for which a lowering of the ratio occurred.

Among different additives evaluated in *B. poitrasii*, isophthalic acid (16 mM) and acetic acid (20 mM) led to decrease in NAD-GDH activity while NADP-GDH activity remained unaffected (GDH ratio ≥0.41) (Table 3.6). At 16 mM of isophthalic acid no germ tube formation was seen during Y-H transition within 6 h, while 56% budding cells were obtained during H-Y transition after 12 h. In *B. poitrasii* acidic pH favours the Y form associated with increased NADP-GDH activity. In case of amphotericin B and H_2O_2 increase in NADP-GDH activity was observed, whereas NAD-GDH was unaltered at their respective concentration that halted Y-H transition (Table 3.6). In the present study GDH was biochemically correlated to Y-H transition that may provide a possible checkpoint for apoptosis. Chemically synthesized additives 18-24B inhibited Y-H transition from 16-95%, while DS16 inhibited germ tube formation from 51.6-95% at their respective concentration. In case of microbial extracts, *Bacillus* culture inhibited Y-H transition by 24-68% while *Pseudomonas* sp. inhibited the same by 36%. The maximum germ tube inhibition was observed for *Bacillus* sp B15 (68%). Yeast form NAD-GDH and NADP-GDH were inhibited by *Bacillus* sp. B1 (42.9%) and B21 (61.54%) respectively. Phenazine-1-carboxamide (PCN, 22-224 µM), isolated and purified from *Pseudomonas* sp. B18 was found to inhibit Y-H transition. PCN (22-67 µM) led to inhibition of germ tube formation by 62.6-82.4% with increasing concentration as compared to control.

During Y-H transition in *B. poitrasii* hyphal form showed >1.5 fold increase in putrescine and spermidine levels and >4 fold increase in spermine levels than yeast form cells (Table 3.1). Similarly increase in polyamine levels during Y-H transition was also reported in case of *C. ulmi*, *Fusarium moniliforme*, *M rouxii*, *Y. lipolytica* whereas reverse trend was reported in *A. parasiticus*, *H. capsulatum*, *M. bacilliformis* and *Mycotypha microspora* (Marshall *et al*., 1979; Calvo-Mendez *et al*., 1987; Guevara-Olvera *et al*., 1993). In *B. poitrasii* yeast cells, polyamine levels decreased on treatment with different additives (DAB, CHA and PCN) (data not shown).

In *M. bacilliformis*, *M. circinelloides*, *M. racemosus*, *P. brasiliensis*, *U. maydis* and *Y. lipolytica*, ODC (a polyamine synthesizing enzyme) was reported to play a significant role in the differentiation (Marshall *et al*., 1979; Garcia *et al.*, 1980; Ruiz-

Herrera *et al.*, 1983; Guevara-Olvera *et al*., 1997; Jimenez-Bremont *et al*., 2001; Blasco *et al*., 2002; Nino-Vega *et al*., 2004; Reyna-Lopez and Ruiz-Herrera, 2004). In *B. poitrasii*, ODC activities were inhibited with increase in concentration of additives. Maximum inhibition (≥85%) was observed in presence of DAB (12 mM), CHA (3 mM), 18B (51 µM), 20B (26 µM), 24B (39 µM), DS16 (87 µM) and PCN (89 µM). As ODC was biochemically correlated to differentiation it may be suggested that this enzyme could be useful as early checkpoint for apoptosis. In the dimorphic fungi *C. albicans* and *U. maydis* the disruption of ODC gene prevented Y-H transition that demonstrated the requirement of polyamines for differentiation (Guevara-Olvera *et al*., 1997; Lopez *et al*., 1997). Different fungi and their morphological forms exhibit different levels of polyamines, which are regulated by polyamine synthesizing enzymes (Calvo-Mendez *et al*., 1987). Inhibition of differentiation, ODC activity and polyamine synthesis was also reported in *C. albicans*, *M. rouxii*, *P. brasiliensis*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* using polyamine synthesis inhibitors like DAB, DFMO and CHA (Rajam and Galston, 1985; Martinez-Pacheco *et al*., 1989; Martinez *et al*., 1990; San-Blas *et al*., 1996b; Pieckenstain *et al*., 2001). The role of polyamine biosynthetic pathway in apoptosis was also well studied using different inhibitors such as CHA, DAB, methylglyoxal-bis-guanylhydrazone (MGBG), difluoromethylornithine (DFMO) (Foster and Walters, 1990; Marton and Pegg, 1995; Kelloff, 2000; Esmat *et al*., 2002; Schipper *et al*., 2000, 2003).

Differentiation was the intermediate step between growth and apoptosis (Denmeade and Isaacs, 1996). The present investigations suggested the ability of the additives to affect morphological transition and their biochemical enzyme correlates *viz*. GDH and ODC. In *B. poitrasii* the additives (DAB, CHA, PCN, 18B, 20B, 24B and DS16) were tested at concentrations that halted Y-H transition (≥50% inhibition) and showed inhibition (≥50%) of ODC (Table 3.3-3.5 and 3.6-3.8 respectively). We suggested that dimorphic transition in fungi can be used as target to identify novel apoptotic inducers. The compounds that halted the Y-H transition and their biochemical correlates were further evaluated as potential apoptosis inducers at their respective apoptotic concentrations using *B. poitrasii* yeast cells.

CHAPTER IV

Biochemical and molecular studies of programmed cell death in *B. poitrasii* and validation studies using yeasts species involved in wine fermentation for PCD

4.1 Effect of different additives on apoptosis and its biochemical correlates in *B. poitrasii*

4.1.1 INTRODUCTION

Programmed cell death (PCD) or apoptosis is a regulated intracellular process that functions during normal cellular development, in response to stress and defence against pathogen attack in plants and animals (Biella *et al*., 2002). Apoptotic cell death a process that was well defined in mammalian cells and was not well studied in yeast and fungi. Apoptosis was considered to be a promising therapeutic target, until recently and it has gained increasing attention as a potential target in fungi (Roze and Linz, 1998; Mousavi and Robson, 2003, 2004; Phillips *et al*., 2003, 2006; Fedorova *et al*., 2005; Semighini *et al*., 2006a). Ultrastructural and biochemical changes that are characteristic of apoptosis were reported in pathogenic fungi, raising the possibility that manipulation of fungal death pathways could have merit as future antifungal therapy. Exposure of *A. nidulans*, *C. albicans* and *M. racemosus* to acetic acid, amphotericin B, farnesol, hydrogen peroxide and lovastatin evoked apoptotic like changes (Roze and Linz, 1998; Phillips *et al*., 2003; Semighini *et al*., 2006a). These changes were suppressed by mutations that block Ras-cAMP-protein kinase A signalling and were accelerated by stimulation of Ras signalling implying a regulatory role for this pathway in the control of fungal apoptosis (Phillips *et al*., 2006).

Like mammalian cells, yeasts cells undergoing apoptosis showed characteristic apoptotic markers: externalization of phosphatidylserine to the outer leaflet of the plasma membrane, DNA cleavage and chromatin condensation (margination) and cytochrome *c* release from mitochondria (Madeo *et al*., 1999). These processes were induced by H_2O_2, acetic acid, aspirin, osmotin, pheromones and defects in DNA replication, glycosylation and cell ageing (Madeo *et al*., 1997, 1999; Laun *et al*., 2001; Ludovico *et al.*, 2001, 2002; Balzan *et al*., 2004; Weinberger *et al*., 2005; Hauptmann *et al*., 2006). Some drugs with known antifungal capacities were identified as yeast-specific apoptosis-inducers such as pradimicin, ciclopiroxolamine, amphotericin B etc. (Hiramoto *et al*., 2003; Kokjohn *et al*., 2003; Morton *et al*., 2007; Almeida *et al*., 2008). The aspartate-specific cysteine proteases that comprise the caspase superfamily are central to apoptosis. Their proteolytic activity led to the ordered disassembly of intracellular components and ultimately, cell death (Uren *et al*., 2000). The caspase superfamily contains three families: the caspases of metazoans, the paracaspases of metazoans and *Dictyostelium*, and the metacaspases of plants, fungi and protozoa (Uren *et al*., 2000). The genome of

Saccharomyces cerevisiae encodes a single metacaspase, Yca1p, which was cleaved in response to oxidative stress (Madeo *et al*., 2002). The yeast apoptotic response is often dependent upon metacaspase indicating an important, but not exclusive, role for metacaspase-dependent programmed cell death in response to the toxic stimuli (Madeo *et al*., 2002; Bettiga *et al*., 2004; Herker *et al*., 2004; Khan *et al*., 2005; Mazzoni *et al*., 2005; Reiter *et al*., 2005; Weinberger *et al*., 2005).

Dimorphic fungi are useful model systems to understand the eukaryotic developmental processes as they differentiate from unicellular yeast to filamentous form for survival and proliferation (Gow, 1995). In case of *C. albicans*, *C. immitis*, *H. capsulatum*, *P. brasiliensis*, *U. maydis* and *Uromyces* sp. and other human and plant pathogens dimorphism was important in pathogenesis. Pathogenic fungi cause plant and human diseases, food spoilage and severe economic damage. An effective way to control dimorphic fungal pathogen is to prevent morphological transition i.e. saprophytic to an invasive form. Y and H forms of dimorphic fungi are often correlated with survival or proliferation. To identify the potential target the study of vital cellular functions in the pathogens is important. In this regard, morphological change can be used as a selective event to visualize their potential to identify new molecular targets for antifungal therapy.

It was suggested that transformation of mammalian cells (normal to cancerous) was the intermediate step between growth and cell death (Denmeade and Isaacs, 1996). The phenomenon of dimorphic transition in fungi may be useful for the study of apoptosis and screening apoptosis inducing agents. It was already reported that different enzyme activities involved in ammonia assimilation and chitin synthesis such as NAD- and NADP-GDH, chitin synthase, chitinase, *N*-acetylglucosaminidase and polyamine synthesizing enzyme ODC showed biochemical correlation with Y-H transition in *B. poitrasii* (Khale *et al*., 1992; Ghormade *et al*., 2000; Chitnis *et al*., 2002; Ghormade *et al*., 2005a). In *M. bacilliformis*, *M. circinelloides*, *M. racemosus*, *P. brasiliensis*, *U. maydis* and *Y. lipolytica*, ODC, was reported to play a significant role in differentiation (Garcia *et al.*, 1980; Ruiz-Herrera *et al.*, 1983; Guevara-Olvera *et al*., 1997; Jimenez-Bremont *et al*., 2001; Blasco *et al*., 2002; Nino-Vega *et al*., 2004; Reyna-Lopez and Ruiz-Herrera, 2004). Recently polyamines were implicated in cell death as polyamine levels alterations, especially in spermidine and spermine, were lethal resulting in cell cycle arrest or apoptosis (Schipper *et al*., 2000; Nitta *et al*., 2002; Pignattin *et al*., 2004). Increasing evidence indicated the close connection of polyamines, cell cycle events and apoptosis (Nitta *et al*., 2002; Pignattin *et al*., 2004). In the chapter III the phenomenon of

dimorphic transition in *B. poitrasii* (and its biochemical correlate) was used to identify potential apoptosis inducers. In this chapter these additives are further evaluated for their apoptotic potential using the yeast form of *B. poitrasii* for characterization of apoptotic markers.

In the present study, apoptosis and its biochemical correlates were studied in *B. poitrasii* using regulators of Y-H transition and biochemical enzyme correlates of morphogenesis like NAD-/NADP-GDH and ODC. The additives assessed were, commercially available compounds- diaminobutanone (DAB, ODC inhibitor), cyclohexylamine (CHA, spermidine synthase inhibitor), isophthalic acid (NAD-GDH inhibitor), amphotericin B (ergosterol synthesis inhibitor), fluconazole (14α-demethylase inhibitor); compound isolated from natural source (phenazine-1-carboxamide); chemically synthesized compounds- triazole linked β-lactam–bile acid conjugates (18, 20 and 24 B), tetrapeptide linked-cholic acid derivative (DS16) and a strobilurin derivative (PC229); acetic acid and H_2O_2. The possible correlation of differentiation and apoptosis in *B. poitrasii* is discussed.

4.1.2 RESULTS

4.1.2.1 Effect of chronological aged yeast and hyphal cells of *B. poitrasii* on ODC activity

Natural ageing in *B. poitrasii* was studied in yeast and hyphal cells. In dimorphic fungi the probable pathway for apoptosis could follow the sequence as- growth, differentiation, apoptosis and finally necrosis. Therefore it was interesting to follow the ODC activities in vegetative Y or H cells aged up to 240 h. Inoculum yeast cells were grown under conditions favouring yeast growth (1% YPG, 37°C). For hyphal formation, inoculum yeasts cells were grown under hypha favouring conditions (0.1% YP at 28°C). For the experiment, $16x10^4$ yeast cells ml^{-1} were inoculated in 1% YPG medium at 37°C, 0.5% YPG and YP medium at 28°C for 6-240 h and allowed to undergo ageing.

ODC activities were highest at 24 h for both Y and H forms and declined till 144 h. ODC activities in 24 h H cells were 3.6 fold higher than 24 h Y cells (Table 4.1). Hyphal cells showed ODC activity upto 192 h. As the hyphal form may be associated with autolysis further analysis for apoptosis was carried out with *B. poitrasii* yeast cells.

Table 4.1 Effect of chronological aged *B. poitrasii* cells on ODC activities under different dimorphic conditions

Time (h)	ODC (U mg^{-1})		
	I	**II**	**III**
6	0.71±0.02	1.05±0.03	1.74±0.081
12	0.90±0.02	1.67±0.08	2.14±0.11
24	1.10±0.001	1.68±0.12	3.98±0.12
48	0.83±0.01	1.09±0.02	3.03±0.09
72	0.56±0.03	0.91±0.05	2.10±0.051
96	0.53±0.01	0.08±0.03	0.88±0.03
144	0.08±0.002	ND	0.55±0.02
192	ND	ND	0.10±0.003
240	ND	ND	ND

B. poitrasii cells grown in; I, 1% YPG at 37ºC; II, 0.5% YPG and III, YP at 28ºC; ND, not detected.

For measuring metacaspase activity, caspase (-1,-3 and -8) like activities were determined because of metacaspase structural similarity to caspases and members of both families contained the caspase-hemoglobinase fold (Bozhkov *et al*., 2010; Carmona-Gutierrez *et al*., 2010b).

4.1.2.2 Chronological aged *B. poitrasii* yeast cells exhibit SOD and caspase like activity

Ageing leads to oxidative damage that accumulates ROS causing cell death. ROS generation was detected by measuring SOD activity. ROS was detected in aged cells (stationary) but not in exponentially grown cells in 1% YPG at 37^{o}C for 24-240 h. SOD activity was found to increase as the cells grow older (Table 4.2). During H-Y (1% YPG, 37^{o}C) transition maximum activity was detected at 144 h.

Elevated intracellular caspase activity was considered to be one of the hallmarks of the apoptotic process. The relative amount of caspase-1, -3, -8 activities in each cell extract was measured using the respective fluorogenic caspase substrates. As shown in Table 4.2, activity was detected within 48 h of growth and maximum activities were observed in 144 h. Correlation coefficient value (r) of SOD with caspases was >0.766 (p= <0.004) at 144 h.

Table. 4.2 SOD and caspase like activities in protein lysates of chronologically aged yeast cells of *B. poitrasii*

Time (h)	SOD (U mg^{-1})	Caspases (Relative fluorescence unit)		
		I	**III**	**VIII**
Control	2.9±0.50	6.69±0.55	10.0±0.80	10.2±0.80
24	3.7±0.04	29.2±1.50	6.05±0.80	5.3±0.50
48	5.7±0.18	32.1±2.00	13.3±1.50	19.2±1.50
72	8.4±0.90	52.1±4.20	26.8±2.10	25.0±2.25
96	22.0±1.50	99.9±8.10	192.9±12.25	101.5±9.00
144	50.0±6.50	405.0±20.10	305.3±20.10	403.7±25.50
192	1.4±0.005	300.0±15.50	204.2±5.25	207.7±10.10
240	ND	4.15±0.90	3.5±0.90	8.6±0.82

B. poitrasii yeast cells were grown in 1% YPG at 37ºC. ND, Not detected.

4.1.2.3 Superoxide dismutase (SOD) and caspase like activities of sporangiospores and zygospores of *B. poitrasii*

Sporangiospores and zygospores are the asexual and sexual propagules of *B. poitrasii*. Natural ageing of these spores were studied as a part of the fungal life-cycle. Formation of sporangiospores and zygospores was carried out as mentioned in materials and methods (section 2.2.3).

Table. 4.3 Time course study of SOD and caspase activities of *B. poitrasii* during sporulation (sporangiospores and zygospores)

Time (d)	SOD (U mg^{-1})	Caspases (Relative fluorescence unit)		
		I	**III**	**VIII**
Sporangiospores				
3	2.7±0.40	75.6±3.50	90.6±5.50	84.2±4.50
4	3.6±0.82	131.8±5.50	152.7±10.50	136.5±12.25
5	4.8±0.35	205.0±15.25	191.4±5.00	150.5±12.50
6	6.6±0.80	434.9±20.50	368.6±12.50	228.3±10.55
7	14.9±0.5	608.0±25.00	491.7±20.10	551.9±25.50
15	9.3±1.50	209.0±10.40	140.7±15.50	142.9±9.00
Zygospores				
3	ND	14.2±0.90	2.0±0.05	40.3±2.50
4	0.83±0.004	30.9±5.50	23.8±1.50	60.9±1.00
5	2.6±0.50	66.4±6.50	66.4±4.50	112.8±10.50
6	3.0±0.05	109.0±3.50	99.3±6.50	151.9±12.45
7	5.3±0.55	236.1±10.65	189.3±10.40	263.8±15.50
15	7.5±1.00	434.9±15.50	292.3±15.00	353.0±25.50
30	16.4±0.80	696.8±25.50	652.7±25.00	673.7±31.45

Sporangiospores and zygospores of *B. poitrasii* were obtained in 1% YPG at 28ºC.

SOD activity was determined in *B. poitrasii* sporangiospores and zygospores samples harvested from 3-30 d. Maximum activity was observed in sporangiospores in 7 d (14.9±0.5), whereas in zygospores in 30 d (16.4±0.8) (Table 4.3). Caspase-like (1,-3,-8) activities were maximum on 7 d for sporangiospores and 30 d for zygospores (Table 4.3). For 7 d old sporangiospores correlation coefficient value (*r*) of SOD with caspases was >0.977 (*p*= 0.000). While for 30 d old zygospores correlation coefficient value (*r*) of SOD with caspases was >0.737 (*p* = 0.006).

4.1.2.4 Effect of different additives on biochemical correlates of apoptosis in *B. poitrasii*

4.1.2.4a Effect of different additives (apoptosis inducers) on biochemical correlates of apoptosis in *B. poitrasii*

In vitro ornithine decarboxylase (ODC) and *in vivo* superoxide dismutase (SOD) and caspase activities were measured in the presence or absence of additives like isophthalic acid (12-18 mM), acetic acid (5-40 mM), H_2O_2 (10-50 mM), amphotericin B (1-6 µM), diaminobutanone (DAB,12-18 mM) and cyclohexylamine (CHA, 3-7 mM).

In vitro addition of DAB (12 mM) and CHA (4 mM) showed maximum inhibition of ODC activity (Table 4.4). Spermidine synthase activity without CHA was 2.52 U mg^{-1} and with 0.5 mM of CHA activity was found to be 0.458 U mg^{-1}. There was no change in ODC levels in presence of acetic acid, H_2O_2, amphotericin B. While *in vitro* NAD-GDH activity inhibited with addition of isophthalic acid and acetic acid was associated with high GDH-ratio ≥0.41 (Table 3.6). *In vitro* addition of H_2O_2 and amphotericin B showed increase in NADP-/NAD-GDH ratio ≥4.1 with increase in concentration (10-50 mM and 1-4 µM respectively) as described in chapter III (Table. 3.7)

SOD activity increased 20 fold at 16 mM **(**isophthalic acid**)** concentration in comparison to control (Table 4.4). In presence of acetic acid (10-40 mM) 39.85 fold increase in activity was observed in comparison to control (Table 4.4). SOD activity of cells treated with H_2O_2 (50 mM) and amphotericin B (4 µM), increased to 20 and 17 fold respectively in comparison to control (1.4±0.005 U mg^{-1}) (Table 4.4).

SOD activity increased with increasing concentration of DAB and CHA by 5.07 and 17.85 fold respectively as compared to control (1.4±0.005 U mg^{-1}) (Table 4.4).

Table. 4.4 Effect of different additives (apoptosis inducers) on biochemical correlates of apoptosis in *B. poitrasii*

Additives	ODC (U mg^{-1})	SOD (U mg^{-1})	Caspase (Relative fluorescence unit)		
			I	**III**	**IV**
Control	0.89±0.11	1.4±0.005	6.69±0.55	10.0±0.80	10.2±0.80
Isophthalic acid (mM)					
12	-	2.1±0.50	111.3±5.50	103.1±1.55	113.4±5.50
15	-	21.8±1.50	206.1±15.00	210.1±5.45	185.7±5.50
16	-	26.0±2.00	462.5±25.00	304.3±4.50	360.5±10.75
18	-	20.0±2.50	142.2±10.54	55.9±2.45	62.0±1.55
Acetic acid (mM)					
5	0.89±0.11	10.5±0.50	232.9±5.50	179.0±10.50	200.8±10.55
10	0.99±0.50	17.6±.80	257.3±1.55	240.9±4.35	237.9±15.60
20	0.91±0.30	23.5±2.00	344.9±20.00	282.6±7.40	312.2±5.51
40	0.91±0.30	21.0±1.50	239.7±15.50	254.0±10.50	184.6±5.70
H_2O_2 (mM)					
10	0.89 ±0.11	16.2±1.00	139.9±5.60	147.2±1.35	78.9±1.50
20	1.01±0.51	19.6±1.85	257.9±10.55	272.0±4.40	219.8±15.00
30	1.02±0.35	25.0±2.50	275.2±10.45	302.0±12.55	238.0±10.10
50	1.15±0.30	20.0±1.00	455.5±15.60	399.5±15.45	276.1±10.55
Amphotericin B (µM)					
1	0.86±0.31	2.5±0.05	249.7±10.50	288.7±20.50	238.8±10.65
2	0.90±0.25	8.4±0.85	270.6±10.75	360.0±15.35	267.9±15.50
4	1.01±0.54	17.0±1.45	154.1±5.50	306.9±5.50	230.7±5.55
6	0.91±0.11	7.9±0.50	85.2±2.50	170.8±2.61	150.4±2.50
DAB (mM)					
12	0.14±0.04 (83.2)	1.6±0.06	89.5±1.50	92.1±3.50	47.7±0.50
15	ND (100)	7.1±1.00	311.3±4.10	219.4±10.45	302.5±25.50
16	ND (100)	2.79±0.08	172.1±5.15	163.6±6.50	166.6±3.45
18	ND (100)	1.2±0.05	8.9±0.80	37.9±1.55	58.3±1.50
CHA (mM)					
3	0.46 ±0.01 (52.0)	11.1±0.90	56.0±2.10	99.8±5.25	116.6±8.50
4	0.04 ±0.001 (95.5)	24.8±1.50	315.0±4.40	195.7±2.50	285.9±12.45
5	ND (100)	6.2±0.50	168.6±5.69	173.9±5.50	159.3±10.00
7	ND (100)	2.0±0.35	42.9±2.50	37.7±1.55	19.6±1.50

B. poitrasii yeast cells were grown in 1% YPG at 37ºC.
The % inhibition of enzyme activities is indicated in the parentheses.
ND, Not detected; -, Not tested.

In case of isophthalic acid (16 mM) caspase-1, -3, -8 activities were found to increase 69.13, 30.30 and 35.34 fold in comparison to their respective controls (Table 4.4). At 20 mM concentration of acetic acid 51.55, 28.14 and 31.20 fold increase in

caspase-1, -3, -8 activities were detected in comparison to their respective controls (Table 4.4). In case of H_2O_2 at 50 mM concentration caspase-1, -3 and -8 activities were increased to 40.44, 39.95 and 27.60 fold in comparison to their respective controls (Table 4.4).

Amphotericin B led to increase in caspase-1, -3 and -8 activities in *B. poitrasii*. Maximum activities were observed at 2 µM concentration (60.8, 35.80 and 26.25 fold respectively) (Table 4.4).

DAB and CHA treated *B. poitrasii* cells led to increased caspase-1, -3,- 8 activities in comparison to respective controls. Maximum activities were observed at 15 mM (46.5, 21.05 and 29.65 fold) and 4 mM (46.53, 21.85, 29.6 fold) concentrations (Table 4.4).

4.1.2.4b Effect of different additives (chemically synthesized and naturally isolated compounds) on biochemical correlates of apoptosis of *B. poitrasii* yeast cells

In vitro ODC and *in vivo* SOD and caspase activities were measured in the presence or absence of additives like 18B (82-110 µM), 20B (79-105 µM), 24B (79-105 µM), DS16 (87-115 µM) and phenazine-1-carboxamide (PCN, 22-224 µM).

ODC activity of *B. poitrasii* yeast cell extract treated with 18B, 20B and 24B was completely inhibited at ≥82, 52 and 79 µM respectively (Table 4.5). *In vitro* addition of DS16 led to inhibition of ODC activity at 87 µM, whereas no activity was observed at ≥101 µM (Table 4.5). *In vitro* inhibition of ODC activity, with and without addition of PCN, increased with increasing concentration (22- 224 µM) (Table 4.5). There was no change in GDH levels in presence of PCN (Table 3.8).

In vivo SOD activity was increased to 26.8, 16.0, 18.5, 22.8 and 15.9 fold in the cells treated with 18B (110 µM), 20B (105 µM), 24B (92 µM), DS16 (115 µM) and PCN (89 µM) respectively in comparison to control (1.4±0.005 U mg^{-1}) (Table 4.5).

Caspase-1, -3 and -8 activities of 18B were increased to 53.65, 27.50 and 29.07 fold till 110 µM in comparison to their respective controls (6.69±0.05, 10.04±0.8, 10.2±0.8) (Table 4.5). At 105 µM concentration of 20B caspase-1, -3 and -8 activities were increased to 47.30, 29.19 and 31.45 fold respectively. In case of 24B at 92 µM caspase-1, -3 and -8 activities were increased (48.64, 20.95 and 27.52 fold respectively) in comparison to their controls. While DS16 (115 µM) led to increase in caspase-1, 3 and 8 activities 68.20, 34.56 and 30.37 fold (Table 4.5). Caspase-1, -3 and -8 activities were

also found to increased to 58.95, 18.55, and 17.7 fold at 89 µM (PCN) in comparison to respective controls (6.69±0.05, 10.04±0.8, 10.2±0.8).

Table. 4.5 Effect of different additives (chemically synthesized and naturally isolated compounds) on biochemical correlates of apoptosis in *B. poitrasii*

Additives (µM)	ODC (U mg^{-1})	SOD (U mg^{-1})	Caspase (Relative fluorescence unit)		
			I	**III**	**IV**
Control	0.891±0.11	1.4±0.005	6.69±0.55	10.0±0.80	10.2±0.80
18B					
82	ND (100)	8.4±0.50	189.8±5.45	235.6±10.55	217.1±10.50
96	ND (100)	33.3±1.35	262.7±10.50	260.0±15.45	251.6±5.00
110	ND (100)	37.6±1.50	358.6±5.00	286.2±5.00	296.6±3.50
20B					
79	ND (100)	4.2±0.80	254.8±2.50	213.5±2.50	182.6±2.50
92	ND (100)	10.8±0.50	273.5±6.55	255.8±7.55	229.0±8.50
105	ND (100)	22.6±2.50	317.0±12.35	303.6±5.55	320.8±10.00
24B					
79	ND (100)	18.8±2.35	89.3±1.45	145.1±3.45	189.5±5.00
92	ND (100)	26.0±1.20	325.4±10.00	210.4±4.50	280.8±10.10
105	ND (100)	21.1±0.55	111.6±2.50	78.0±4.00	135.6±5.50
DS16					
87	0.11± 0.010 (87.6)	14.6±1.35	167.4±5.00	180.3±1.00	161.5±4.50
101	ND (100)	14.7±1.00	192.5±5.45	263.6±11.50	242.0±2.00
115	ND (100)	32.0±2.00	456.3±15.00	347.0±5.50	309.7±5.61
PCN					
22	0.323±0.03 (63.7)	2.1±0.61	28.5±1.50	27.4±0.80	23.6±1.00
44	0.244±0.02 (72.6)	3.2±0.75	69.7±4.50	72.9±2.50	64.4±1.50
67	0.162±0.01 (81.8)	8.3±0.90	128.7±2.55	127.0±5.61	121.4±0.51
89	0.078±0.007(91.2)	21.8±1.50	394.4±5.50	186.3±2.50	180.6±2.50
224	0.008±0.000 (99)	20.0±0.89	31.0±1.21	28.4±1.00	15.7±0.50

B. poitrasii yeast cells were grown in 1% YPG at 37ºC.
The % inhibition of enzyme activities is indicated in the parentheses.
ND, Not detected.

4.1.2.5 Chronologically aged *B. poitrasii* yeast cells die exhibiting markers of apoptosis

The chronologically aged *B. poitrasii* yeast cells grown in 1% YPG at 37°C collected at different time point from 6-240 h and were analysed for markers of apoptosis. Initial 6, 12 and 24 h cells did not exhibit any apoptotic phenotype. A relatively large proportion of the cells grown for 144 h in 1% YPG medium showed bright fluorescence around the cellular circumference upon staining with annexin V binds specifically to phosphatidylserine (PS) but did not take up propidium iodide, indicating an intact membrane (Fig. 4.1 F). PI has often been used as a probe for non-

viable cells. Dead or dying cells with injured plasma membranes can incorporate PI (PI (+) cells), which stains double-stranded nucleic acids (Haugland, 1996).

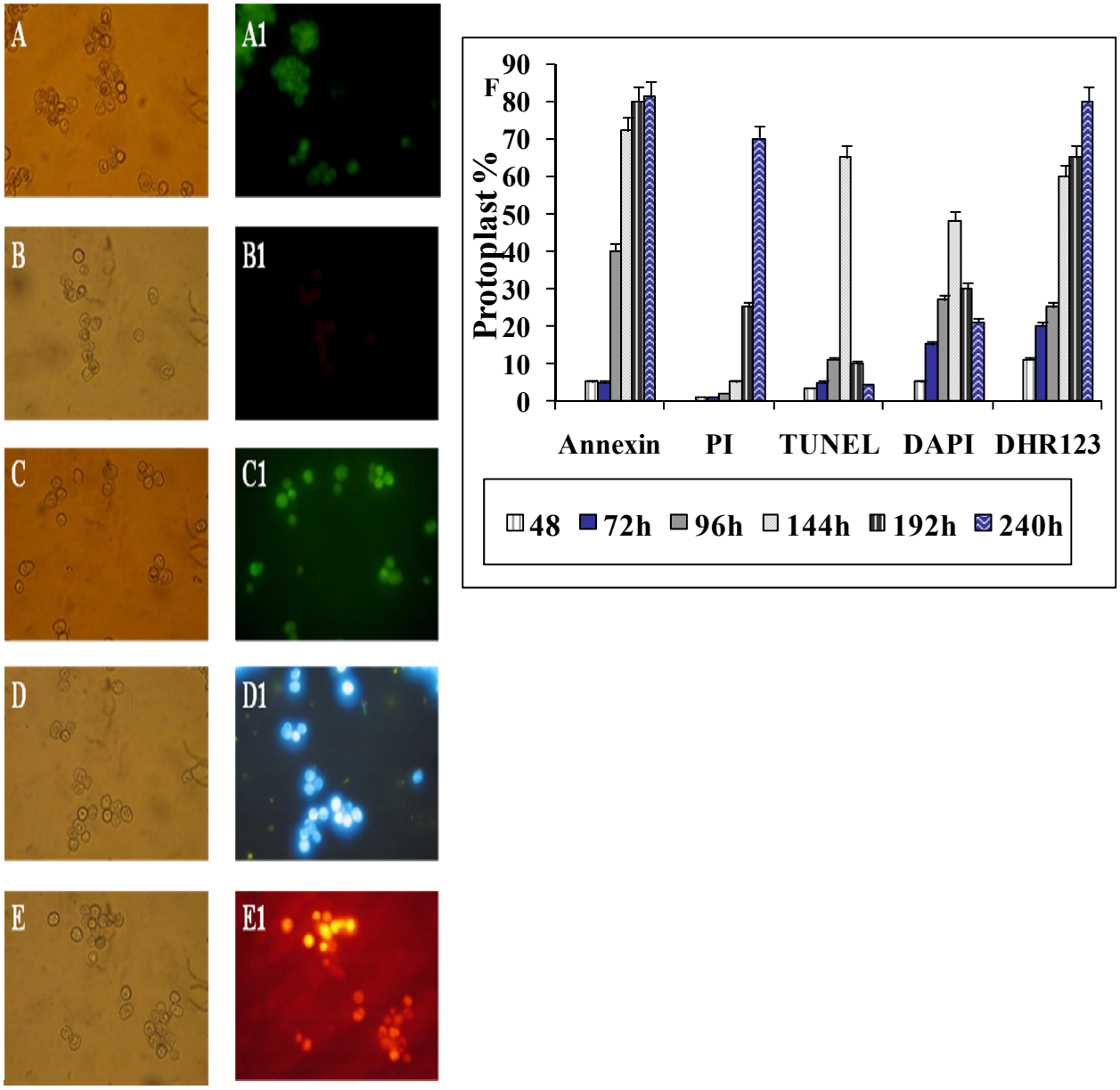

Fig. 4.1 Chronologically aged *B. poitrasii* yeast cells die exhibiting markers of apoptosis. (A1-B1) Annexin V-PI staining. (C1) TUNEL reaction. (D1) DAPI staining. (E1) DHR 123 staining of *B. poitrasii* grown for 144h. (A-E) Bright field image of respective test. (F) Percentage of protoplasts displaying Annexin +, loss of membrane integrity (PI+), DNA strand breaks (TUNEL+), chromatin condensation (DAPI+), DHR123 in *B. poitrasii* grown for 144h.

At 144 h 65% of protoplasts were TUNEL positive, which exhibited an intense green nuclear staining indicating massive DNA fragmentation (Fig. 4.1 F). 48% protoplasts showed DAPI staining, indicating chromatin condensation in 144 h cells. 90% cells were DHR positive at 240 h indicating cells with increased mitochondrial ROS production. Correlation coefficient value (r) of TUNEL with SOD and caspases was >0.800 (p = <0.002).

4.1.2.6 Apoptosis in *B. poitrasii* under different dimorphic condition

B. poitrasii yeast cells were grown under different dimorphic conditions (0.5 and 0.1% YPG and YP at 28°C) from 24-192 h and analysed for apoptotic phenotype. Glucose administration results in a diminished efficacy of cells to enter differentiation, finally causing superoxide-mediated replication stress and apoptosis. Results showed that number of cells undergoing apoptosis increased with increase in glucose concentration (Fig. 4.2).

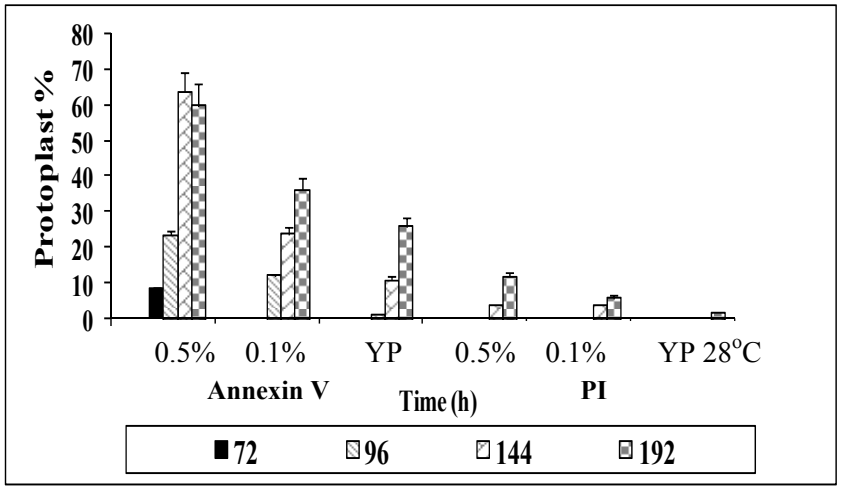

Fig. 4.2 Apoptosis in *B. poitrasii* under different dimorphic condition.
B. poitrasii, annexin V-PI staining (0.5 and 0.1% YPG and YP at 28°C for 48-192 h).

4.1.2.7 Effect of different additives on apoptosis of *B. poitrasii*

All the apoptosis experiments for the detection of its classical markers were performed using *B. poitrasii* yeast cells grown in 1% YPG at 37ºC for 200 min in presence or absence of additives as mentioned in section 2.2.3 and 2.3.1. Cells harvested were analysed for apoptotic markers like exposure of phosphatidylserine by annexin V FITC, DNA fragmentation by TUNEL, chromatin condensation by DAPI, ROS generation by DHR123 and loss of membrane integrity by PI.

4.1.2.7a Effect of different additives (apoptosis inducers) on apoptosis of *B. poitrasii*

The effect of varied concentrations of isophthalic acid (12-18 mM), acetic acid (5-20 mM), H_2O_2 (10-50 mM) and amphotericin B (1-4 µM) on apoptosis in *B .poitrasii* yeast cells was studied.

At 16 mM isophthalic acid maximum annexin V-FITC stained protoplasts were observed, while PI staining displayed low number of stained protoplast. At 18 mM concentration of isophthalic acid protoplasts entered into necrotic phase. The yeast cells obtained after H-Y transition carried out in 0.1% YPG at 28ºC for 12 h with and without isophthalic acid showed different levels of apoptotic cells. For instance, in the presence

of 12 and 16 mM of isophthalic acid, 21 and 66%, cells showed apoptosis respectively as visualized by DHR staining. While in control the apoptotic cells were not seen. Increased TUNEL, DAPI and DHR123 positive protoplasts were also observed at 16 mM concentration (Table 4.6; annexure II-Fig. 1). Correlation coefficient value (*r*) of annexin and TUNEL with SOD and caspases was 0.796 ($p = 0.002$), while annexin and DHR123 with SOD showed 0.978 ($p = 0.000$) at 16 mM isophthalic acid (Table 4.4 and 4.6).

Acetic acid (5-20 mM) treated cells showed increased annexin and TUNEL positive protoplasts (10-50%). Chromatin condensation and production of ROS were highest at 20 mM concentration (Table 4.6; annexure II-Fig. 2). At 20 mM concentration of acetic acid) correlation coefficient value (*r*) of TUNEL with SOD and caspases was >0.830 ($p = 0.001$) (Table 4.4 and 4.6).

Cells treated with H_2O_2 (10-50 mM) showed increased annexin and TUNEL positive protoplasts (10-70%) (Table 4.6; annexure II-Fig. 3). At 50 mM concentration maximum cells were stained with DAPI and DHR123 (Table 4.6). Correlation coefficient value (*r*) of TUNEL with SOD and caspases was >0.866 ($p = 0.000$) at 50 mM concentration of H_2O_2.

Amphotericin B (2 µM) treated cells exhibited high proportion of annexin (+) PI (-) and TUNEL positive protoplasts (Table 4.6; annexure II-Fig. 4). At 2 µM concentration of amphotericin B showed elevated DAPI and DHR staining (Table 4.6). Correlation coefficient value (*r*) of TUNEL to SOD and caspases was 0.998 ($p = 0.000$) at 2 µM concentration of amphotericin B.

Apoptotic phenotypes were also assessed in presence of DAB and CHA (Table 4.6; annexure II-Fig. 5). Cells treated with DAB (12 mM) showed annexin V-FITC-positive protoplasts (15%), while there was a small increase in PI and TUNEL positive protoplasts (Table 4.6). 35-40% of protoplasts stained positive for annexin V-FITC when incubated with 15 mM concentration of DAB. DAB at 15 mM induced nuclear degradation in 40% of protoplasts (TUNEL-positive). At 15 mM concentration of DAB correlation coefficient value (*r*) of TUNEL with SOD and caspases was >0.766 ($p = <0.004$).

Annexin V-FITC showed high number of stained protoplasts at 4 mM CHA while low PI staining which indicated membrane integrity. At same concentration TUNEL, DAPI and DHR 123 indicated high number of fragmented DNA, condensed chromatin and ROS production (Table 4.6; annexure II-Fig. 6). Correlation coefficient

value (r) of TUNEL to caspases was >0.766 (p = 0.003) at 4 mM (CHA) (Table 4.4 and 4.6).

Table.4.6 Effect of different additives (apoptosis inducers) on apoptosis of *B. poitrasii*

Additives	Apoptotic protoplasts %				
	Annexin	**TUNEL**	**DAPI**	**DHR123**	**PI**
Control	ND	ND	ND	ND	ND
Isophthalic acid (mM)					
12	35.0±2.8	10.0±0.5	30.0±5.0	15.0±1.5	5.0±1.0
15	60.0±5.0	15.0±2.8	31.0±4.0	20.0±1.1	5.0±0.5
16	85.0±5.7	25.0±4.0	72.0±7.5	30.0±4.0	7.0±0.5
18	77.0±7.8	5.0±0.5	15.0±1.0	25.0±2.2	41.0±5.0
Acetic acid (mM)					
5	20.0±2.8	10.0±0.05	10.0±1.0	30.0±1.7	2.0±0.0
10	30.0±2.8	30.0±0.5	30.0±4.0	50.0±5.0	2.0±0.05
20	45.0±5.5	50.0±0.5	50.0±5.7	70.0±8.6	5.0±0.5
40	70.0±5.0	20.0±3.0	30.0±1.1	40.0±2.5	30.0±2.5
H_2O_2 (mM)					
10	10.0±1.5	30.0±5.0	7.0±1.5	20.0±2.1	2.0±0.5
20	40.0±5.1	50.0±4.5	20.0±2.1	50.0±5.7	5.0±0.5
30	60.0±5.0	65.0±2.8	35.0±5.0	70.0±3.0	5.0±0.6
50	80.0±9.4	70.0±5.0	50.0±3.0	40.0±3.5	10.0±0.5
Amphotericin B (µM)					
1	10.0±1.1	50.0±3.5	20.0±1.0	10.0±0.6	5.0±0.5
2	80.0±4.0	55.0±5.0	60.0±5.7	35.0±3.0	10.0±0.5
4	50.0±5.0	20.0±0.5	40.0±3.5	30.0±1.5	15.0±0.6
6	60.0±5.5	10.0±1.0	15.0±0.8	10.0±0.5	25.0±1.2
DAB (mM)					
12	15.0±2.0	15.0±0.6	15.0±0.6	15.0±1.5	3.0±0.6
15	35.0±2.5	40.0±2.0	60.0±6.5	40.0±3.0	5.0±0.5
16	45.0±5.0	25.0±3.0	42.0±5.0	30.0±2.5	15.0±2.0
18	60.0±7.6	15.0±1.7	20.0±3.5	30.0±2.0	30.0±2.5
CHA (mM)					
3	30.0±2.5	15.0±1.0	30.0±1.0	5.0±0.6	5.0±0.5
4	55.0±1.5	65.0±2.5	70.0±5.5	70.0±6.5	5.0±0.6
5	75.0±5.5	40.0±3.5	30.0±0.5	30.0±2.0	25.0±1.5
7	80.0±10.0	20.0±2.5	10.0±0.5	50.0±3.5	40.0±2.5

B. poitrasii yeast cells were grown in 1% YPG at 37°C for 200 min in the presence or absence of additives. ND, Not detected.

4.1.2.7b Effect of different additives (chemically synthesized and naturally isolated compounds) on apoptosis of *B. poitrasii* yeast cells

The chemically synthesized compounds, 18B (82-110 µM), 20B (79-105 µM), 24B (79-105 µM), DS16 (87-115 µM) and PCN (22-224 µM) were used to study apoptosis in *B. poitrasii* yeast cells. Annexin positive protoplasts were highest at 110 µM, 105 µM and 92 µM concentrations of 18B, 20B and 24B respectively, whereas PI positive protoplasts were 5-15% (Table 4.7; annexure II-Fig. 7, 8, 9). At 110 µM (18B), 105 µM (20B) and 92 µM (24B) concentrations maximum numbers of protoplasts

exhibited TUNEL, DAPI and ROS positive staining. Correlation coefficient value (*r*) of TUNEL with SOD and caspases was >0.769 (p = <0.003) at 110 µM (18B).

For 20B (105 µM) correlation coefficient value (*r*) of TUNEL to caspases was >0.765 (P = <0.004) whereas for 24B (92 µM) correlation coefficient value (*r*) of TUNEL with caspases I was >1.00 (p = 0.00), while DHR showed >0.589 (p = <0.04).

DS16 treated cells at 115 µM concentration showed maximum annexin and TUNEL positive protoplasts (60%) (Table 4.7; annexure II-Fig. 10). DAPI (40%) and DHR123 (50%) positive protoplasts were observed at 115 µM concentration of DS16. Correlation coefficient value (*r*) of TUNEL to caspases 8 was 0.796 (p = 0.002), while annexin and DHR123 with SOD showed >0.763 (p = <0.004).

Apoptotic phenotype was also studied using varied concentrations of PCN (22-224 µM). Annexin V-FITC, PI and DHR123 showed increased number of stained protoplasts with increased concentrations (Table 4.7; annexure II-Fig. 11). At 89 µM, 40% of protoplasts were stained positive for annexin-V, while PI stained protoplasts were ≥5%. At 89 µM concentration TUNEL and DAPI showed that highest number of stained protoplasts with DNA fragmentation and chromatin condensation respectively (Table 4.7). Correlation coefficient value (*r*) of TUNEL with SOD was 0.803 (p = 0.002) whereas no correlation was seen with caspases at 89 µM (PCN).

4.1.2.7c Effect of fluconazole and strobilurin derivative (PC229) on Y-H transition and apoptosis of *B. poitrasii*

Fluconazole and PC229 did not inhibit differentiation and also failed to induce apoptosis at any tested concentration (22-418 µM) while it lead to necrosis (Table 4.8). A relatively large proportion of the cells showed phenotypic markers of necrosis i.e. annexin V (+) and PI (+) staining at higher concentration of fluconazole (209 and 418 µM) and PC229 (178 and 356 µM).

Table. 4.7 Effect of different additives (chemically synthesized and naturally isolated compounds) on apoptosis of *B. poitrasii* yeast cells

Additives (μM)	Apoptotic protoplasts %				
	Annexin	**TUNEL**	**DAPI**	**DHR123**	**PI**
Control	ND	ND	ND	ND	ND
18B					
82	30.0±4.0	20.0±1.5	40.0±4.0	30.0±5.0	10.0±1.5
96	40.0±2.5	25.0±3.4	65.0±5.0	50.0±6.6	10.0±0.6
110	70.0±8.6	60.0±5.0	75.0±8.6	60.0±5.0	15.0±2.0
20B					
79	20.0±2.0	10.0±0.5	30.0±5.0	5.0±0.5	5.0±0.6
92	40.0±2.5	25.0±1.7	55.0±6.0	30.0±1.1	5.0±0.6
105	80.0±5.7	40.0±4.6	60.0±5.0	60.0±1.1	10.0±0.5
24B					
79	40.0±2.5	50.0±5.0	10.0±1.7	45.0±4.0	5.0±0.8
92	55.0±4.0	75.0±8.1	30.0±2.4	60.0±7.0	5.0±0.5
105	65.0±7.0	20.0±2.4	20.0±2.5	30.0±2.4	40.0±2.3
DS16					
87	25.0±1.1	20.0±3.5	25.0±1.1	20.0±2.5	5.0±1.0
101	40.0±2.5	45.0±4.0	35.0±2.8	30.0±4.1	10.0±1.1
115	60.0±5.6	60.0±5.0	40.0±3.6	50.0±5.5	10.0±0.6
PCN					
22	4.0±1.2	7.0±0.8	4.0±1.1	8.0±1.0	1.0±0.3
44	15.0±3.0	15.0±0.6	8.0±1.1	10.0±0.6	2.0±0.8
67	25.0±1.5	20.0±0.5	38.0±2.0	40.0±5.5	2.0±0.6
89	40.0±4.6	25.0±1.5	50.0±4.5	60.0±0.2	5.0±1.0
224	60.0±7.5	15.0±1.5	30.0±5.0	35.0±5.0	35.0±2.8

B. poitrasii yeast cells were grown in 1% YPG at 37°C for 200 min in the presence or absence of additives. ND, Not detected.

Table 4.8 Effect of fluconazole and strobilurin derivative (PC229) on Y-H transition and apoptosis of *B. poitrasii*

Apoptotic inducer (μM)	Germ tube %	% Annexin + protoplasts	% PI + protoplasts	Necrosis
Control	59.1±3.0	ND	1.0±0.5	ND
Fluconazole				
26	58.0±3.5	ND	3.0±0.6	ND
52	57.2±4.0	ND	3.0±0.5	ND
104	57.0±6.5	ND	12.0±1.0	ND
209	58.0±5.0	40.0±2.5	15.0±1.0	+
418	57.0±7.5	60.0±5.5	40.0±2.5	+
PC229				
22	58.0±2.5	ND	ND	ND
44	58.0±3.0	ND	2.0±1.5	ND
89	56.0±3.0	35.0±2.5	15.0±1.0	+
178	57.0±4.5	70.0±5.0	40.0±3.5	+
356	55.0±3.5	50.0±3.5	40.0±1.5	+

Transition was observed in YP media at 28ºC for 6h.

B. poitrasii yeast cells were grown in 1% YPG at 37°C for 200 min in the presence or absence of additives. ND, Not detected.

4.1.3 DISCUSSION

Dimorphic fungi are useful model systems to understand the eukaryotic developmental processes as they differentiate from unicellular yeast to filamentous form for survival and proliferation (Gow, 1995; Deacon, 2006). In fungi, differentiation, a survival mechanism shares a number of biochemical correlates common with apoptosis. Therefore a link between differentiation and apoptosis may be possible. If so, the understanding of differentiation *per se* could contribute towards design of apoptosis inducers for the control of fungal pathogenesis.

Apoptosis was reported in a number of filamentous fungi and the compounds such as farnesol, acetic acid, H_2O_2, amphotericin B, the antifungal protein PAF and sphingoid bases triggered this response (Cheng *et al*., 2003; Mousavi and Robson, 2004; Chen and Dickman, 2005; Leiter *et al*., 2005). The signalling and effector pathways that regulate fungal apoptosis are yet to be elucidated (Madeo *et al*., 2004; Carmona-Gutierrez *et al*., 2010a). *S. cerevisiae* displayed hallmarks of apoptosis, in response to various harsh treatments (Madeo *et al*., 1999; Ligr *et al*., 2001; Ludovico *et al*., 2001; Phillips *et al*., 2003). The physiological role of suicide in a unicellular organism was suggested to increase the fitness, genetic diversity and dynamicity of the whole cell community (Skulachev, 2001; Fabrizio *et al*., 2004a; Knorre *et al*., 2005). The potential apoptotic inducers were identified using *B*. *poitrasii* on the basis of their potential to halt Y-H transition *via* their biochemical correlates and ability to induce the hallmarks of apoptosis. For comparison to the reported hallmarks of apoptosis in the *S. cerevisiae* we performed experiments in Y form of *B. poitrasii*. All pro-apoptotic treatments in *B*. *poitrasii* led to arrest of morphogenesis by inhibition of Y-H transition and their biochemical correlates *viz*. GDH and ODC described in chapter III. In the present study, *B*. *poitrasii* was used as a model to study the effect of low fungicidal doses of different additives on biochemical correlates of apoptosis and their ability to induce apoptosis.

In fungi, it was shown that the biochemical correlates of differentiation and apoptosis are common. In *C*. *albicans* and *S*. *cerevisiae* the Ras and MAPK signalling networks were shared by the differentiation and apoptotic pathways (Gourlay *et al*., 2006; Phillips *et al*., 2006). The *Ras* deletion mutants of *C. albicans* and *S. cerevisiae* failed to show apoptosis in presence of apoptotic stimuli. In *B*. *poitrasii*, cAMP phosphorylation/dephosphorylation regulated the relative proportion of NADP-/NAD-GDH that determined the cell wall chitin content in the yeast or hyphal forms (Khale and Deshpande, 1993). The biochemical correlates of differentiation in *B*. *poitrasii* such as

glutamate dehydrogenase, chitin synthase, chitinase, *N*-acetyl glucosaminidase are well characterized (Khale and Deshpande, 1993; Deshpande, 1998b; Ghormade *et al*., 2000; Chitnis *et al*., 2002; Amin *et al*., 2004a). Another biochemical correlate of differentiation in *B*. *poitrasii*, ornithine decarboxylase, involved in polyamine synthesis, showed increased activity and polyamine levels in the H form as compared to the Y form (Ghormade *et al*., 2005a).

The role of polyamine biosynthetic pathway in apoptosis was well studied using different inhibitors such as CHA, DAB, methylglyoxal-bis-guanylhydrazone (MGBG), difluoromethylornithine (DFMO) (Foster and Walters, 1990; Marton and Pegg, 1995; Kelloff, 2000; Esmat *et al*., 2002; Schipper *et al*., 2000, 2003). In *B*. *poitrasii* the additives (DAB, CHA, 18B, 20B, 24B, DS16 and PCN) were tested at concentrations that halted Y-H transition and showed inhibition (≥50%) of ODC (Table 4.4 and 4.5). Y-H transition may precede apoptosis and can be used as a checkpoint. As ODC was biochemically correlated to differentiation it may be suggested that this enzyme could be useful as biochemical marker for apoptosis. Inhibition of differentiation, ODC activity and polyamine synthesis was also reported in *C*. *albicans*, *M*. *rouxii*, *P*. *brasiliensis*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* using polyamine synthesis inhibitors like DAB, DFMO and CHA (Rajam and Galston, 1985; Martinez-Pacheco *et al*., 1989; Martinez *et al*., 1990; San-Blas *et al*., 1996b; Pieckenstain *et al*., 2001).

Recent studies indicated linkages between polyamines and apoptosis (Faaland *et al*., 2000; Erez *et al*., 2002). Alteration in the polyamines in mammalian cells correlated with decreased mitochondrial membrane potential *via* increased activity of polyamine oxidase (PAO) (Erez *et al*., 2002). Altered PAO activity released mitochondrial cytochrome *c*, which stimulated caspase activation that directly causes apoptosis (Obaya *et al*., 1999; Mitchell *et al*., 2000). The intracellular molecules like cAMP, Ca-calmodulin, and others involved in the signal transduction exhibited concentration dependent correlation with the morphological outcome (Deshpande, 1998a). In *B*. *poitrasii* the cAMP dependent phosphorylation of NAD- and NADP-dependent glutamate dehydrogenase (GDH) was reported which influenced Y-H transition (Khale and Deshpande, 1993). Polyamines were also reported to inhibit GDH activity in *B*. *poitrasii* (Ghormade *et al*., 2005a). In *S*. *cerevisiae* pro-apoptotic treatments lead to arrest of the cell cycle before entering apoptosis (Kornitzer *et al*., 2001). Treatments used to induce apoptosis in yeasts could damage DNA directly through the production of ROS causing G2-M arrest. In *C*. *albicans* treatment with pro-apoptotic doses of H_2O_2 and

acetic acid stimulated Y-H transition (Phillips *et al*., 2003). It was suggested that hyphal growth or differentiation may provide escape route from apoptosis (Phillips *et al*., 2003).

To protect against oxidative damage, cells contain effective defence mechanisms including enzymes such as catalase, superoxide dismutase and glutathione peroxidase and antioxidants such as glutathione (GSH) and vitamins C and E (Gessler *et al*., 2007). Such protective mechanisms were reported in *A. fumigatus*, *C. albicans*, *S. cerevisiae* and *S. pombe* in response to the antibiotic amphotericin B and mutations affecting replication initiation (Gessler *et al*., 2007; Perrone *et al*., 2008). Increased ROS production were associated with copper, cadmium and arsenic toxicity and alleviated by over-expression of *Sod2*, encoding the mitochondrial manganese superoxide dismutase. Increased SOD activity (10-20 folds) was observed during asexual and sexual spore germination, ageing of yeast cells and with increase in concentration of additives in *B. poitrasii* (Table 4.2-4.5). Similar increase was reported in *N. crassa*, *F. decemcellulare* and other filamentous fungi (Gessler *et al*., 2007; Perrone *et al*., 2008).

Caspases are regulator of apoptosis; functional homologues of mammalian caspases were recently identified as *S. cerevisiae* and *A. nidulans* metacaspases and were shown to be involved with the apoptotic response (Uren *et al*., 2000; Narsimhan *et al*., 2001; Madeo *et al*., 2002; Wissing *et al*., 2004). The role of metacaspase during growth and stress response was examined in *B. poitrasii* (Table 4.2-4.5). In *B. poitrasii* caspase like activities increased during sporulation (Table 4.3). Thrane *et al*. (2004) reported caspase activities in protein extracts of *A. nidulans* during sporulation. Increase in caspase like activity upon induction of apoptosis was also reported in *A. fumigatus*, *Penicillium chrysogenum* and *S. cerevisiae* (Sami *et al*., 2001; Mousavi and Robson, 2003; Herker *et al*., 2004; Richie *et al*., 2007). These activities were suppressed by including caspase inhibitor *z*-VAD-fmk into the medium, implicating caspase like proteins in the process. The inducers used in this study were caspase dependent. Caspase like activities increased with rising concentrations of additives but decreased during necrosis. In *B. poitrasii* amphotericin B mediated stress were caspase dependent. While in *A. fumigatus* and *C. albicans* apoptosis was caspase independent (Phillips *et al*., 2003; Mousavi and Robson, 2004).

Apoptosis under natural conditions was evaluated in *B. poitrasii*. *B. poitrasii* yeast cells grown for 144 h showed apoptotic markers with decreased ODC activity. While, hyphal cells showed decline in activity at 240 h, that may be due to continuous apical growth (Table 4.1). The 144 h old yeast cells demonstrated hallmarks of apoptosis

(Fig. 4.1). The *B. poitrasii* yeast cells grown under condition promoting differentiation (low glucose and temperature) showed fewer apoptotic cells as compared to cells grown in high glucose and temperature (Fig. 4.2). Ruckenstuhl *et al*. (2010) reported that in *S. cerevisiae* glucose triggered apoptosis during ageing. This may be due to hyperactivation of the Ras protein, which is part a of signalling pathway upstream from PKA activation, increases apoptosis. In *S. cerevisiae* the production of acetate molecules and low pH caused chronologically aged cultures to die exhibiting markers of apoptosis (Ludovico *et al.*, 2001; Fabrizio *et al*., 2003, 2004; Herker *et al*., 2004).

The compounds that halted the Y-H transition were further evaluated as potential apoptosis inducers using *B. poitrasii* yeast cells. Additives used in the study were commercially available compounds (DAB, CHA, isophthalic acid, amphotericin B and fluconazole), PCN, chemically synthesised compounds (18B, 20B, 24 B and DS16), strobilurin derivative (PC229), acetic acid and H_2O_2. It is reported for the first time that dying *B. poitrasii* yeast cells exhibited key markers of apoptosis, after exposure to low fungicidal doses of apoptotic inducers. *B. poitrasii* displayed chromatin condensation (DAPI), exposure of phosphatidylserine, nuclear fragmentation (TUNEL) and ability to exclude PI, ROS production as well as the dependency on caspase activity. Similar results were reported in *S. cerevisiae*, filamentous fungi and animal cells (Rao *et al*., 1996; Madeo *et al*., 2002; Carmona-Guterrez *et al*., 2010). The saprophyte *B. poitrasii* can now be added to the growing list of eukaryotic microbes known to display apoptosis. Inhibition of differentiation (Y-H transition) or enhanced H-Y transition may lead eventually to apoptosis. Inhibition of GDH activity and Y-H differentiation was observed in presence of isophthalic acid (16 mM) and acetic acid (20 mM). At their respective apoptotic concentration isophthalic acid and acetic acid induced apoptosis (Table 4.6). Interestingly it was reported that isophthalic acid induced apoptosis in leukemia cells (Galkin *et al*., 2008). Apoptosis markers induced by isophthalic acid showed high correlation with SOD and caspases ($r = >0.693$, $p = <0.006$). *B. poitrasii* undergoes apoptosis in presence of acetic acid and H_2O_2 (Table 4.6). Previously exposure to low doses of acetic acid and H_2O_2 were reported to induce apoptosis in *C. albicans*, *S. cerevisiae* and *Zygosaccharomyces bailii* (Ludovico *et al*., 2001, 2003; Phillips *et al*., 2003). Killing by weak acids was attributed to intracellular increase in acidification and ADP/ATP ratio (Bracey *et al*., 1998; Bradbury *et al*., 2000). Weak acids are used as preservatives for prevention of microbial contamination of foodstuffs and also for the treatment of *C. albicans* and other fungal infections (Fleet, 1992; Jain and Agarwal, 1994).

Amphotericin B treated cells of *S. cerevisiae* and *C. albicans* showed accumulation of ROS with reduced intracellular pH (Bracey *et al*., 1998). In *B. poitrasii* apoptotic concentrations of amphotericin B is 2-4 μg ml^{-1} while it was reported to be 0.5-2.0 μg ml^{-1}in *C. albicans* (Phillips *et al*., 2003) (Table 4.6). AMB showed correlation with SOD and caspases (r=0.826, *p*=0.001) while annexin failed to show any correlation (Table 4.4 and 4.6).

In response to various antifungals, oxidative stress was activated *via* ROS (Costa and Moradas-Ferreira, 2001). Oxidative stress in *S. cerevisiae* caused protein and lipid peroxidation, reduced mitochondrial enzyme activities and DNA damage (Costa and Moradas-Ferreira, 2001). ROS induced cell death in *B. poitrasii* yeast form cells. Intracellular ROS accumulation was apparent at the apoptotic concentrations of inhibitors that were examined in *B. poitrasii*. At higher concentration, necrosis was observed that was indicated by loss of membrane integrity. The induction of apoptosis at lower concentrations and necrosis by high concentrations of cytotoxic substances was reported in *S. cerevisiae* and *C. albicans* (Ligr *et al*., 1998; Madeo *et al*., 1999; Ludovico *et al*., 2001; Phillips *et al.*, 2003). DAB and CHA induced apoptosis in *B. poitrasii* by reducing the polyamine level that eventually led to ROS induced cell death (Table 4.7). Apoptotic markers (PI and TUNEL) induced by DAB and CHA showed correlation with SOD and caspases r = >0.844 (*p* = 0.001). In case of H_2O_2, 18B, 20B, 24B and DS16 apoptosis in *B. poitrasii* was pleiotropic as they are known for cell wall associated target as well as inhibition of biochemical correlates (Table 4.6 and 4.7). 18B showed no correlation with caspase 8. 20-24B and DS16 showed no correlation with caspases -3 while strong correlation with SOD r=>0.866 (*p*=0.000). Madeo *et al.* (1999) and Narasimhan *et al.* (2001) suggested that ROS stimulate some unknown pro-apoptotic regulatory machinery in *S. cerevisiae*. Recent work on *Aspergillus nidulans* and *C. albicans* indicated that intracellular ROS may be indispensable for fungal apoptosis (Helmerhorst *et al*., 2001; Cheng *et al*., 2003).

In *B. poitrasii*, cycloheximide blocked the apoptotic phenotype and attenuated apoptotic effect. Similar results were reported in case of *Z. bailii*, *S. cerevisiae* and *A. fumigatus* (Madeo *et al*., 1999; Ludovico *et al*., 2003; Mousavi and Robson, 2003).

In the presence of fluconazole and PC229, Y-H differentiation in *B. poitrasii* was not inhibited and apoptosis was bypassed as the cell went directly into necrosis (Table 4.8). Amin *et al*. (2004b) reported that itraconazole, an inhibitor from the azole group, did not induce apoptosis in *A. fumigatus*. Therefore it may be suggested for dimorphic

fungi that differentiation precedes apoptosis and may be targeted for antifungal strategies.

The present investigations suggested the ability of the different additives to induce apoptosis and affect biochemical enzyme correlates of apoptosis *viz*. ODC, SOD and caspases. In *B. poitrasii* conditions promoting differentiation prolonged apoptosis suggesting the possibility of Y-H transition as an effective antifungal target. As these biochemical correlates were associated with induction of apoptosis they could be helpful to control fungal pathogenesis and food spoilage organisms. This study will be useful to explore the development of antifungal agent that will be helpful in medicinal chemistry and identification of antifungal compounds that can target the morphogenesis and induce apoptosis. This study could lead towards the better understanding of fungal morphogenesis and apoptosis and help to establish correlation between differentiation and apoptosis.

4.2 Molecular studies of ODC and metacaspases

4.2.1 INTRODUCTION

Polyamine metabolism is known to play a key role in differentiation of dimorphic fungi, particularly during the process of dimorphic transition (Calvo-Mendez *et al*., 1987; Martinez-Pacheco *et al*., 1989; Ruiz-Herrera, 1994; Guevara-Olvera *et al*., 1997). The first step in the biosynthesis of polyamines is the decarboxylation of ornithine by ornithine decarboxylase (ODC) (Tabor and Tabor, 1984).

Morphological changes in dimorphic fungi are expressed mainly as a transition between yeasts and hyphal forms. This event is accompanied by an increase in ODC activity during the early stages of mycelial formation, such as in the *B. poitrasii*, *C. albicans*, *Mucorales* and *Y. lipolytica* (Calvo-Mendez *et al*., 1987; Martinez-Pacheco *et al*., 1989; Guevara-Olvera *et al*., 1993; Lopez *et al*., 1997; Jimenez-Bremont *et al*., 2001; Ghormade *et al*., 2005a). Dimorphic transition was repressed by the addition of DAB (ODC inhibitor) in many fungi, including *P. brasiliensis* and *B. poitrasii* (Martinez-Pacheco *et al*., 1989; Guevara-Olvera *et al*., 1993; San-Blas *et al*., 1996a; Ghormade *et al*., 2005a). Recently polyamines were implicated in cell death as alteration in polyamine levels was often lethal, resulting in cell cycle arrest or apoptosis (Schipper *et al*., 2000). In *S. cerevisiae*, a polyamine induced degradation of ODC was reported (Toth and Coffino, 1999). Alteration in polyamine levels, especially in spermidine and spermine, was shown to be a common feature in apoptosis induced by a variety of stimuli (Schipper *et al*., 2000). Though number of reports showed that polyamines, cell cycle events and apoptosis are closely connected, their correlation has not been clearly elucidated.

Apoptosis, a promising therapeutic target for human cancer cells, has gained attention as a potential target for antifungal treatments (Mousavi and Robson, 2003, 2004; Phillips *et al*., 2003, 2006; Semighini *et al*., 2006a; Richie *et al.*, 2007). Apoptosis is regulated by conserved signalling pathways that activate caspase proteases leading to target protein cleavage and finally apoptosis. Recently Uren *et al*. (2000) identified the presence of metacaspases in fungi. Metacaspase mediated apoptosis was observed in a variety of apoptogenic situations following treatment with hydrogen peroxide (H_2O_2) and acetic acid in *S. cerevisiae* (Madeo *et al*., 2002; Frohlich *et al*., 2007). Recently, specific peptide substrates with a fluorogenic group were designed to differentiate the activities of Caspase -1, -3 and -8 (Richie *et al*., 2007). Caspase -3 and -8 were detected in cellular extracts from *A. nidulans* (Thrane *et al*., 2004). In the chapter IV it was shown that the additives that halted the Y-H transition in *B. poitrasii* also affected ODC activity and

caused activation of caspase -1, -3 and -8 activities. Caspases also function as inductive signal for other cellular processes such as cell cycle progression, proliferation, cell migration and differentiation in mammalian cell lines (Fernando and Megeny, 2007). Knowledge of the mechanisms involved in the dimorphic process and apoptosis and their correlation may lead to the identification of potential molecular targets for novel antifungal agents. The ODC and metacaspase encoding genes from several fungi, *A. nidulans*, *C. albicans*, *C. immitis*, *Neurospora crassa*, *S. cerevisiae* and *U. maydis* were previously isolated, permitting a molecular approach to the problem (Fonzi and Sypherd, 1987; Williams *et al*., 1992; Guevara-Olvera *et al*., 1997; Lopez *et al.,* 1997; Guevara-Olvera *et al*., 2000; Madeo *et al*., 2002; Thrane *et al*., 2004). The isolation and molecular analysis of the *ODC* (*BpODC*) and metacaspase gene from *B. poitrasii* was carried out.

4.2.2 RESULTS

4.2.2.1 Isolation of ODC gene from *B. poitrasii*

The PCR amplification of the genomic DNA of *B. poitrasii* was carried out using primers designed from conserved regions of *ODC* genes of different fungi (section 2.4.5). The primer set F-R was designed according to Jimenez-Bremont *et al*. (2001). The PCR amplification using primer set F-R amplified a 300 bp fragment from genomic DNA. The primer set F4-R3 was able to amplify an 850 bp size product. In order to amplify larger *ODC* gene fragments additional primer sets were designed. The primer set F1-R1 designed from the 5′ and 3′ ends respectively of *Y. lipolytica* amplified the full length *ODC* gene. The PCR amplification using this primer set gave a single amplicon of 1349 bp (Fig. 4.3). The purified PCR product was cloned into pGEM-T easy vector and sequenced (section 2.4.6).

The sequence revealed the presence of 1349 bp (Fig. 4.4). The nucleotide sequences of ODC clones were searched in NCBI GenBank database and they showed sequence similarity with other reported fungal *ODC* gene (Table 4.9). The deduced amino acid sequence for this gene was compared with the other protein sequences from the database (Table 4.9). The deduced amino acid sequence of *BpODC* fragment showed >45% similarity to the other ODC in the database (Table 4.9, Fig. 4.5). Maximum similarity (97%) was observed with *Y. lipolytica*. The putative BpODC protein showed pyridoxal binding region at position 101-119 (aa) and the substrate binding region at 257-274 (aa). Six cysteine residues were found between the position 341-410 amino acids.

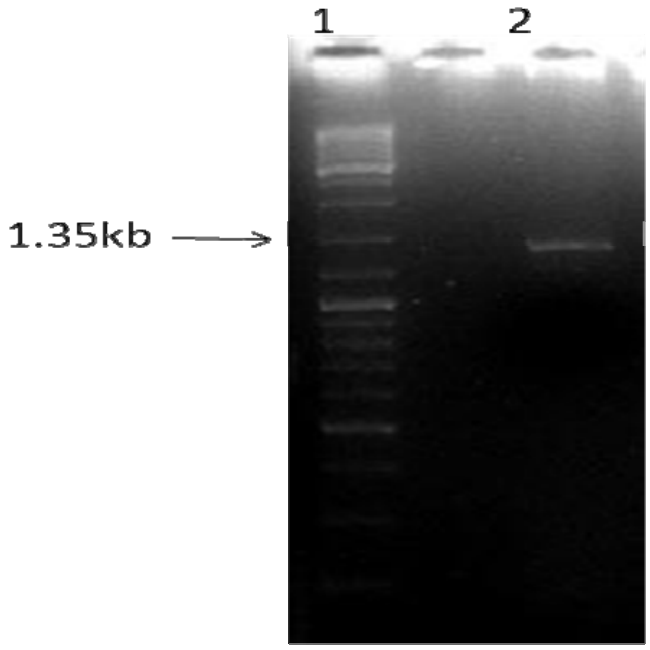

Fig. 4.3 PCR amplification of *B. poitrasii* genomic DNA using F1 and R1 primers. Lane 1, DNA size marker and lane 2, PCR amplified product.

```
   1 ATGGCCCCTC ACATCCTTGG CCCCGAGCCC GTCCAGGCCG ACTCATCCTT
  51 TCTCGGCACA CGACCGTGCC CCACTCTGAG CTACCCTGCT TCTTTTGTGG
 101 ATGAGGTCTC TGCTTCTATT GACCAAGGCC TCTCTGAGTC CGAGAAGCTC
 151 ATTGCTAACG CCATGAAGAC CCGAATCGAG GCTATCGATC TCGAGACCTG
 201 CTCTCCTGGA TCCGAAGACT CCTTCTTCGT GGCTGACCTC GGAATCGTCT
 251 ACTCGCAGTT CATGCGATGG AGTAACTGCC TTCCCCGAGT CACCCCCTTC
 301 TACGCCATGA AGTGTAACGG TGACCCCAAG GTTCTCCAGC TTCTCGCAGC
 351 CCTCGGCACC GGTTTCGACT GCGCCTCCCG AAACGAGATC CAGACAATTC
 401 TGGACCTCGG TGTCTCCCAG GACCGAATCA TCTACGCGCA TCCCTGCAAG
 451 GTGGCGTCCT ACATTCGATA CGCCTCCTCT GTGGGCGTGG AGAAGATGGT
 501 GTTTGACAAC GCCGAGGAGC TCTACAAGTG TGCAAAGTAC CACCCTACCG
 551 CCAAGCTGTT CCTCCGAATT GTCACCGACG ACTCCCAGTC TCTGTGTCAG
 601 TTTTCTGTCA AGTACGGCGC TGCTCTGGAG AACACCCAGT CGCTGCTCCA
 651 GCTTGCTAAG GATCTCGGTC TGAACGTGGC CGGTGTGTCT TTCCATGTGG
 701 GATCCGGCGC TGGAGACCCC AACGCCTTCC TGGACGCTGT GCGAAACGCT
 751 AAGCGGGTGT TTGACCAGGG AGCTGCCATT GGCATGCACC TCAACACTCT
 801 GGATGTCGGC GGAGGCTTCT CGGACGATTC CTTTGAGTCC TCTGCTGCCG
 851 TTCTGGGGGA CGCTCTTGAC AGTACTTCCC AGAGGAGAGC GAGTAACCTC
 901 ATGGCTGAGC CCGCGGTTCT TTGTGTCCGA GCCTTCACCA TTGCCTCTCA
 951 TGTATTGCTC GACGATCATT GAGAACCAGA ACAGCATGCT CTACCTCCAC
1001 CGACGGTGTT CTACGAACAT GACTGCATTC TGTTTGACCA CAAGGAGCCT
1051 TGCCAGTTCT GACTCTACAG CAAGTTCATT GTACCGGTAA GCGATATCCG
1101 AGAGCGGAGA GCTCTCCTCG CCGAGAGACG CGGATCTCTG TTGGGGCCCC
1151 ACCTGCGACG GTATCGACTG CATTTCTAAG TCGTGCCAGC TACCTGTTCT
1201 TCTCGATGTC GGTGACTGGA TGTACTTCAC TTCCTTTGGT GCCTACACCG
1251 TGGCAGCCTC CACCACCTTC AACGGTTTCA ACAGCGATTG TGAGACCCTG
1301 TACGTTTGCT CTAAGAAGGA GGTTGAGCAG TACATCAAGT TGTAAGTAT
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Fig. 4.4 Nucleotide sequence (1349 bp) of ornithine decarboxylase from *B. poitrasii*. The pyridoxal phosphate binding region is underlined and the substrate binding region is shown by the dotted line.

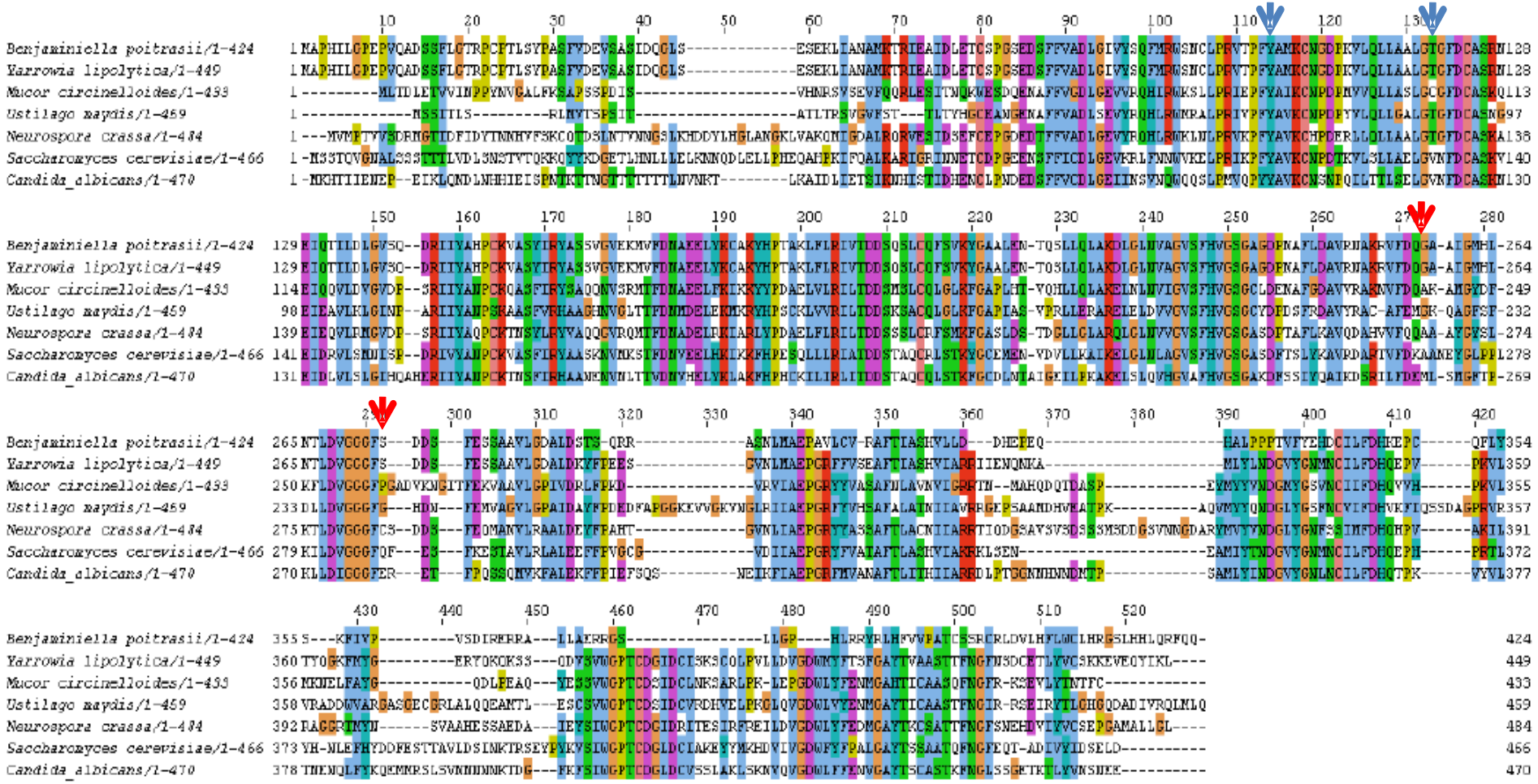


Fig. 4.5 Comparison of the deduced amino acid sequences of *B. poitrasii* ornithine decarboxylase with other fungi using Clustal W 1.8 program. Blue arrows indicate pyridoxal binding site and the red arrows indicate the substrate binding region. Gene bank accession nos.: *Y. lipolytica* (AJ237707), *M. circinelloides* (CAB61758), *U. maydis* (CAA61274), *N. crassa* (AAA33604.1), *S. cerevisiae* (P08432), *C. albicans* (*CAA64451*).

Table. 4.9 Comparison of different ornithine decarboxylase (ODC) gene fragments

Organism	Accession No.	% Similarity (bp)	% Identity (aa)
Aspergillus flavus	XM002377008	(687/1350) 51	(228/424) 49
Chaetomium globosum	XP001229110	(731/1380) 53	(244/459) 53
Candida albicans	CAA64451	(155/1413) 11	(136/470) 29
Epichloe festucae	ABM55741	(709/1338) 53	(231/445) 52
Gibberella zeae	XP386079	(714/1347) 57	(175/448) 39
Magnaporthe oryzae	XP365739	(759/1380) 55	(252/459) 55
Metarhizium anisopliae	CBK55649	(494/830) 53	(138/276) 50
Mucor circinelloides	CAB61758	(456/1302) 35	(208/433) 48
Nectria haematococca	EEU44489	(714/1347) 57	(232/448) 52
Neurospora crassa	M68970	(771/1455) 53	(271/484) 56
Podospora anserina	XP001911010	(828/1533) 54	(260/510) 51
Paracoccidioides brasiliensis	AAF34583	(659/1344) 49	(201/447) 45
Saccharomyces cerevisiae	P08432	(686/1401) 49	(228/466) 49
Tuber melanosporum	XP002842313	(685/1347) 52	(228/438) 52
Ustilago maydis	CAA61274	(400/1380) 29	(138/459) 30
Yarrowia lipolytica	AJ237707	(1309/1350) 97	(395/449) 88

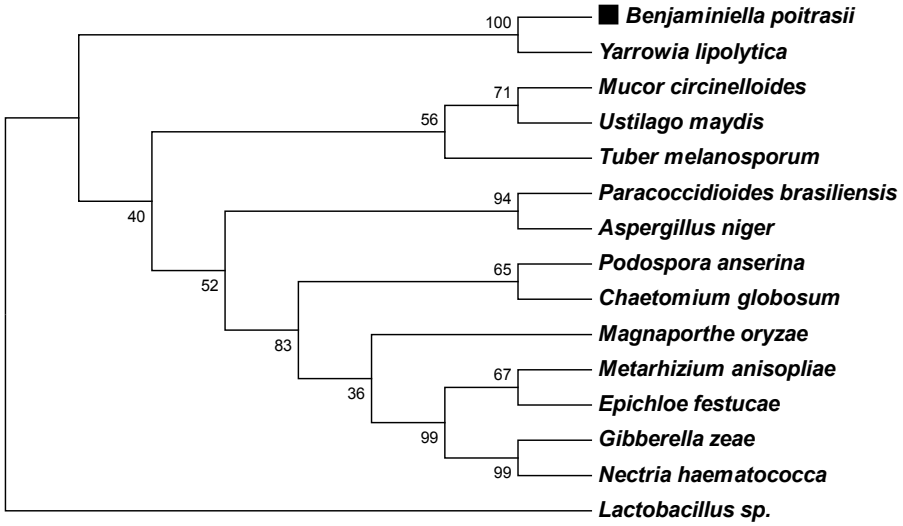


Fig. 4.6 Phylogenetic tree derived from amino acid sequence of *B. poitrasii* ornithine decarboxylase and other ODC proteins. The tree shows the consensus out of 1000 phylogenies constructed by maximum parsimony. Gene bank accession nos.: *Y. lipolytica* (AJ237707), *G. zeae* (XP386079), *N. haematococca* (EEU44489), *E. festucae* (ABM55741), *M. anisopliae* (CBK55649), *C. globosum* (XP001229110), *P. anserine* (XP001911010), *M. grisea* (XM365739), *A. niger* (XP001401610), *T. melanosporum* (XP002842313), *U. maydis* (CAA61274), *M. circinelloides* (CAB61758), *Lactobacillus* (AAA64830).

For the taxonomic and phylogenetic study, analysis of the deduced amino acid sequence (321 amino acids) of *B. poitrasii* ODC gene was carried out with the amino acid sequences of other ODC genes from Genbank. Maximum parsimony and bootstrap method included in the Clustal W program was used for phylogenetic tree construction and alignment of amino acid sequences as mentioned in materials and methods (section 2.4.10 and 2.4.11). The phylogenetic tree showed that the ODC sequence of *Y. lipolytica* was closely related to *B. poitrasii* (Fig. 4.6).

4.2.2.2 Isolation of metacaspase gene from *B. poitrasii*

The PCR amplification of the genomic DNA of *B. poitrasii* was carried out with primers designed from conserved regions in the metacaspase genes of different fungi (section 2.4.5). The PCR amplification using primer set AF1-AR1 amplified a 467 bp fragment(Fig. 4.7). The PCR products obtained using primer sets were purified and cloned into pGEM-T easy vector and sequenced (section 2.4.6). The nucleotide sequences of metacaspase clones were searched for sequence similarity in NCBI GenBank database. The amplicon obtained using AF1-AR1 primer set showed similarity with other reported fungal metacaspase genes. The nucleotide sequences and amino acid sequences for the cloned metacaspase gene obtained using AF1-AR1primer set are shown in the Fig. 4.8 and Fig. 4.9 respectively. The deduced amino acid sequence of *B. poitrasii* metacaspase fragment contained the p20 domain that was characteristics of fungal metacaspases and the universally conserved catalytic cysteine-histidine diad or the p10 domain. The amino acid sequence of *B. poitrasii* showed >65% similarity with metacaspase proteins from other fungi in p20 domain (Table 4.10). Maximum identity (>90%) was observed with *A. fumigatus*, *A. clavatus*, *A. nidulans*, *N. fischeri*, *P. marneffei* and *T. stipitatus* (Table 4.10).

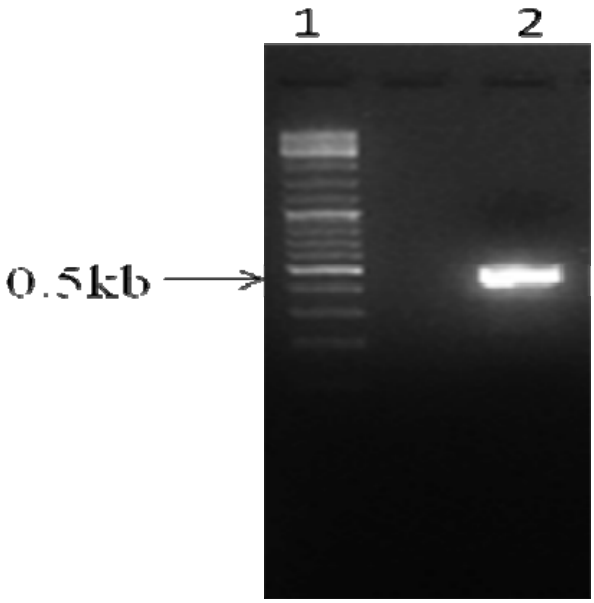


Fig. 4.7 PCR amplification of *B. poitrasii* genomic DNA using AF1 and AR1 primers. Lane 1, DNA size marker and lane 2, PCR amplified product.

```
  1 TGATTGGAAT CAACTATTTC GCTTTGAGGG ACAGTTGCGC GGGTCCTCAA
 51 CGATGTGAAG AACATGCCCA CATACCTCAA CCAGAATTTT GGCTATGCCC
101 GTGAAGATAT GGTGCTCCTG ACTGATGACC AACAAAACCC GATGAGCCAA
151 CCAACGAAGG CGAATATCCT GCGCGCGAGG GAGTGGTTGG TCAAAGACGC
201 ACAGCCCAAC AATTCCCTCT TTTTCCATTA CTCTGGACAC GGTGGACCGA
251 CCCCTGACTT GGACGGGGAC GAGTATGACG GATATGACGA AGTTATCTAC
301 CCGGTGGTTT TCCTTTGTGG CGGGGACCCA TAGTCCGATG ATGAGGATGC
351 ATCCGCACCA TGGTGAAGCC TCCTGAGGCC AGGCGTGCGA CTCACAGCGA
401 TCGTCGAGGT CGTGTCATTC AGGCTCTGTT TTGGACCTGC CGTTACTTCT
451 ACTTCTACAC AGGGTTT
```

Fig. 4.8 Nucleotide sequence (467 bp) of metacaspase from *B. poitrasii*. The p20 domain is underlined and the cysteine-histidine diad is highlighted.

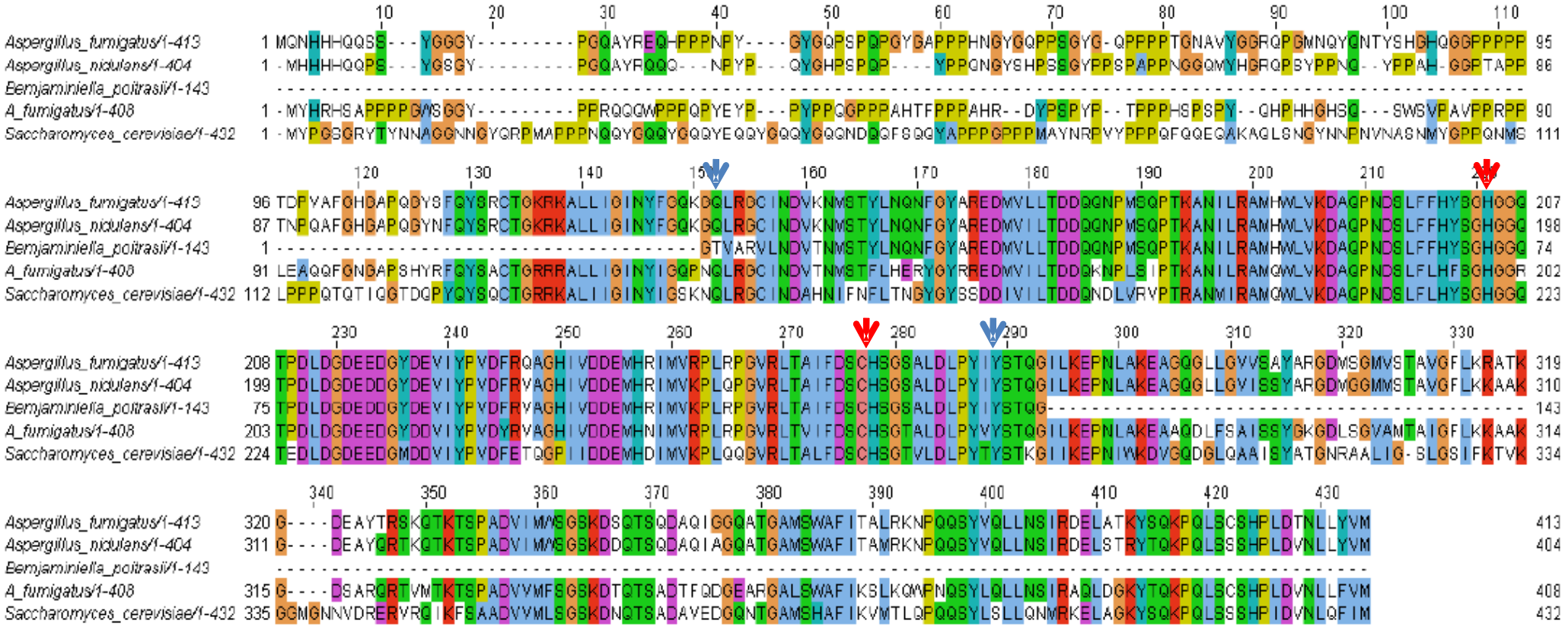


Fig. 4.9 Comparison of the deduced amino acid sequences of *B. poitrasii* metacaspase with other fungi using Clustal W 1.8 program. Blue arrows indicate p20 domain and the red arrows indicate conserved location of the predicted cysteine - histidine diad. Gene bank accession nos.: *A. fumigatus CasA* (ABF71662), *A. fumigatus CasB* (ABF71662), *A. nidulans* (AAO13381), *S. cerevisiae* (NP014840).

Table. 4.10 Comparison of different metacaspase gene fragments

Organism	Accession No.	% Similarity (bp)	% Identity (aa)
Aspergillus clavatus	XP001269487	(373/467) 80	(134/143) 93
Aspergillus fumigatus CasA	ABF71662	(373/467) 80	(134/143) 93
Aspergillus nidulans	CBF81352	(396/467) 85	(134/143) 93
Neosartorya fischeri	XM001264993	(372/467) 79	(134/143) 93
Neurospora crassa	XP959720	(331/467) 71	(118/143) 82
Penicillium marneffei	EEA27784	(332/467) 72	(134/143) 93
Paracoccidioides brasiliensis	XP002790160	(355/467) 76	(128/143) 89
Saccharomyces cerevisiae	NP014840	(267/467) 57	(119/143) 83
Schizosaccharomyces pombe	NP588503	(280/467) 60	(94/143) 65
Talaromyces stipitatus	XP002341251	(365/467) 78	(131/143) 91
Yarrowia lipolytica	Q6C2Y6	(350/467) 75	(94/143) 65

For the taxonomic and phylogenetic study an analysis of the deduced amino acid sequences (143 amino acids) of *B. poitrasii* metacaspase was carried out with the amino acid sequences of other metacaspase genes from Genbank. The alignment was carried out using maximum parsimony and bootstrap method included in the Clustal W program. The phylogenetic tree showed that the metacaspase sequence of *B. poitrasii* can be clubbed with *N. fischeri* and *A. clavatus* (Fig. 4.10).

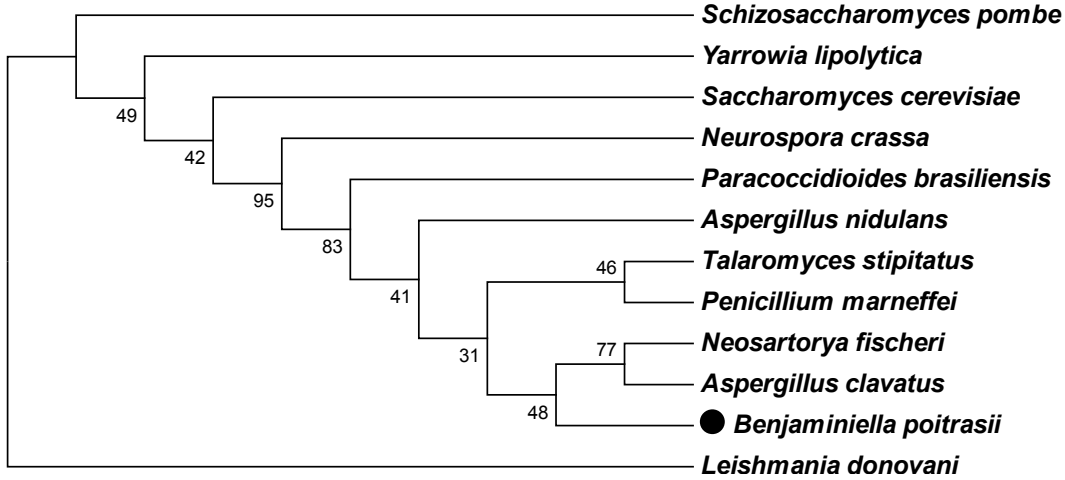


Fig. 4.10 Phylogenetic tree derived from amino acid sequence of *B. poitrasii* metacaspase and other metacaspase proteins. The tree shows the consensus out of 1000 phylogenies constructed by maximum parsimony. Gene bank accession nos.: *T. stipitatus* (XP002341251), *P. marneffei* (EEA27784), *A. nidulans* (CBF81352), *N. fischeri* (XM001264993), *A. clavatus* (XP001269487), *P. brasiliensis* (XP002790160), *N. crassa* (XP959720), *S. cerevisiae* (NP014840), *Y. lipolytica* (Q6C2Y6), *S. pombe* (NP588503), *L. donovani* (DQ367530).

4.2.3 DISCUSSION

ODC, a polyamine synthesizing enzyme was reported to play a significant role in the differentiation and apoptosis (Ruiz-Herrera *et al.*, 1983; Garcia *et al.*, 1980; Guevara-Olvera *et al*., 1997; Schipper *et al*., 2000, 2003; Blasco *et al*., 2002). ODC converts ornithine into putrescine, which was subsequently converted into spermidine and spermine. Our observations showed that additives used in the study inhibited Y-H transition and ODC enzyme and also induced apoptosis. In other fungi such as *M. circinelloides*, *P. brasiliensis*, *U. maydis* and *Y. lipolytica* deletion of the ODC gene was shown to affect differentiation (Guevara-Olvera *et al*., 1997; Jimenez-Bermont *et al*., 2001; Blasco *et al*., 2002; Nino-Vega *et al*., 2004). Polyamines are regulators of cellular functions, promoting proliferation or cell death depending on the cell type and on environmental signals. Schipper *et al*. (2000) suggested the role of polyamines in apoptosis though its mechanism was not clear.

Similar to ODC, caspases are also known to be involved in normal cellular processes as well as apoptosis (Richie *et al*., 2007). The yeast metacaspase *Yca1p* and *A. fumigatus* metacaspase are implicated in an apoptotic-like cell death that is triggered by exposure to a wide variety of adverse environmental stimuli (Madeo *et al*., 2002; Richie *et al*., 2007). In *S. cerevisiae*, loss of metacaspase activity confers resistance to oxidative stress-induced death suggesting a role for *Yca1p* in a caspase-dependent cell death programme (Madeo *et al*., 2002; Wissing *et al*., 2004; Khan *et al*., 2005).

It was an emerging idea among higher eukaryotes that many apoptosis-executing proteins have normal functions besides cell death and these functions are exploited by the apoptotic machinery under certain types of stress conditions. Thus, an understanding of the normal function of caspases is essential to understand how they contribute to cell death pathway. The molecular characterization of ODC and metacaspase gene from *B. poitrasii* displayed typical conserved gene features.

The *B. poitrasii ODC* gene was 1.349 kb long and the putative protein sequence contained 424 aa. *Y. lipolytica ODC* gene was 1.35 kb and the putative protein sequence contained 449 aa (Jimenez-Bremont *et al*., 2001). *B. poitrasii* showed the presence of the conserved pyridoxal 5-phosphate attachment sequence (101-119 aa) and substrate binding site (257-274 aa). The presence of these domains was also reported in *M. circinelloides*, *P. brasiliensis* and *Y. lipolytica ODC* gene (Jimenez-Bremont *et al*., 2001; Blasco *et al*., 2002; Nino-Vega *et al*., 2004).

Analysis of metacaspase gene fragment of *B. poitrasii* showed that the depicted amino acid sequence (143) share 65-93% sequence identity with other fungal metacaspases. The deduced *B. poitrasii* metacaspase protein sequence contains a p20 and p10 domain that includes the universally conserved catalytic cysteine- histidine diad that is required for catalysis by cysteine proteases (Fig. 4.9) (Uren *et al*., 2000; Richie *et al*., 2007). The sequence of the proline rich domain is the most variable region among the reported metacaspase proteins (Uren *et al*., 2000).

A phylogenetic tree was constructed using ODC and metacaspase genes from different fungi belonging to zygomycete ascomycete and basidiomycete. This is consistent with the traditional taxonomy methods based on morphological features. The phylogenetic tree demonstrates that the *ODC* gene of *B. poitrasii* was closely related to *Y. lipolytica* whereas metacaspase gene showed relatedness to *A. clavatus* and *N. fischeri* (Fig. 4.6 and 4.10). A similar phylogenetic tree to study the taxonomic relatedness of fungi was also reported using *ODC* gene from *P. brasiliensis* (Nino-Vega *et al*., 2004).

The molecular characterization of *B. poitrasii ODC* and metacaspase genes will be useful for further studies to design specific probes for southern analysis and to study the cause- effect relationship by reverse genetics.

4.3 Screening of chemically synthesized compounds and microbial metabolites using fungal strains

4.3.1 Effect of chemically synthesized compounds and microbial metabolites on fungal growth

All the newly synthesized triazole-linked β-lactam–bile acid conjugates (B), bile acid dimers linked with triazole and bis- β-lactam (D) and tetrapeptide linked-cholic acid derivatives (S) were tested *in vitro* for antifungal activity. The antifungal activity was tested using fungal strains saprophytes *B. poitrasii*, *Y. lipolytica*, human pathogens *C. albicans*, *C. neoformans* and plant pathogen *F. oxysporum*. The MIC and IC_{50} values were determined using standard broth microdilution technique described by NCCLS (materials and methods, section 2.2.6.3). The compounds 17 -24 B, SB09, 32-39, 15, 17, 19, 20D and DS16 were active against all the strains tested, whereas SB51-52 and DS 14 were found to be inactive (Table 4.11).

Table. 4.11 Effect of triazole linked β-lactam, dimers and cholic acid derivatives on fungal growth

Inhibitor	Minimum Inhibitory Concentration (μg ml^{-1}) Fungal strains				
	B.p	***Y.l***	***C.a***	***C.n***	***F.o***
17B	32	64	64	32	64
18B	32	4	128	128	16
19B	32	8	128	32	128
20B	128	64	32	32	16
21B	64	16	32	128	32
22B	64	128	32	32	64
23B	32	16	64	32	128
24B	8	32	16	64	32
SB09	80	50	50	50	30
SB32	16	96	32	128	64
SB34	24	128	128	128	32
SB36	24	128	32	128	64
SB37	32	128	64	128	64
SB39	16	128	64	128	32
SB51	50	100	ND	ND	100
SB52	80	ND	80	ND	ND
15D	94	10	85	17	64
17D	12	16	25	36	27
19D	32	35	16	18	25
20D	17	128	32	51	19
DS14	43	128	128	128	75
DS16	12	15	25	36	16

B.p – *Benjaminiella poitrasii*, **Y.l-***Yarrowia lipolytica*, **C.a** – *Candida albicans*, **C.n-** *Crytococcous neoformans*, **F.o** – *Fusarium oxysporum*; ND, inhibition not detected; Compounds were dissolved in DMSO (2.5%, v/v); Triazole-linked β-lactam–bile acid conjugates (B), bile acid dimers linked with triazole and bis- β-lactam (D) and cholic acid derivatives (S)

Among the screened microbial metabolites (50 µl) 5 bacterial cultures showed 60-70% hyphal tip bursting. These cultures showed ≥ 5 mm zone of inhibition and were used for screening of potential glutamate dehydrogenase and ornithine decarboxylase inhibitors (Table 4.12).

Table. 4.12 Screening of bacterial cultures as potential inhibitor producers

Inhibitors	*B. poitrasii* zone of inhibition (mm)	HTB
Bacillus sp (B1)	13-14	70.0±3.0
Bacillus sp (B2)	9-10	70.0±5.0
Bacillus sp (B14)	11-12	ND
Bacillus sp (B15)	14-15	60.0±3.0
Bacillus sp (B17)	9-10	25.0±4.0
Bacillus sp (B21)	8-9	65.0±3.0
Bacillus sp (B22)	11-12	ND
Pseudomonas sp (B18)	15-16	62.0±4.0
Streptomyces sp	5-6	45.0±7.0

HTB, Hyphal tip bursting; The culture filtrates used for plate assay were 10 times concentrated.

Based on inhibition of Y-H transition (Table 3.5), inhibition of GDH and ODC activities (Table 3.8) and above mentioned results for zone of inhibition (Table 4.12) in *B. poitrasii*, *Pseudomonas* sp. B18 was carried forward for isolation and purification of potential microbial secondary metabolite discussed in annexure I.

Along with this purified compound other selected chemically synthesized compounds were further studied for their role as effectors of morphogenesis and apoptosis inducer. This study could be useful to develop and design antifungal drugs against human and plant pathogen by using differentiation and their biochemical correlates and apoptosis as a strategy.

4.4 Effect of phenazine-1-carboxamide (PCN) on yeasts species involved in wine fermentation for apoptosis

4.4.1 INTRODUCTION

Yeasts belonging to the genus *Dekkera/Brettanomyces* and *Mechnikowia* represented one of the major problems in the wine industry (Sponholz, 1993). These yeasts are natural inhabitants of the grape berry surface and fermenting must (Ibeas *et al*., 1996). Under the availability of oxygen and carbon and energy sources, they can produce unpleasant odours and tastes which affect the wine aroma (Fugelsang, 1997; Ciani *et al*., 2003;Dias *et al*., 2003). Spoilage of foods and beverages caused by yeast leads to change in taste, aroma, off colour etc. (Thomas, 1993; Tudor and Board, 1993). Spoilage by *Dekkera/Brettanomyces* can be prevented by adequate cellar hygiene, sulfitation, and ageing at low temperatures. In the wine industry, killer strains of *S. cerevisiae* are currently used as starter cultures and the utilisation of the killer toxin of *Kluyveromyces phaffii* was proposed to control apiculate wine yeasts that are able to counteract undesired organisms in wine (Van Vuuren *et al*., 1992; Ciani and Fatichenti, 2001). The cytotoxic effects induced by weak acids in yeast, may affect cell viability and result in cell death. This further insight into the mechanisms that determine different susceptibilities of yeast to weak carboxylic acids compared to other microorganisms and will lead to the improvement or design of new strategies for food and beverage preservation. The toxins and weak acids control spoilage organisms by induction of apoptosis. Inhibition of *Cryptococcus albidus*, *Dekkera bruxellensis*, *Pichia membranifaciens*, *S. cerevisiae*, *Z. bailii* and *Zygosaccharomyces bisporus* was observed by antifungal hexapeptides (PAFs) and lactoferrin (LF)-derived peptides (Enrique *et al*., 2007, 2008).

PCN was isolated and screened for its apoptotic induction properties using *B. poitrasii* as a model. This compound was further evaluated for its potential to prevent food spoilage. Apoptosis is an essential process in multicellular as well as unicellular organisms. This study would facilitate the development of novel antifungal agents that switch on endogenous cell suicide mechanisms in spoilage yeasts involved in wine fermentation.

In this study, we reported that cell death in wine spoilage yeasts *D. bruxellensis*, *M. pulcherrima*, *Zygosaccharomyces rouxii*, *Candida krusei*, *Debaryomyces hansenii* and *Pichia anamola* induced by PCN is associated with the apoptotic phenotype.

4.4.2 RESULTS

4.4.2.1 Effect of phenazine-1-carboxamide (PCN) on biochemical correlates of apoptosis in yeasts flora of grapes

The *in vitro* ODC activity was performed with and without addition of PCN, concentrations used in the study was decided on MIC values and ≤ IC_{50} values (Table 4.13). The yeast cells were grown in 1% YPG at 28°C for 24 h and cell extracts were used as enzyme source. Different levels of ODC activities from 3.52±0.004 to 0.332± 0.062 U mg^{-1} protein, were observed for *D. bruxellensis*, *M. pulcherrima*, *Z. rouxii*, *C. krusie*, *D. hansenii* and *P. anamola* (Table 4.13). PCN inhibited ODC activity of *D. bruxellensis* to 85.9% at 89.6 µM (PCN), whereas in *M. pulcherrima* activity decreased to 69.5% at 89.6 µM (PCN) as compared to control (Table 4.13).

In *Z. rouxii* ODC activity was inhibited to 93.9% at 112 µM concentration of PCN. In *C. krusei* ODC activity decreased at 71.7 µM of PCN (76.7%), while no activity was observed at ≥89.6 µM. In *D. hansenii* ODC activity was inhibited from 43.3-86.7% with increasing concentration of PCN (112-269 µM) (Table 4.13). In case of *P. anamola* ODC activity inhibited to 90.8% at 143 µM.

Intracellular caspase like activities were induced in *D. bruxellensis* with the increase in concentrations of PCN and maximum activity was observed at 89.6 µM of PCN. At 89.6 µM concentration of PCN activities increased by 39.31, 22.41 and 17.33 fold, in comparison to respective controls (Table 4.13). PCN treated *M. pulcherrima* yeast cells showed increased caspase-1, -3 and -8 activities by 74.29, 66.28 and 68.83 fold at 89.6 µM concentration in comparison to respective controls (Table 4.13).

Caspase-1, -3 and -8 activities of *Z. rouxii* treated yeast cells increased by 82.38, 426.14 and 82.15 fold at 112 µM (PCN) in comparison to respective control (Table 4.13). At 89.6 µM concentration of PCN in *C. krusei* caspase-1, -3 and -8 activities were increased by 34.60, 26.47 and 71.07 fold in comparison to respective controls (Table 4.13).

Intracellular caspase-1, -3 and -8 activities increased by 24.47, 21.34 and 105.47 fold at 224 µM (PCN) in comparison to respective controls in *D. hansenii* (Table 4.13). Whereas caspase activities in *P. anamola* increased by 251.22, 42.76 and 113.02 fold at 71.7 µM concentration of PCN in comparison to respective controls (Table 4.13).

Table. 4.13 Effect of phenazine-1-carboxamide (PCN) on biochemical correlates of apoptosis in yeasts flora of grapes

PCN (µM)	ODC ($U\ mg^{-1}$)	Caspase (Relative fluorescence unit)		
		I	**III**	**IV**
D. bruxellensis				
Control	0.924±0.05	10.7±0.04	12.3±0.09	36.9±2.50
44.8	0.130±0.02 (67.8)	220.6±5.50	96.7±1.50	180.4±5.00
89.6	0.030±0.005 (85.9)	420.7±25.20	276.1±5.50	641.0±21.00
179.0	ND (100)	259.2±4.40	191.8±2.50	162.3±3.50
M. pulcherrima				
Control	0.812±0.06	3.7±0.05	5.4±0.80	5.7±0.80
71.7	0.551±0.04 (32.4)	148.1±1.25	157.9±1.50	148.6±1.50
89.6	0.248±0.02 (69.5)	272.6±5.00	357.9±10.50	391.0±5.00
179.0	ND (100)	156.9±5.00	238.7±2.00	192.8±2.20
Z. rouxii				
Control	3.52±0.09	3.3±0.05	1.04±0.008	6.1±0.80
71.7	2.80±0.9 (20.4)	156.9±1.50	171.9±5.00	193.7±3.50
112.0	0.21±0.005 (93.9)	272.7±10.50	426.1±12.50	505.2±10.80
179.0	ND (100)	26.3±1.50	178.4±1.50	156.3±2.50
C. krusei				
Control	1.28±0.06	14.7±0.50	12.3±2.0	6.5±0.80
71.7	0.297±0.01 (76.7)	186.0±2.40	213.9±10.50	194.8±5.25
89.6	ND (100)	509.8±5.00	325.6±5.50	462.0±5.00
179.0	ND (100)	37.8±1.00	136.2±1.00	157.2±1.80
D. hansenii				
Control	0.332±0.04	13.9±2.0	14.7±0.80	5.4±0.50
112.0	0.110±0.004 (43.3)	72.8±3.50	164.9±2.50	173.9±1.10
224.0	0.088±0.005 (66.8)	340.3±10.20	314.6±5.25	568.5±2.50
269.0	0.044±0.005 (86.7)	150.9±5.50	221.8±4.00	170.6±4.40
P. anamola				
Control	2.63±0.03	1.8±0.005	9.2±0.90	6.5±0.80
53.8	1.64±0.80 (37.6)	211.1±10.50	189.4±10.50	277.1±5.00
71.7	0.564±0.03 (78.5)	459.7±15.00	395.6±10.00	740.0±25.0
143.0	0.241±0.02 (90.8)	144.6±2.53	182.8±2.50	149.0±5.00

B. poitrasii yeast cells were grown in 1% YPG at 28ºC.
The % inhibition of enzyme activities is indicated in the parentheses.
ND, Not detected.

4.4.2.2 Effect of PCN on apoptosis of yeast flora of grapes

To assess whether PCN induced cell death in wine yeasts *D. bruxellensis*, *M. pulcherrima*, *Z. rouxii*, *C. krusei*, *D. hansenii* and *P. anamola*, the percentage of positively stained protoplasts was determined with annexin V-FITC, TUNEL, DAPI, DHR123 and PI, by addition of PCN. Concentrations used to study its effect on ODC and apoptosis was based on MIC values and $\leq IC_{50}$ values.

Table. 4.14 Effect of phenazine-1-carboxamide (PCN) on apoptosis of yeasts flora of grapes

PCN (µM)	Apoptotic protoplasts %				
	Annexin	**TUNEL**	**DAPI**	**DHR123**	**PI**
Control	ND	ND	ND	ND	ND
D. bruxellensis					
44.8	15.0±1.1	10.0±1.0	35.0±1.1	30.0±5.0	3.0±0.5
89.6	30.0±2.8	25.0±1.5	60.0±5.0	60.0±5.0	7.0±1.0
179.0	30.0±5.0	15.0±0.5	28.0±5.0	55.0±5.0	30.0±0.6
M. pulcherrima					
71.7	20.0±2.3	20.0±1.1	30.0±2.5	40.0±2.5	5.0±0.5
89.6	25.0±2.8	35.0±2.5	70.0±2.5	70.0±5.7	8.0±0.6
179.0	40.0±5.0	25.0±2.8	40.0±2.8	40.0±5.0	25.0±2.4
Z. rouxii					
71.7	5.0±1.0	15.0±1.0	35.0±2.5	40.0±5.1	2.0±0.5
112.0	25.0±1.7	45.0±5.0	50.0±5.0	70.0±8.6	5.0±0.5
179.0	30.0±2.5	15.0±2.8	20.0±1.0	50.0±6.0	20.0±2.8
C. krusei					
71.7	20.0±2.8	2.0±0.6	10.0±0.0	20.0±0.5	5.0±0.5
89.6	30.0±3.0	5.0±0.5	45.0±0.6	70.0±5.7	10.0±1.0
179.0	45.0±5.0	2.0±0.5	30.0±3.0	55.0±5.0	30.0±5.0
D. hansenii					
112.0	7.0±0.5	30.0±5.0	25.0±2.8	40.0±2.5	1.0±0.5
224.0	15.0±1.0	50.0±5.7	55.0±5.0	75.0±5.0	5.0±1.0
269.0	10.0±1.0	10.0±1.0	30.0±5.13	55.0±7.6	20.0±2.0
P. anamola					
53.8	5.0±0.6	1.0±0.5	20.0±1.5	35.0±5.0	2.0±0.5
71.7	15.0±1.0	5.0±1.0	40.0±5.0	70.0±5.0	5.0±1.0
143.0	40.0±5.0	3.0±0.5	30.0±0.5	55.0±5.7	25.0±3.4

B. poitrasii yeast cells were grown in 1% YPG at 28°C for 200 min in the presence or absence of additives. ND, Not detected.

In *D. bruxellensis* apoptosis was studied using PCN (44.8-179 µM). Annexin V-FITC, TUNEL, DAPI and DHR123 positive stained protoplasts increased till 89.6 µM concentration (Table 4.14 and annexure II-Fig. 12). Correlation coefficient value (*r*) of annexin, TUNEL, DAPI and DHR123 with caspases was >0.91 ($p = 0.000$) at 89.6 µM concentration of PCN.

In *M. pulcherrima* apoptosis was studied at 71.7-179 µM concentrations of PCN. At 89.6 µM concentration of PCN, 40% of protoplasts were stained positive for annexin, while PI stained protoplasts were >10%. At 89.6 µM concentration highest number of protoplasts showed DNA fragmentation, chromatin condensation and ROS production as detected by TUNEL, DAPI and DHR123 respectively (Table 4.14 and annexure II-Fig. 13). Correlation coefficient value (*r*) of TUNEL and DAPI with caspases was >0.86 ($p = 0.000$) at 89.6 µM concentration of PCN.

PCN at concentrations 71.7-179 µM was used to study apoptosis in *Z. rouxii*. Annexin, TUNEL, DAPI and DHR123 stained protoplasts were maximum at 112 µM concentration of PCN, whereas PI positive protoplasts were ~5% (Table 4.14 and annexure II-Fig. 14). Correlation coefficient value (*r*) of annexin, TUNEL, DAPI and DHR123 with caspase 1 and 8 was >0.80 (p = <0.002) at 112 µM concentration of PCN.

In *C. krusei* PCN at concentrations 71.7-179 µM was used to study apoptosis. Annexin, DAPI (+) protoplasts and generation of ROS were highest at 89.6 µM concentration (45%), while PI (+) cells was ~10%. DNA fragmentation observed by TUNEL showed very low staining (Table 4.14 and annexure II-Fig. 15). Correlation coefficient value (*r*) of annexin, TUNEL, DAPI and DHR123 with caspases was >0.86 (p = 0.000) at 89.6 µM concentration of PCN.

D. hansenii yeast cells were studied for apoptotic phenotype using PCN at concentrations 112-269 µM. At 224 µM concentration maximum number protoplasts were stained positive for annexin V-FITC, DAPI (chromatin condensation) and DHR124 (ROS production) while PI positive cells were 5% (Table 4.14 and annexure II-Fig. 16). At 224 µM concentration of PCN correlation coefficient value (*r*) of TUNEL and DHR123 with caspases was >0.72 (p = <0.007).

In *P. anamola* increase in annexin and PI positive protoplasts was observed at143 µM (PCN), while maximum apoptosis was seen at 71.7 µM (PCN). Small increase in TUNEL positive protoplasts was observed (~5%). Increased chromatin condensation and ROS production was observed at 71.7 µM concentration (Table 4.14 and annexure II-Fig. 17). Correlation coefficient value (*r*) of annexin, DAPI and DHR123 with caspase 1 was >0.73 (p = <0.006) at 71.7 µM concentration of PCN.

4.4.3 DISCUSSION

Wine spoilage yeasts cause economic losses and are difficult to control. Apoptotic inducers may contribute to the control of such spoilage organisms due to their fungicidal effect (Madeo *et al*., 1999). Weak acids and garlic extracts are widely used as preservatives to prevent the growth of microorganisms on foodstuffs and are also used in the treatment of *C. albicans* and other fungal infections (Fleet, 1992; Jain and Agarwal, 1994; Lemar *et al*., 2002). Many cytotoxic substances can induce apoptosis or necrotic cell death in yeast, depending on their concentration *viz.* acetic acid, mutations, pheromone, sugar etc. (Ligr *et al*., 1998; Ludovico *et al*., 2001; Severin and Hyman, 2002; Madeo *et al*., 2004; Ludovico *et al*., 2005). Phenazine was reported to function as

an antibiotic and inducer of apoptosis by enhancing production of oxygen radical (Baron and Rowe, 1981; Dwivedi and Johri, 2003).

In this study, we showed that low concentration of PCN inhibited ODC activity and induced cell death in spoilage yeasts *D. bruxellensis*, *M. pulcherrima*, *Z. rouxii*, *C. krusei*, *D. hansenii* and *P. anamola* exhibiting markers of apoptosis like phosphatidylserine externalization, DNA fragmentation, chromatin condensation and ROS production as well as the dependency on caspase like activity.

ODC is a rate limiting enzyme in the regulation of the levels of polyamines within the living cells. Different morphological forms of fungi exhibited different levels of polyamines (Calvo-Mendez *et al*., 1987). Our results showed inhibition of ODC activity in spoilage yeasts involved in wine fermentation (Table 4.13). These polyamines uptake/transport systems are used in cancer therapy can interfere or block key cell proliferation events (Wallace *et al*., 2003). Alteration of these polyamines caused induction of apoptosis by caspase activation (Obaya *et al*., 1999; Faaland *et al*., 2000; Mitchell *et al*., 2000; Erez *et al*., 2002; Seiler and Raul, 2005).

High percentage of annexin V-FITC and low PI staining identified the apoptotic concentration of PCN. At these PCN concentrations, high TUNEL, DAPI and DHR123 staining was also observed (Table 4.14). The induction of apoptosis at low concentration and necrosis at high concentrations by cytotoxic substances was a well known phenomenon and was reported in *C. albicans*, *S. cerevisiae* and *Z. bailii* treated with acetic acid and H_2O_2 (Madeo *et al*., 1999; Ludovico *et al*., 2001, 2003; Phillips *et al*., 2003). Different from the mode of action of acetic acid and H_2O_2, the first step that PCN takes in triggering apoptosis was by inhibiting differentiation and their biochemical enzyme correlates *viz.* ODC. This resulted in the rapid depletion of intracellular polyamine levels leading to the burst of ROS and sequential cell death.

Representative strains of spoilage yeasts *D. bruxellensis*, *M. pulcherrima*, *Z. rouxii*, *C. krusei*, *D. hansenii* and *P. anamola* showed hallmarks of apoptosis induced by PCN. Nuclear chromatin condensation precedes both phosphatidylserine exposures and endonuclease DNA cleavage in the test organism used. Similar observations were reported in apoptosis in immature thymocytes (Sun *et al*., 1994; Darzynkiewicz *et al*., 1997). The apoptotic events taking place in *Z. rouxii* and *D. hansenii* like DNA fragmentation (Table 4.14), were similar to those reported in ageing carbon-depleted cultures of *A. fumigatus*, *A. nidulans* and *S. cerevisiae* (Mousavi and Robson, 2003; Herker *et al*., 2004; Emri *et al*., 2005).

Results with PCN showed accumulation of ROS in all the test strains used, whereas higher staining of DHR 123 was found near to necrotic concentration and this may be attributed to the loss of mitochondrial potential (Table 4.14). Similar involvement of intracellular accumulation of ROS were also reported to be involved in the initialization of apoptosis in fungi (Madeo *et al*., 1999; Karaffa *et al*., 2001; Laun *et al*., 2001; Cheng *et al*., 2003; Phillips *et al*., 2003; Emri *et al*., 2004; Mousavi and Robson, 2004; Leiter *et al*., 2005).

Caspase could be activated directly or indirectly by ROS and in turn triggered apoptosis (Madeo *et al*., 2004). Caspase like activities (400-800 fold) were reported in lysates of saturated cultures and sporulating cultures of *A*. *fumigatus* and *A*. *nidulans* suggesting direct role of the metacaspase in the process (Mousavi and Robson, 2003; Thrane *et al*., 2004; Richie *et al*., 2007). In our study, PCN induced apoptosis in *B*. *poitrasii* was found to be caspase dependent. We have examined the contribution of metacaspase to the growth and stress response of the food spoilage yeasts, based on increasing evidence of the role of metacaspase in apoptosis. Our results showed that PCN induced death of spoilage yeasts was caspase dependent. Caspase like activities were found to increase with increase in concentration of PCN.

From the present investigations, it can be suggested that PCN inhibited ODC activity and induced apoptosis in spoilage yeasts that may be useful for control of food spoilage yeasts. This study could lead us towards the better understanding and correlation of fungal morphogenesis and apoptosis. Studies suggested that differences in architecture of the death-regulating machineries of mammals and fungi exist, making the chances of finding novel targets for antifungal therapy (Richie *et al*., 2007).

CHAPTER V

CONCLUSIONS

Programmed cell death (PCD) or apoptosis is a regulated intracellular process that functions during normal cellular development, in response to stress and as defence against pathogen attack in plants and animals (Biella *et al*., 2002). Fungi are known causal agents of food spoilage and their control is important to reduce economic losses. Wine spoilage yeasts such as *Dekkera/Brettanomyces* and others are usually controlled by adequate cellar hygiene, sulfitation, and ageing at low temperatures. Spoilage fungi such as *Issatchenkia orientalis*, *I. terricola* and *P. membranifaciens* showed Y- H transition. Several antifungal agents are known to affect Y-H transition and induce apoptosis. Antifungal agents *viz* acetic acid, amphotericin B, farnesol, viscosinamide etc. are known to induce apoptosis in *C. albicans*, *R. solani*, *S. cerevisiae*, *Z. bailii*, (Madeo *et al*., 2002; Phillips *et al*., 2003; Semighini *et al*., 2006a).

B. poitrasii, a dimorphic zygomycete is a model to understand dimorphism. In *B. poitrasii* it was already reported that different enzymes involved in ammonia assimilation and chitin synthesis such as NAD- and NADP- dependent glutamate dehydrogenases (GDHs), chitin synthase, chitinase, *N*-acetylglucosaminidase and polyamine synthesizing enzyme ODC were biochemically correlated with Y-H transition (Khale *et al*., 1992; Ghormade *et al*., 2000; Chitnis *et al*., 2002; Ghormade *et al*., 2005a). Among these, the relative proportion of NADP-/NAD- dependent GDH regulated by phosphorylation/ dephosphorylation was found to have a significant correlation with Y-H transition (Khale and Deshpande, 1993; Khale *et al*., 1992). Furthermore, *B. poitrasii* possessed three GDHs, one requiring NAD while the other two were form specific which use NADP as a cofactor (Amin *et al.*, 2004a). Morphological shift from Y-H form was accompanied with the change in GDH and ODC activities. The NAD-GDH and ODC enzyme levels were lower in the *B. poitrasii* yeast form as compared to the hyphal form cells whereas the NADP-GDH enzyme levels were higher in yeast form cells (Khale and Deshpande, 1993; Ghormade *et al*., 2005a).

In the present investigation the apoptotic inducers were identified on the basis of their potential to either decrease Y-H transition or increase H-Y transition in *B. poitrasii* *via* their biochemical correlates, GDH and ODC. The additives assessed were commercially available compounds such as diaminobutanone (DAB, ODC inhibitor), cyclohexylamine (CHA, spermidine synthase inhibitor), isophthalic acid (NAD-GDH inhibitor), amphotericin B (ergosterol synthesis inhibitor), fluconazole (14α-demethylase inhibitor); naturally isolated compound like phenazine-1-carboxamide (PCN, isolated and purified from *Pseudomonas* sp. B18) and chemically synthesized compounds like

triazole linked β-lactam–bile acid conjugates (18, 20 and 24 B) and tetrapeptide linked-cholic acid derivative (DS16), strobilurin derivative (PC229). Traditionally used apoptosis inducers acetic acid and H_2O_2 were also tested. The possible correlation of differentiation and apoptosis in *B. poitrasii* was studied.

During Y-H transition NAD-GDH activity increased by 11 fold whereas NADP-GDH activity decreased by 6 fold, while *vice a versa* was observed during H-Y transition. In case of ODC, the activity increased by 4.5 fold. NADP-GDH ($r = -0.862$, $p = 0.0001$) and NAD-GDH ($r = 0.862$, $p = 0.0001$) activities were correlated significantly with ODC activity. Y-H transition in *B. poitrasii* was associated with increase in polyamine levels (>1.5 fold, putrescine and spermidine and 4 fold, spermine, higher in H cells than Y form cells). Similar increase in polyamine levels during Y-H transition was also reported earlier in case of other fungi such as *C. ulmi*, *Fusarium moniliforme*, *M rouxii*, *Y. lipolytica* (Marshall *et al*., 1979; Calvo-Mendez *et al*., 1987; Guevara-Olvera *et al*., 1993).

The additives analyzed in this study inhibited Y-H transition (80-94%). At their inhibitory concentration the *in vitro* NAD-GDH activity decreased in case of isophthalic acid (16 mM), and acetic acid (20 mM), while NADP-GDH activity was unaltered. In presence of H_2O_2 (50 mM) and amphotericin B (4 µM) NADP-GDH activity was induced in Y form of *B. poitrasii*, while there was no change in NAD-GDH activity. Above observations were evident with increase in NADP-/NAD-GDH ration (≥0.41). Chemically synthesized additives 18-24B inhibited Y-H transition by 16-95% at their respective concentration, while DS16 inhibited germ tube formation by 51.6-95%. In case of microbial extracts *Bacillus* culture inhibited Y-H transition by 24-68% while *Pseudomonas* sp. inhibited by 36%. The maximum germ tube inhibition was observed for *Bacillus* sp B15 (68.2%). Yeast form NAD-GDH and NADP-GDH were inhibited by *Bacillus* sp. B1 (42.9%) and B21 (61.5%) respectively.

ODC activities were inhibited with increase in concentration of additives. Maximum inhibition (≥85%) was observed in presence of DAB (12 mM), CHA (3 mM), 18B (51 µM), 20B (26 µM), 24B (39 µM) and DS16 (87 µM). In presence of microbial culture filtrates ODC activities were inhibited from 14.7 to 87.9%, whereas B14 showed no inhibition. *Bacillus* B1, B15 and *Pseudomonas* B18 showed 87.9, 85.7 and 93.5% inhibition of ODC activity respectively.

All the above additives which affected Y-H transition and biochemical correlates (GDH and ODC) were found to induce apoptosis with characteristics of apoptotic

phenotype. Apoptotic concentration were determined by maximum annexin V-FITC, TUNEL, DAPI and DHR123 positive protoplast for isophthalic acid (16 mM), acetic acid (20 mM), H_2O_2 (50 mM), amphotericin B (2 µM), DAB (15 mM), CHA (4 mM), 18B (110 µM), 20B (105 µM), 24B (92 µM) and DS16 (115 µM). While at the higher concentration (more than apoptotic concentration) increase in number of necrotic cells was observed with high number of PI stained cells. SOD and caspase-like activities also increased with increasing additives concentration. Maximum activity was observed at apoptotic phase, whereas decrease in activity was seen at necrotic phase. Chronologically aged cells of *B. poitrasii* showed increase in apoptotic phenotype as they approached the stationary phase, with increase in SOD and caspase-like activity. Similar trend was observed during sporulation in *B. poitrasii*. These results showed that differentiation or Y-H transition may precede apoptosis and can be used as a checkpoint to study linkage between morphogenesis and cell death.

Isolation and characterization of ODC and metacaspase genes of *B. poitrasii* was carried out. The sequence analysis of *BpODC* gene revealed the presence of pyridoxal phosphate, substrate binding site and catalytic active site. In case of metacaspase sequence p20, p10 domain and catalytic active site (cysteine-histidine diad) was observed. The study of cause-effect relationship by analyzing the expression of *BpODC* and metacaspase genes in *B. poitrasii* wild type and disruptant strains will further clarify the role of these genes in the differentiation and apoptosis. Guevara-Olvera *et al*. (1997) and Jimenez-Bermont *et al*. (2001) reported the role of ODC in dimorphism by showing that ODC null mutant in *U. maydis* and *Y. lipolytica* failed to show Y-H transition and differentiation occurred when polyamine was supplemented exogenously.

Madeo *et al*. (2004) demonstrated that metacaspase Yca1p was involved in apoptosis in *S. cerevisiae*. Furthermore *A. fumigatus* metacaspase-deficient mutants showed no sensitivity towards the apoptotic stimuli reported to initiate apoptosis (Richie *et al*., 2007).

ODC regulated the levels of polyamines (putrescine, spermidine and spermine) in the cell (Ruiz- Herrera and Martinez-Espinoza, 1998). These polyamines are regulators of cellular functions, promoting proliferation or cell death depending on the cell type and on environmental signals. Recently these polyamines were implicated in cell death because depletion of polyamines is often lethal and results in cell cycle arrest or apoptosis (Schipper *et al*., 2000, 2003). Such alterations, especially in spermidine and spermine, were shown to be a common feature in apoptosis (Schipper *et al*., 2000, 2003).

In *B. poitrasii* yeast cells polyamine levels decreased on treatment with different additives (DAB, CHA and PCN). Number of evidences indicates that polyamines, cell cycle events and apoptosis are closely connected (Schipper *et al*., 2000; Bachrach *et al*., 2001; Nitta *et al*., 2002; Pignattin *et al*., 2004). In fungi a number of biochemical correlates of differentiation and apoptosis like ODC are common and there is a possibility of a link between differentiation and apoptosis (Fig. 5.1). If so, then understanding of differentiation, *per se* could be useful to design apoptosis inducers for the control of fungal pathogenesis.

Differentiation in fungi is also regulated by biosignalling pathways. In *C. albicans* and *S. cerevisiae* it was shown that the Ras and MAPK signalling networks were shared by the differentiation and apoptotic pathways (Gourlay *et al*., 2006; Phillips *et al*., 2006). The *Ras* deletion mutants of *C. albicans* and *S. cerevisiae* failed to show apoptosis in presence of apoptotic stimuli. Chou *et al*. (2004) showed that down-regulation of PKC levels induced apoptosis *via* caspase activation in cells by antisense treatment or expression of dominant-negative constructs. Protein kinases (PKCs), family of phospholipid-dependent serine/threonine kinases that have important role in signal transduction pathways (Ras and MAPK) are involved in growth, differentiation and cell death (Chou *et al*., 2004). In budding yeast MAPK cascade responsible for cell integrity mediates cell cycle regulation and cell wall synthesis also showed responding to signals like pheromone and external osmolarity. MAPK related sequences were also observed to correlate to hyphal formation in *C. albicans* (Felipe *et al.*, 2005). In several pathogenic and nonpathogenic fungi, Ras was involved in filamentation, pseudohyphal/ hyphal growth and mating. Ras-related transcript was identified in *P. brasiliensis*, but further studies are required to elucidate its function in Y-H transition and in the mechanism of pathogenicity (Felipe *et al.*, 2005). The cAMP/protein kinase A cascade regulated the fungal development and virulence. In *P. brasiliensis* cAMP addition led to inhibit filamentation. In dimorphic fungi, cAMP- and calcium-calmodulin-calcineurin dependent pathways seem to be involved in differentiation (Felipe *et al.*, 2005).

In *B. poitrasii*, cAMP pathway was reported to regulate GDHs. Previously, it was suggested that GDH activities in *B. poitrasii* were regulated by phosphorylation/ dephosphorylation in a Ca-CaM and, or cAMP dependent processes that affected the Y-H transition directly or indirectly (Khale and Deshpande, 1993; Ghormade *et al*., 2005a) (Fig. 5.1). Polyamines, essential for the normal cell growth, are reported to control GDH activity in mammalian tissue (Jarzyna *et al*., 1994). Adenylate cyclase, responsible for

synthesis of cAMP was inhibited by polyamines thereby affecting the maintenance of specific morphology (Khan *et al*., 1990). In *B. poitrasii*, it was observed that putrescine and spermidine inhibited the yeast form specific NADP-GDH activity (Ghormade *et al*., 2005a) (Fig. 5.1). The inhibitory action of polyamines was showed to reverse by the addition of NADPH, suggesting that the enzyme has common binding site for both. Insel and Fenno (1978) reported decrease in ODC activity in lymphoma cells when incubated with cAMP which was correlated with cAMP-dependent protein kinase activity and arrest of cell growth at G1phase.

The studies mentioned in chapter III and IV indicated that in *B. poitrasii* decreased GDHs and ODC activities were shown to be involved in the Y-H differentiation as well as in the apoptosis. On the basis of present investigations a model is proposed for the possible linkage of differentiation and apoptosis in *B. poitrasii* (Fig. 5.1). Alterations of polyamines led to DNA fragmentation, chromatin condensation and generation of reactive oxygen species (ROS) associated with apoptotic death (Fig. 5.1). In ageing yeast, spermidine treatment triggered deacetylation of histone H3 through inhibition of histone acetyltransferases (HAT), suppressing oxidative stress and apoptosis (Eisenberg *et al*., 2009). These observations suggested linkage of glutamate dehydrogenase and ODC activity with apoptosis. Differentiation can also be used as a target for identification of antifungal compounds that cause apoptosis.

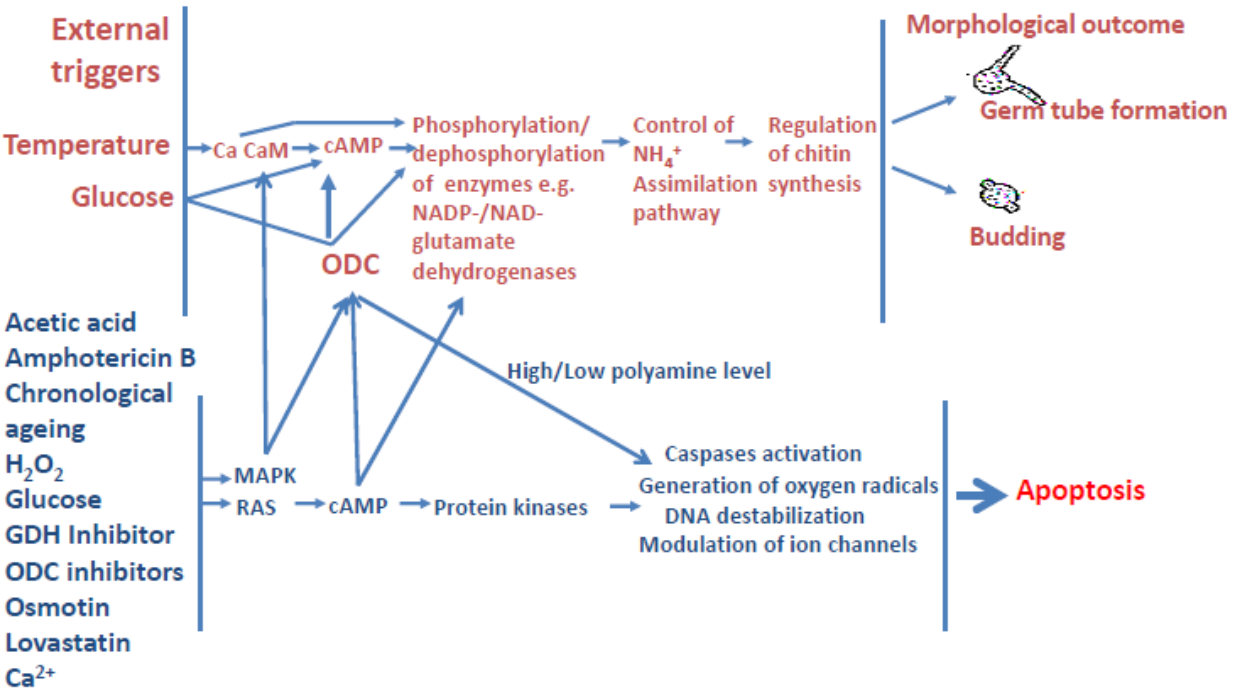


Fig. 5.1 Possible correlation of differentiation with apoptosis

A promising antifungal metabolite from *Pseudomonas* sp. B-18 was isolated. Antifungal activity in active fraction (D) was identified using plate assay against pathogens *F. oxysporum*, *D. oryzae*, *M. grisea* and saphrophyte *B. poitrasii*. The purified compound was isolated as pale yellow needles in methanol, which showed formula weight of 223 using ESI-MS spectrum with molecular formula $C_{13}H_9N_3O$ (phenazine-1-carboxamide, PCN). Amide nature of compound was detected by FT-IR. Purified PCN was studied for its effect on Y-H transition inhibition (22-224 μM). PCN led to inhibition of germ tube formation from 62.6-82.3% with increasing concentration (22-67 μM) as compared to control in *B. poitrasii*. PCN also inhibited >90% of ODC activity and increase in apoptotic cells was observed from 22-89 μM in *B. poitrasii*.

Further, PCN induced apoptosis in yeasts involved in wine fermentation such as *D. bruxellensis*, *M. pulcherrima*, *Z. rouxii*, *C. krusei*, *D. hansenii* and *P. anamola* with 71.7-224 μM (MIC, ≥143 μM). PCN also inhibited ODC activities in these wine spoilage yeasts at the above mentioned concentrations. Inhibition of ODC and induction of apoptosis in *B. poitrasii* and various wine yeasts demonstrated connection between the commitment of yeasts cells to undergo morphogenesis and to die. The agents identified using differentiation as target that caused apoptosis can be used for the control of wine spoilage yeasts.

During the course of the work, it was found that Y-H transition and the biochemical correlates of *B. poitrasii* served to screen antifungal agents/inducers of apoptosis. Studies using effectors of morphogenesis showed that Y-H transition and its biochemical correlates GDH and ODC could serve as a check point to study apoptosis. PCN, 18B, 20B and 24B were identified as potential ODC inhibitors and apoptotic inducers, which can be used as potential agent for control of food spoilage yeasts. The isolation of ODC and metacaspase gene in the present investigation will be helpful to investigate its possible role in differentiation and apoptosis. Purification and isolation of PCN was done from *Pseudomonas* sp. B18 because of its ability to inhibit morphogenesis and to induce apoptosis. PCN was also found to inhibit ODC activity and induced apoptosis in wine yeasts making it useful to control wine spoilage yeasts.

ANNEXURE I

1. Extraction, purification and identification of phenazine-1-carboxamide from *Pseudomonas* sp. B-18

1.1 Isolation of the hydrophobic antifungal compound produced by strain B-18

Pseudomonas strain B-18 is a fluorescent pseudomonad. Colonies showed green-blue pigment after prolonged incubation (96h). For the isolation of antifungal compound, *Pseudomonas* sp. was incubated in medium mentioned in materials and methods (section 2.2.5) for 96h. Maximum level of antifungal metabolite was observed at 4 d. These results are consistent with a report of antifungal antibiotics viscosinamide and furanones (Paulitz *et al*., 2000). Broth of strain B-18 inhibited growth of saphrophyte *B. poitrasii* and plant pathogenic fungi *F. oxysporum*, *M. grisea* and *D. oryzae* (Table 1). The potential antifungal compound was found concentrated in acetone extracts of cells. The acetone extract of cell biomass was fractionated using methanol and the residue was purified using silica gel chromatography (200-300 mesh) with methanol: chloroform (5:95) mixture for elution. Antifungal activity in fraction D was tested using plate assay against *B. poitrasii* and *F. oxysporum,* growth inhibition was observed at concentration (50 µg) (Table 1).

Table. 1 *In vitro* antifungal activity detection by using disc diffusion method

Fraction (50 µg)	Zone of inhibition (mm)			
	B. poitrasii	*M. grisea*	*D. oryzae*	*F. oxysporum*
Control	[a] -	-	-	-
Crude extract	32	20	25	20
A	2	[b]NT	NT	2
B	2	NT	NT	2
C	2	NT	NT	-
D	4	NT	NT	6
E	-	-	-	-
F	-	-	-	-
PCN	30	30	20	25

Negative control, DMSO (50 % v/v); [a] No inhibition, [b] Not tested; A, acetone extract; B, ethyl acetate extract; C, Residue of broth ; D, chloroform filtrate; E, ethyl acetate soluble of cell biomass and F , residue .

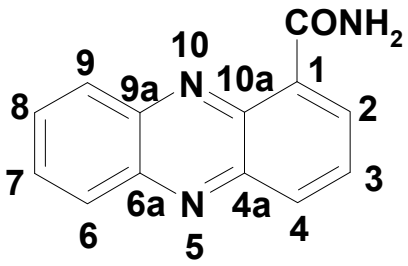
1.2 Physico-chemical characterization of phenazine-1-carboxamide (PCN)

The chemical structure of the antifungal was confirmed by comparison of its physicochemical and spectral properties with those of standard PCN (Chin-A-Woeng *et al*., 1998). Active fraction D, 0.45g was fractionated by column chromatography using silica gel (200-300 mesh) with elution gradient of 2 to 10% methanol in chloroform. Total 10 fractions were collected, fractions 2-4 were pooled and further purified by preparative TLC to get purified compound. Further, purified compound was crystallized

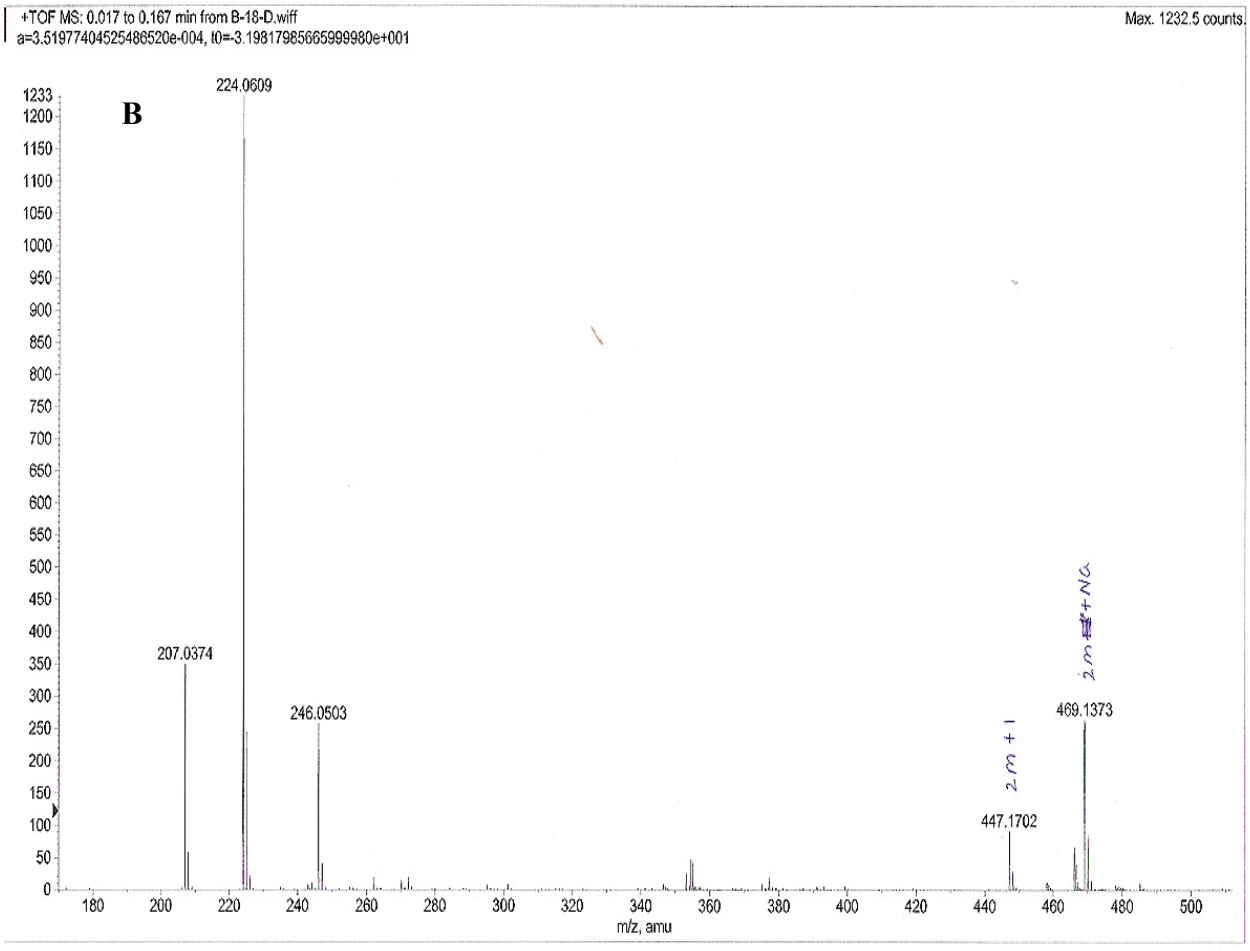
as pale yellow needles in methanol and was characterized by IR, ^{1}H-NMR,^{13}C NMR, ESI-MS.

ESI-MS spectrum showed $[M+H]^{+}$ peak at 224 and $[M+Na]^{+}$ peak at 246, indicating the molecular weight of the compound 223 corresponding to molecular formula $C_{13}H_9N_3O$. Peak at *m/z* 207 was due to loss of NH_3 suggested a fragmentation typical of amide containing compound (Fig. 1 B). This was confirmed by absorption peaks in IR spectrum, ^{1}H-NMR (Fig. 1 C and Table 2 A). The melting point of the purified compound was found to be 245 °C which corresponds to the melting point of PCN 246 °C (Chin-A-Woeng *et al*., 1998) (Fig. 4.1 A).This data along with ^{13}C NMR spectrum indicated purified compound being PCN (Fig. 1 D and Table 2 B).

A



Phenazine-1-carboxamide (PCN)
($C_{13}H_9N_3O$; M^{+} 224)



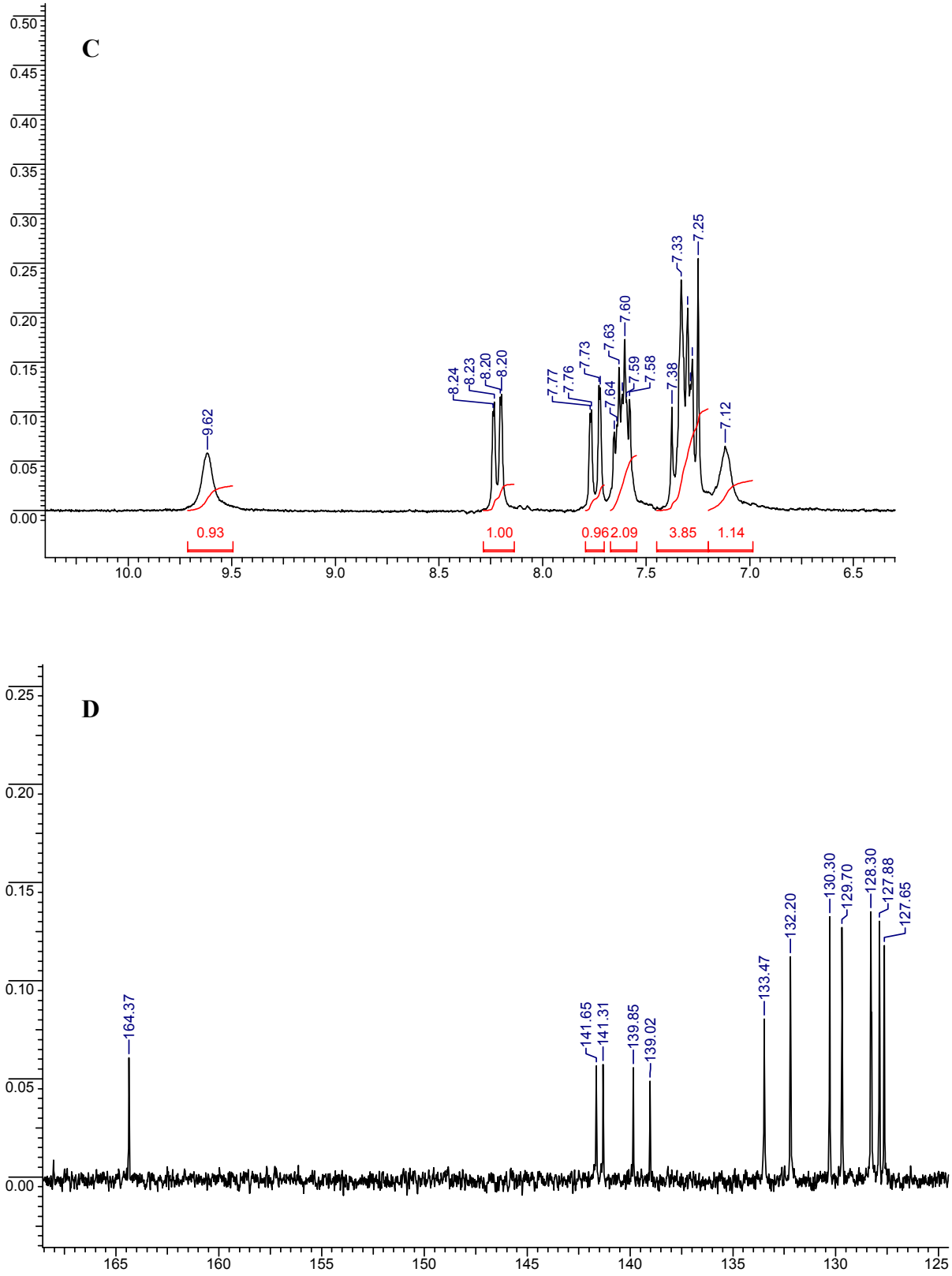


Fig. 1 Spectroscopic studies of phenazine-1-carboxamide (PCN). (A) Structure of PCN as deduced from (B) EI mass spectrum (C) ^{1}H-NMR (D) ^{13}C-NMR signals

Table. 2 Nuclear magnetic resonance (NMR) signals from phenazine-1-carboxamide (A) ^{1}H-NMR (B) ^{13}C-NMR

A.

Proton	Chemical shift δ (ppm)	Splitting pattern [coupling constants (*Hz*)]
NH2	10.72	bs
	6.32	bs
H-2	8.54	dd (8.7; 1.3)
H-3	8.015	dd (8.7; 7.0)
H-4	8.97	dd (7.0; 1.3)
H-6	8.29	ddd (8.0; 1.5; 0.5)
H-7	7.97	ddd (8.0; 7.5; 1.3)
H-8	8.04	ddd (7.5; 7.5; 1.5)
H-9	8.34	ddd (7.5; 1.3; 0.5)

B.

Carbon	Chemical shift δ (ppm)
CONH2	166.54
C-1	128.93
C-2	134.35
C-3	129.12
C-4	136.00
C-4a	141.55a
C-5a	143.56a
C-6	131.75b
C-7	129.81c
C-8	129.90c
C-9	131.07b
C-9a	143.20a
C-10a	140.85a

ANNEXURE II

A. Microscopic observations of apoptotic *B. poitrasii* yeast cells

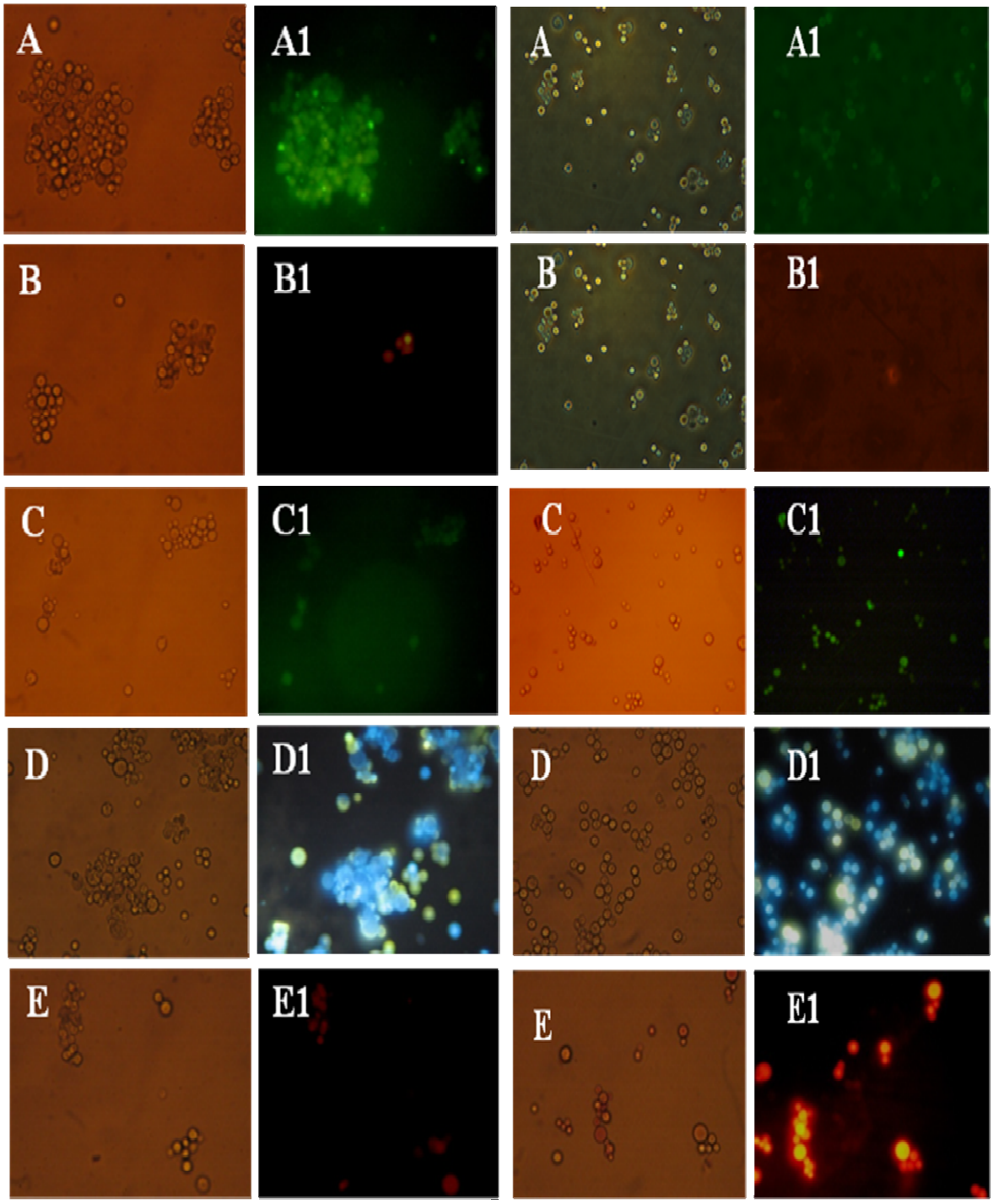


Fig. 1. Isophthalic acid induced apoptosis in *B. poitrasii.* (A1-B1) Annexin V-PI staining. (C1) TUNEL reaction. (D1) DAPI staining. (E1) DHR 123 staining of *B. poitrasii* with 16 mM isophthalic acid. (A-E) Bright field image of respective test.

Fig. 2. Acetic acid induced *B. poitrasii* yeast cell death. (A1-B1) Annexin V-PI staining. (C1) TUNEL reaction. (D1) DAPI staining. (E1) DHR 123 staining of *B. poitrasii* with 20 mM acetic acid. (A-E) Bright field image of respective test.

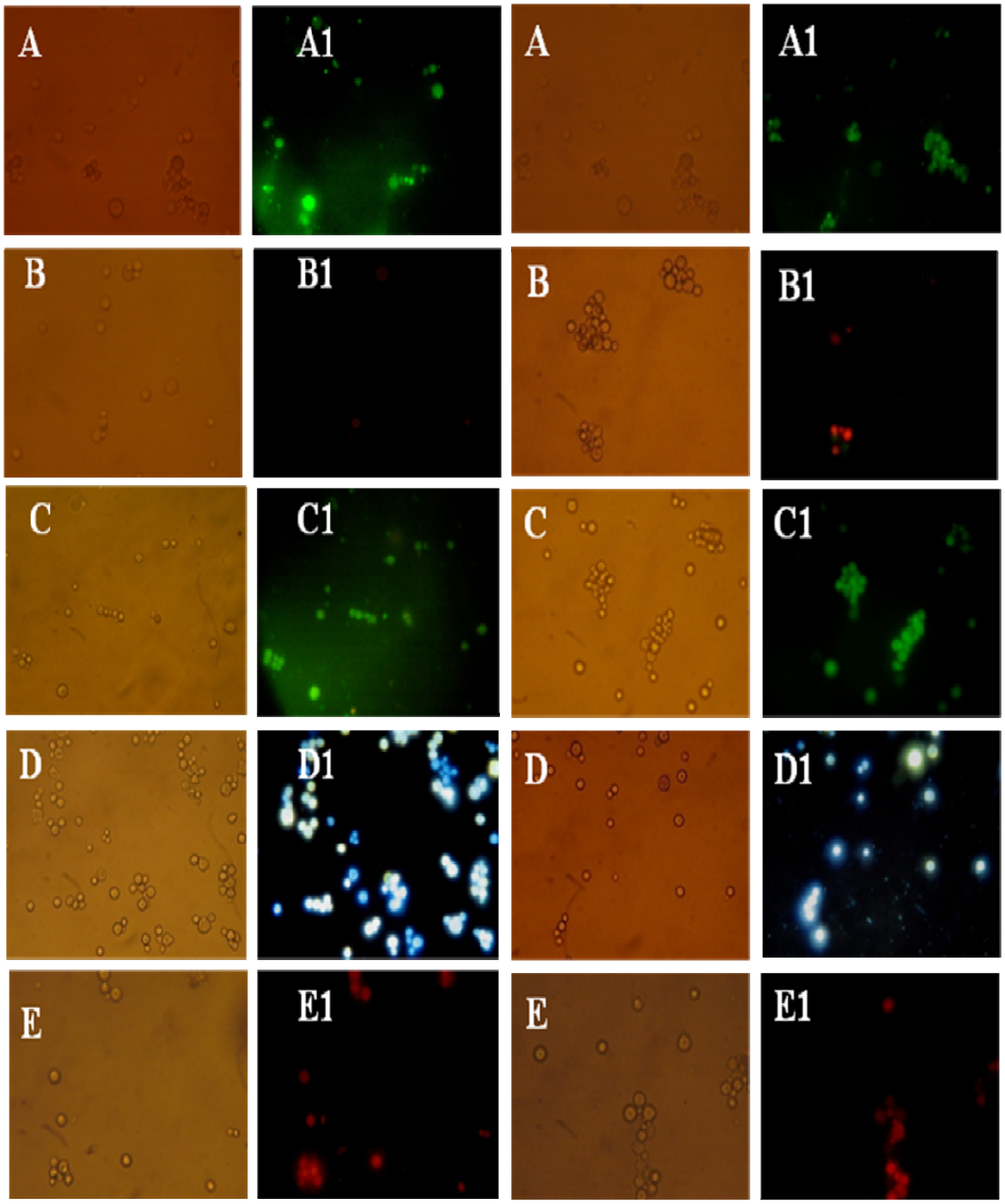


Fig. 3. H_2O_2 induced apoptosis in *B. poitrasii*. (A1 and B1) Annexin (+) and PI (-). (C1) TUNEL (+). (D1) Chromatin condensation by DAPI. (E1) ROS production by DHR123. (A-E) Bright field image showing non stained cells.

Fig. 4. Amphotericin B induced apoptosis in *B. poitrasii* yeast cells. (A1 and B1) Annexin (+) and PI (+). (C1) Damaged DNA (revealed by TUNEL assay). (E1) Chromatin condensation (revealed by DAPI staining). (E1) ROS (revealed by DHR staining) after treatment with 2 µM AMB. (A-E) Bright field image showing non stained cells.

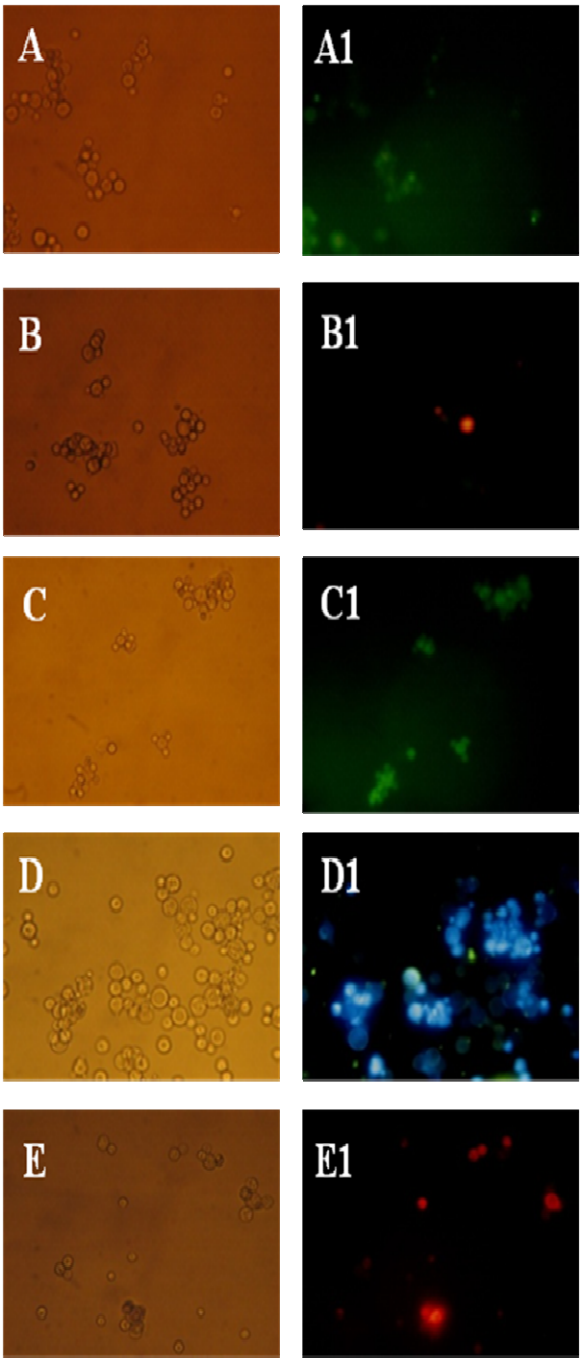


Fig. 5. DAB induced apoptosis in *B. poitrasii* yeast cells. (A1-B1) Annexin V-PI staining. (C1) TUNEL reaction. (D1) DAPI staining. (E1) DHR 123 staining of *B. poitrasii* with 15 mM DAB. (A-E) Bright field image of respective test.

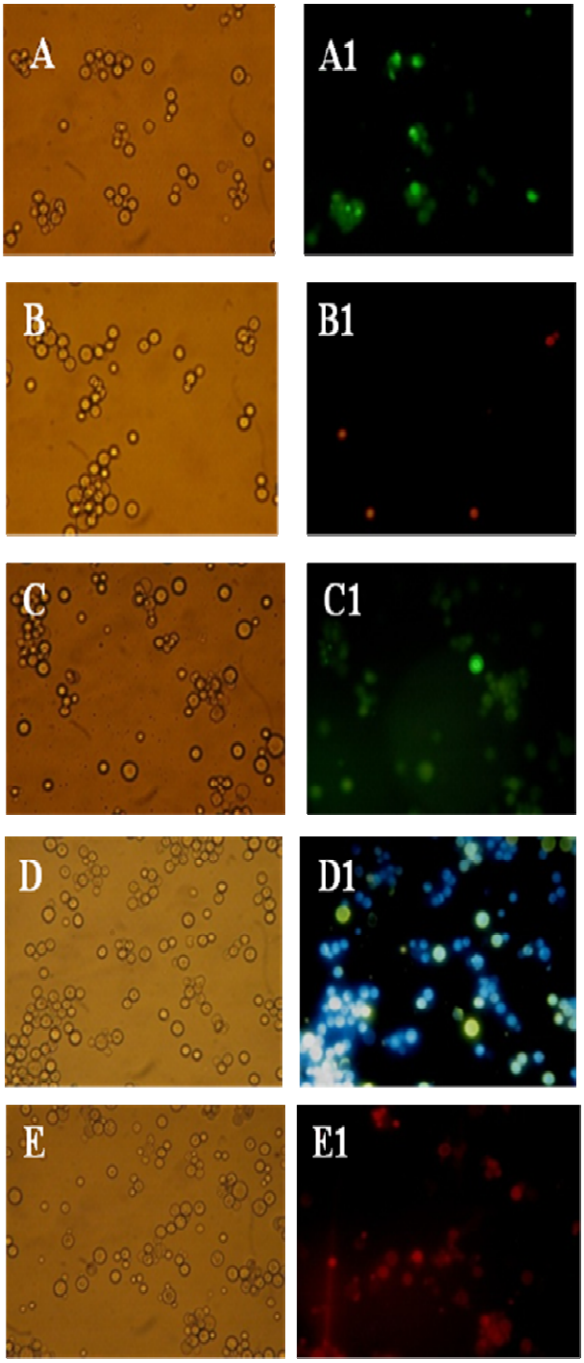


Fig. 6. CHA induced apoptosis in *B. poitrasii* yeast cells. (A1-B1) Annexin V-PI staining. (C1) TUNEL reaction. (D1) DAPI staining. (E1) DHR 123 staining of *B. poitrasii* with 4 mM CHA. (A-E) Bright field image of respective test.

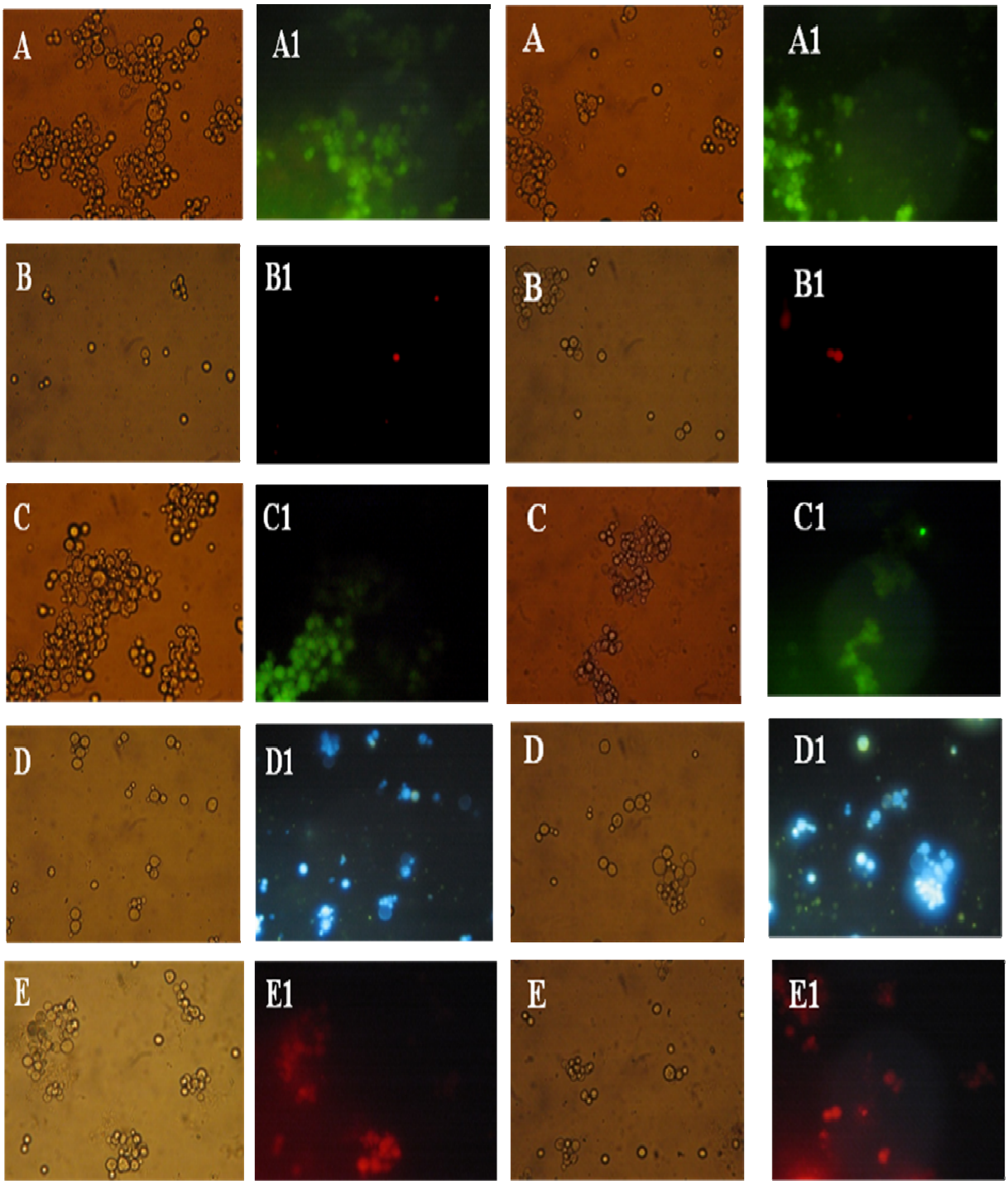


Fig. 7. 18B induced apoptosis in *B. poitrasii* yeast cells. (A1-B1) Annexin V-PI staining. (C1) TUNEL. (D1) DAPI staining. (E1) DHR 123 staining of *B. poitrasii* yeast cells exposed to 110 µM 18B. (A-E) Bright field image of respective test.

Fig. 8. 20B induced apoptosis in *B. poitrasii* yeast cells. (A1-B1) Annexin V-PI staining. (C1) TUNEL reaction. (D1) DAPI staining. (E1) DHR 123 staining of *B. poitrasii* with 105 µM 20B. (A-E) Bright field image of respective test.

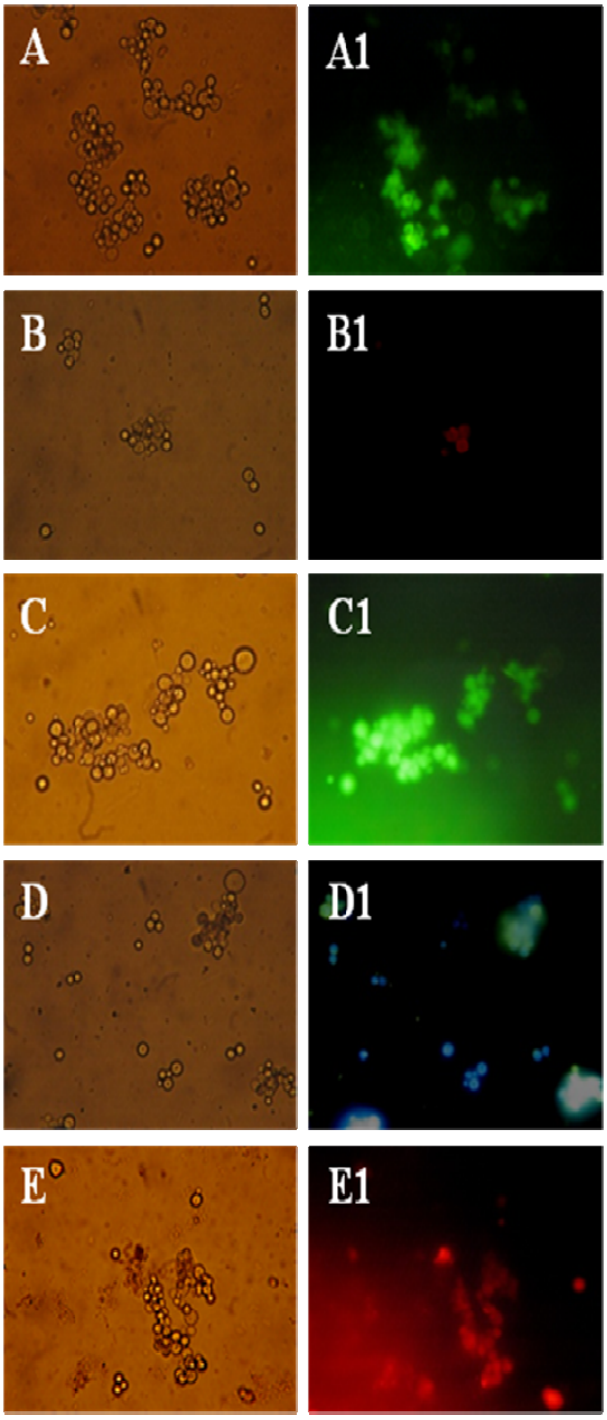


Fig. 9. 24B induced apoptosis in *B. poitrasii* yeast cells. (A1-B1) Annexin V-PI staining. (C1) TUNEL reaction. (D1) DAPI staining of *B. poitrasii* with 92 µM 24B. (A-E) Bright field image of respective test. (E1) DHR 123 staining of *B. poitrasii*. (A-E) Bright field image of respective test.

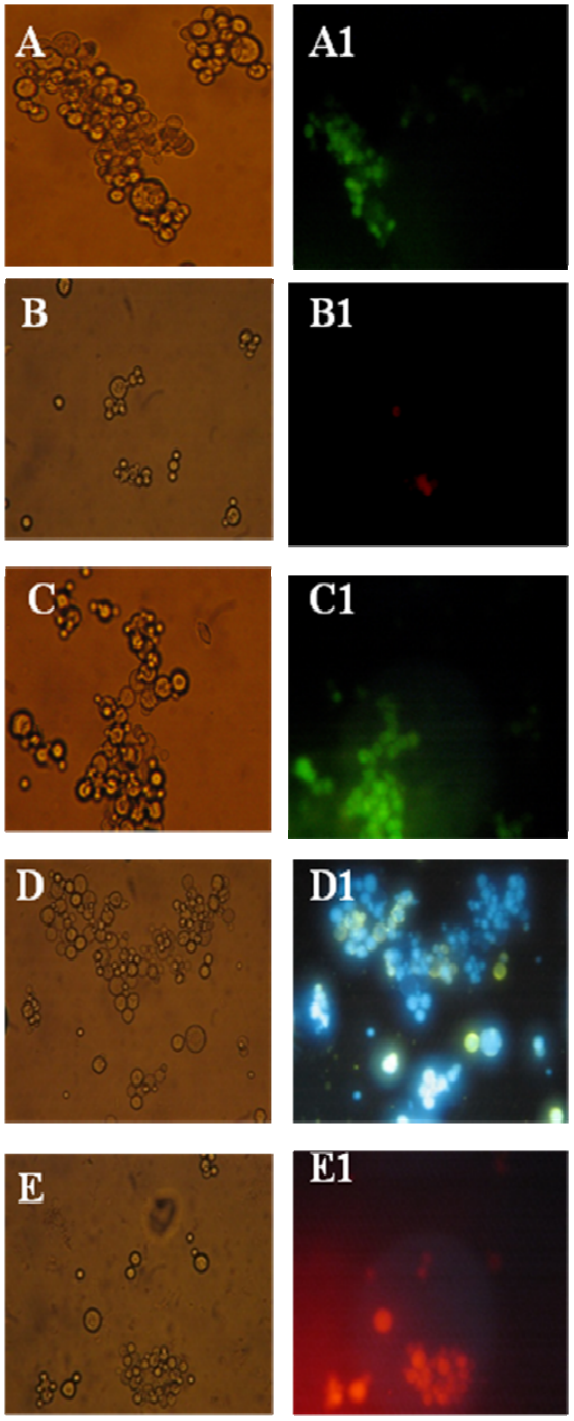


Fig. 10. DS16 induced apoptosis in *B. poitrasii* yeast cells. (A1-B1) Annexin V-PI staining. (C1) TUNEL (D1) DAPI staining (E1) DHR 123 of *B. poitrasii* with 115 µM DS16. (A-E) Bright field image of respective test.

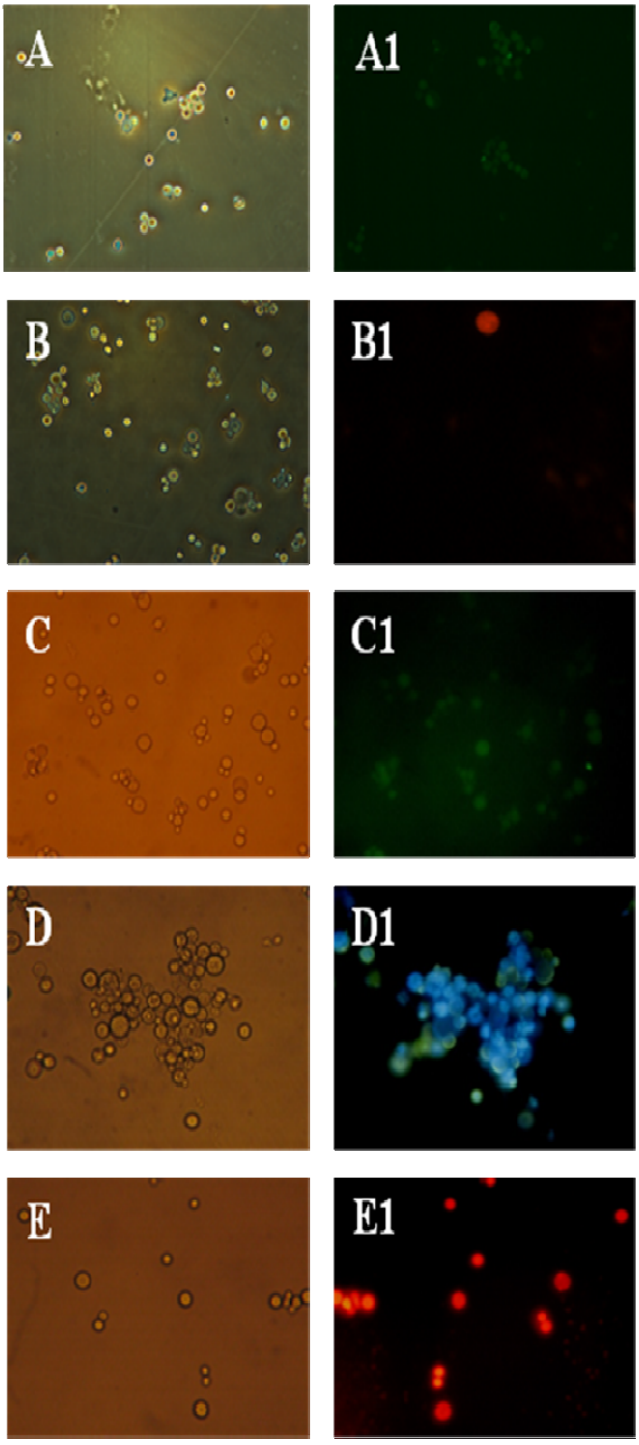


Fig. 11. PCN induced apoptosis in *B. poitrasii* yeast cells. (A1-B1) Annexin V-PI staining. (C1) TUNEL reaction. (D1) DAPI staining. (E1) DHR 123 staining of *B. poitrasii* treated with 89 µM PCN. (A-E) Bright field image of respective test.

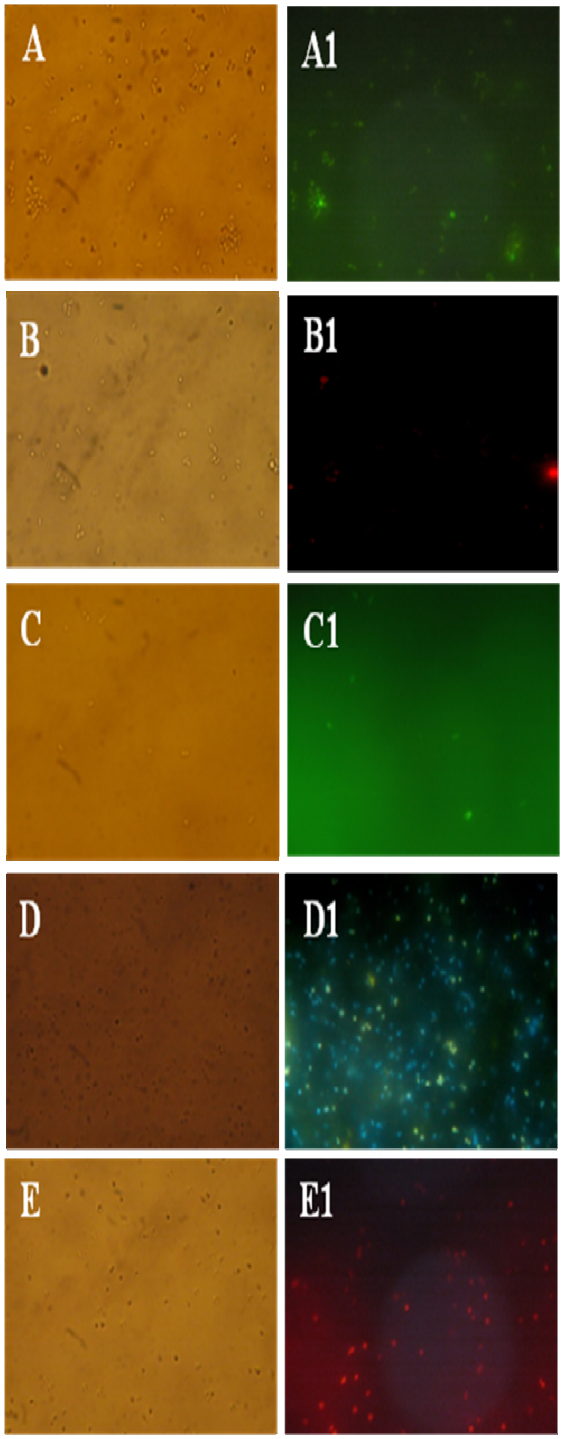


Fig. 12. PCN induced apoptosis in *D. bruxellensis.* (A1-B1) Annexin V-PI staining. (C1) TUNEL reaction. (D1) DAPI staining. (E1) DHR 123 of *D. bruxellensis* with 89.6 µM PCN. (A-E) Bright field image of respective test.

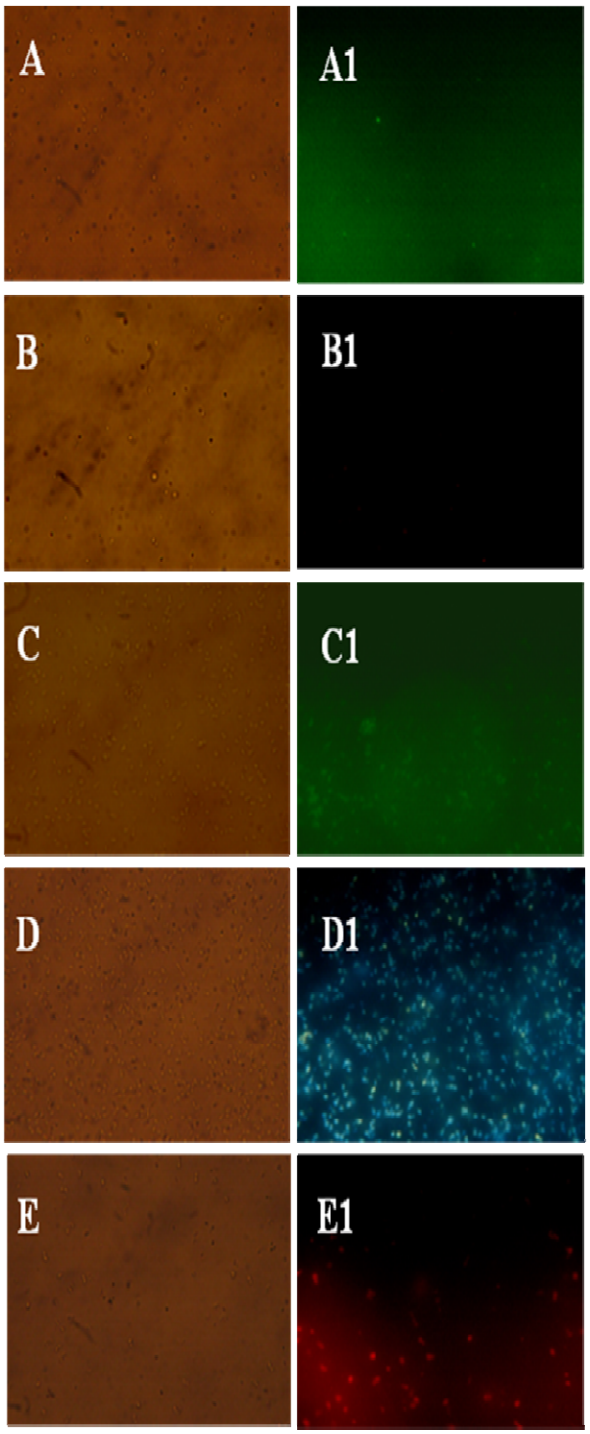


Fig.13. PCN induced apoptosis in *M. pulcherrima*. (A1-B1) AnnexinV-PI staining. (C1) TUNEL reaction. (D1) DAPI staining. (E1) DHR123 of *M. pulcherrima* with 89.6 µM PCN. (A-E) Bright field image of respective test.

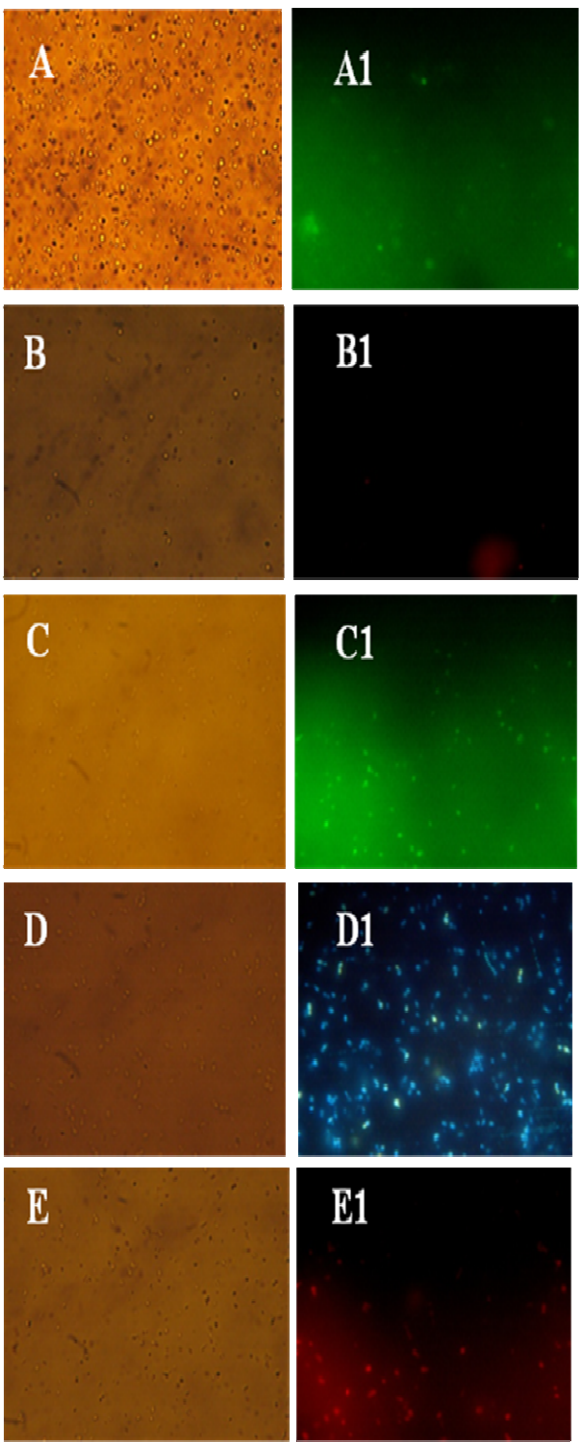


Fig.14. PCN induced apoptosis in *Z. rouxii*. (A1-B1) Annexin V-PI staining. (C1) TUNEL reaction. (D1) DAPI staining. (E1) DHR 123 staining of *Z. rouxii* with 112 µM PCN. (A-E) Bright field image of respective test.

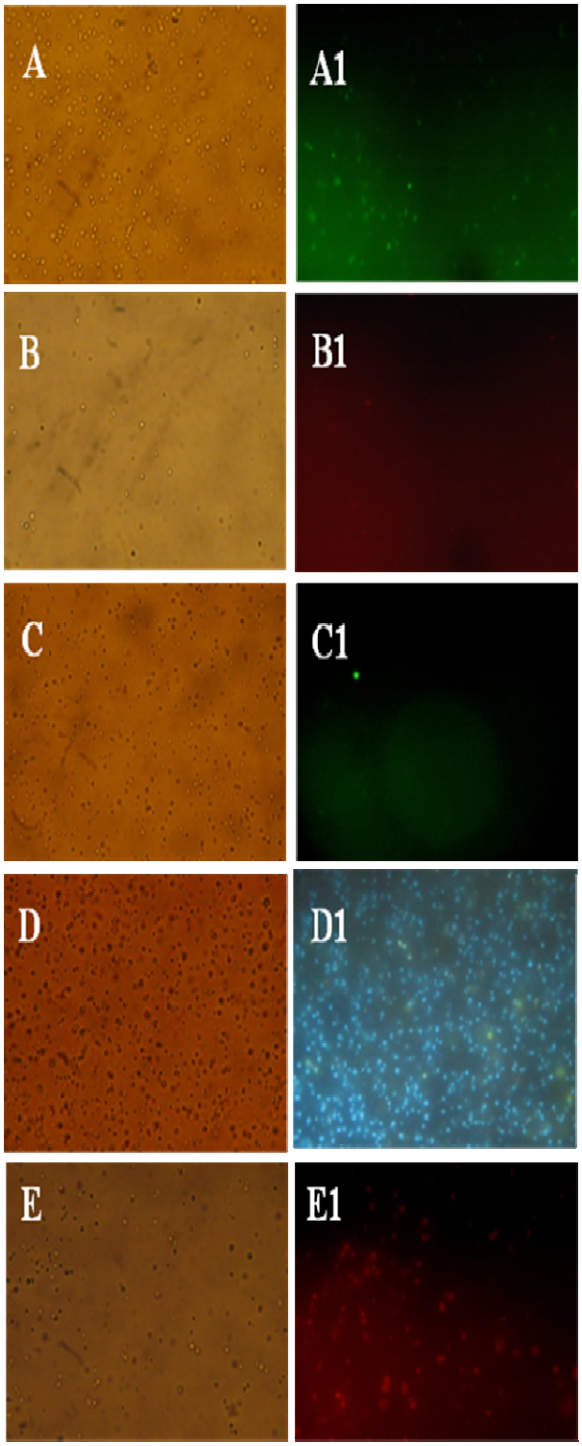


Fig.15. PCN induced apoptosis in *C. krusei*. (A1 and B1) annexin (+) and PI (+) protoplasts. (C1) TUNEL (+) protoplasts. (D1) Chromatin condensation by DAPI. (E1) ROS production by DHR123 after exposure to 89.6 µM PCN. (A-E) Bright field image showing non stained cells.

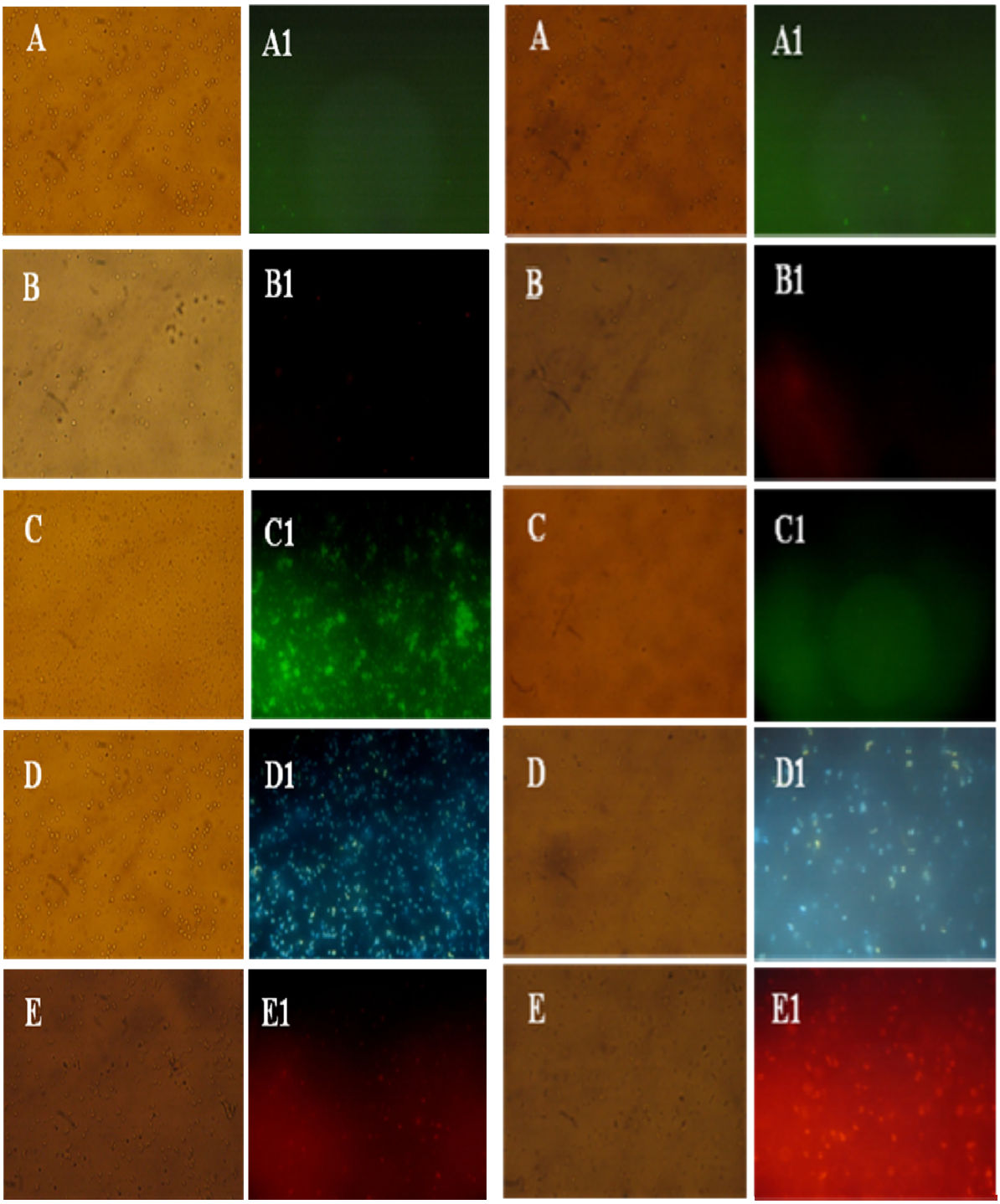


Fig.16. PCN induced apoptosis in *D. hansenii* cells. (A1 and B1) annexin (+) and PI (+) protoplasts. (C1) Damaged DNA (revealed by TUNEL assay). (D1) DAPI staining. (E1) DHR staining, after treatment with 224 µM PCN. (A-E) Bright field image showing non stained cells.

Fig.17. PCN induced apoptosis in *P. anamola*. (A1 and B1) annexin (+) and PI (+) protoplasts. (C1) TUNEL assay. (E1) DAPI staining. (E1) DHR staining after treatment with 71.7 µM PCN. (A-E) Bright field image showing non stained cells.

CHAPTER VI

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List of Publications/presentations

1. **Shirazi F.**, Kulkarni M. and Deshpande M.V. (2007) A rapid and sensitive method for screening of chitinase inhibitors using Ostazin Brilliant Red labeled chitin as a substrate for chitinase estimation. *Lett Appl Microbiol* **44**:660–665.
2. Gholap A.R., Toti K.S., **Shirazi F**., Kumari R., Bhat M.K., Deshpande M.V. and Srinivasan K.V. (2007) Synthesis and evaluation of antifungal properties of a series of the novel 2-amino -5-oxo-4-phenyl-5, 6,7,8- tetrahydroquinoline- 3-carbonitrile and its analogues. *Bioorg Med Chem* **15**:6705–6715.
3. Vatmurge N.S., Hazra B.G., Pore V.S., **Shirazi F**., Chavan P.S. and Deshpande M.V. (2007) Synthesis and antimicrobial activity of β-lactam–bile acid conjugates linked via triazole. *Bioorg Med Chem* **18**:2043–2047.
4. Bavikar S.N., Salunke D.B., Hazra B.G., Pore V.S., Dodd R.H., Thierry J., **Shirazi F**., Deshpande M.V., Kadreppa S. and Chattopadhyay S. (2008) Synthesis of chimeric tetrapeptide-linked cholic acid derivatives: Impending synergistic agents. *Bioorg Med Chem Lett* **18**:5512–5517.
5. Gholap A.R., Toti K.S., **Shirazi F**., Deshpande M.V. and Srinivasan K.V. (2008) Efficient synthesis of antifungal pyrimidines via palladium catalyzed Suzuki/ Sonogashira cross-coupling reaction from Biginelli 3,4-dihydropyrimidin- 2(1H)-ones. *Tetrahedron* **64**:10214–10223.
6. Vatmurge N.S., Hazra B.G., Pore V.S., **Shirazi F**., Deshpande M.V., Kadreppa S., Chattopadhyay S. and Gonnade R.G. (2008) Synthesis and biological evaluation of bile acid dimers linked with 1,2,3-triazole and bis-β-lactam. *Org Biomol Chem* **6**:3823–3830.
7. Chaudhary P.M., Chavan S.R., **Shirazi F**., Razdan M., Nimkar P., Maybhate S.P., Likhite A.P., Gonnade R., Hazara B.G., Deshpande M.V. and Deshpande S.R. (2009) Exploration of click reaction for the synthesis of modified nucleosides as chitin synthase inhibitors. *Bioorg Med Chem* **17**:2433–2440.
8. Deshpande S.R., Deshpande M.V., **Shirazi F**., Chaudhary P.M., Sharma M.B., Nath N., Rao N.M., Kumar B.A., Kaliannan G., Sanjoy P., Raj K., Rao B.V., Bodhanrao G.B., Reddy V.V.N. and Singh Y.J. (2009) Substituted 1,4-dioxa-8-azaspiro[4,5]decanes useful as fungicides and a process for the preparation thereof. (March.2009, Nf-40) (612/DEL/2009) 27/3/2009.

Papers presented in Symposia/Conferences/Meetings

1. Doiphode N.R., Kaur G.J., **Shirazi F**., Manimaran U. and Deshpande M.V. (2005) Chitin metabolizing enzymes: novel target for antifungal agents. The poster was presented in "International Conference on the Biology of yeasts- 2005" held at Indian Institute of Science, Bangalore, Sep. 27–29.
2. **Shirazi F**., Chavan P.S., Dahiya A., Kaur G.J., Panchanadhikar V. and Deshpande M.V. (2007) *Benjaminiella poitrasii:* a model system to study differentiation and its linkage to programmed cell death. The poster was presented in International Conference on the Biology of yeasts and Filamentous Fungi held at National Chemical Laboratory, Pune, Feb. 15–17.
3. Chavan P.S., Dahiya A., **Shirazi F**., Sawargave S.P., Phalgune U., Joshi S.P. and Deshpande M.V. (2007) Biocontrol of plant pathogenic fungi using phenazine 1-carboxamide from *Pseudomonas fluorescence* strain B-18. The poster was presented in International Conference on the Biology of yeasts and Filamentous Fungi held at National Chemical Laboratory, Pune, Feb. 15–17.
4. **Shirazi F**., Chavan P.S., Chaudhary P., Sawargave S.P., Joshi S.P. and Deshpande M.V. (2009) Phenazine 1-carboxamide induced programmed cell death/apoptosis in yeast involved in wine spoilage. The poster was presented at Research Scholar Meet held at National Chemical Laboratory, Pune, Feb. 25–28.
5. **Shirazi F**. and Deshpande M.V. (2009) *Benjaminiella poitrasii*: a model system to study possible correlation between fungal differentiation and apoptosis. The oral presentation was given in National Conference on Frontiers in Biological Sciences held at Sardar Patel University, Vallabh Vidyanagar, Gujarat, Feb. 27–28.