DIMORPHISM IN *BENJAMINIELLA POITRASII:* ROLE OF NAD-DEPENDENT GLUTAMATE DEHYDROGENASE IN YEAST-HYPHA TRANSITION

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Affectionately dedicated to ……

My Parents

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DECLARATION

Certified that the work incorporated in the thesis entitled **"Dimorphism in *Benjaminiella poitrasii:* Role of NAD-dependent glutamate dehydrogenase in yeast-hypha transition"** submitted by Ms. Namita Doiphode was carried out under my supervision. Such material as has been obtained from other sources has been acknowledged in this thesis.

Research Guide

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National Chemical laboratory

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List of Abbreviations

Abbreviation	Full Form
ATP	Adenosine triphosphate
AU	Arbitrary units
BSA	Bovine serum albumin
cAMP	Adenosine 3′5′-cyclic monophosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EMS	Ethyl methane sulfonate
h	Hour(s)
IEF	Isoelectric focusing
µg	Microgram
µl	Microlitre
µmol	Micromole
min	Minute(s)
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
MNNG	*N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine
PAGE	Poly acrylamide gel electrophoresis
PMSF	Phenyl methane sulphonyl fluoride
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultra violet light

Abstract

Abstract

Various fungi from different taxonomic groups have the ability to grow reversibly as unicellular yeast (Y) or as filamentous hypha (H). This reversible change in the morphological forms of fungi in response to environmental perturbations is termed as dimorphism. As the differentiation in general is unidirectional, reversible morphogenesis in fungi has become a model system to study differentiation in eukaryotes. Another aspect is that some of the human pathogens like *Candida, Histoplasma* and *Paracoccidiodes* show yeast – hypha transition for their survival and proliferation in the host tissue. In other words, it is a saprophytic to pathogenic change, which is exhibited by these pathogens. In the present study, *Benjaminiella poitrasii* a saprophytic zygomycetous fungus, an NCL isolate was used as a model organism to understand the biochemical basis of dimorphism and to screen different antifungal agents. It exhibited exclusively yeast form at higher temperature (37 ˚C) and higher glucose concentration (>5 %) whereas 28 ˚C and <1 %, glucose concentration favored hypha formation. The yeast to hypha transition was rapid and without any intermediate forms which made it easy to pinpoint the correlation of biochemical events with morphological outcome. One of the ways to understand morphogenesis is the isolation of morphological mutants. Therefore stable monomorphic yeast form mutants (Y-2 and Y-5), which remained in yeast form even under hypha triggering conditions, were isolated earlier (Khale *et al.*, 1990). Recently it has been shown that in *B. poitrasii* the asexual and sexual spores also responded to the dimorphism triggering conditions as the vegetative cells did (Ghormade and Deshpande, 2000).

So far different biochemical correlates for the morphological outcome reported include: enzymes of cell wall synthesis and degradation, enzymes of carbon and nitrogen metabolism, intracellular effectors like polyamines, cAMP and Ca-calmodulin, etc. However the correlation of biochemical changes with morphogenesis is still not clear, probably due to the strain dependent variations in stimuli inducing such changes. The major structural component of the fungal cell wall, a determinant of the shape is chitin. It was reported that in *B.poitrasii* the hyphal form cell wall had three times more chitin content than the yeast form cell wall. Both carbon and nitrogen metabolism pathways are involved in the chitin synthesis. The enzymes involved in ammonium assimilation are: NAD-dependent (E.C 1.4.1.2.) and NADP-dependent (E.C 1.4.1.4) glutamate dehydrogenase (GDH), glutamate synthase (GOGAT, E.C 1.4.1.13) and glutamine synthetase (GS, E.C 6.3.1.2). Both the NAD-GDH and NADP-GDH are present in the yeast and hyphal form and also in the yeast mutants of *B. poitrasii* (Khale *et al.*, 1992). The relative proportion of the two enzymes expressed as NADP-/NAD- GDH ratio showed correlation with the morphological outcome *viz*. high GDH ratio (> 0.15) was prevalent in the yeast form while low GDH ratio was found in the hyphal form. However the other ammonium assimilating enzymes glutamate synthase and glutamine synthetase did not show any appreciable correlation with the chitin contents and with the morphological outcome (Khale *et al.*, 1992).

Recently it has been shown that a common NAD-GDH was present in both the forms of *B.poitrasii* while two different NADP- GDHs were present in the Y and H form. (Amin *et al.*, 2004). Peters and Sypherd, (1979) reported both NAD - and

NADP- dependent GDHs in *Mucor racemosus*. In *M. racemosus* the expression of NAD-GDH was correlated with the morphology. As only NAD-GDH showed form specific difference in the activity level. The NAD-GDH activity was 10 fold higher in hypha than in the yeast form. Moreover, LeJohn (1971) reported the significant contribution of NAD-GDH in the understanding of evolution of fungi. More than forty species of lower fungi, myxomycetes and phycomycetes, were found to possess only an NAD-linked glutamate dehydrogenase while the higher fungi deuteromycetes, ascomycetes and basidiomycetes showed two distinct enzymes *viz*. one NAD-linked and other NADP-linked glutamate dehydrogenase. However, Le John (1971) emphasized the importance of NAD-GDH regulation in the evolution of phycomycetes genera. Phosphorylation and dephosphorylation was reported to be involved in the regulation of GDH activities in *B. poitrasii* (Amin *et al.,* 2004; Khale and Deshpande, 1993). The present investigations are therefore carried out to understand the role of glutamate dehydrogenase with special reference to NAD-GDH in the dimorphic behavior of *B.poitrasii.*

The extensive literature survey on eukaryotic differentiation, different models of morphogenesis in fungi, biochemical correlates of morphogenesis such as enzymes involved in cell wall synthesis - chitin synthase and endochitinase, enzymes involved in ammonium assimilation such as glutamate dehydrogenase, glutamine synthetase and glutamate synthase, intracellular effectors involved in signal transduction pathway such as cAMP, Ca-calmodulin has been illustrated in the **Introduction (Chapter I)**. The significance of ammonium assimilating enzymes

with respect to evolution in fungi and in the development of antifungal agents has also been discussed.

The **Materials and Methods (Chapter II)** used during the investigations and sources of chemicals and equipments have been described. Media used for the maintenance, growth and transition studies have been described. The different microbial cultures have been isolated for their potential antifungal activity. The media for the maintenance and antifungal agent production using these organisms have also been included. The commonly used microbiological techniques like light microscopy, shake flask experiments, mutagenesis using physical mutagen such as ultraviolet light, chemical mutagens like *N*-methyl-*N′* - nitro- *N* nitrosoguanidine and ethyl methane sulfonate and antifungal assay have been given. Biochemical techniques used for purification of glutamate dehydrogenase are: cell lysis by homogenizer, DEAE chromatography, affinity chromatography, ultrafiltration and isoelectric focusing. The enzyme purity was checked using activity staining of glutamate dehydrogenase and protein staining after polyacrylamide gel. The other techniques described are enzyme assays: spectrophotometric assay of glutamate dehydrogenase, glutamate synthase and glutamine synthetase and chitin deacetylase. *B. poitrasii* is a zygomycetous fungus, having chitosan in its cell wall. The biochemical correlation of GDH, chitin and chitosan content was also studied. Solid state NMR for chitosan detection has also been described.

Different environmental factors like temperature, presence of oxygen and nutritional factors like addition of glucose or metal ions in the medium trigger dimorphism in different fungi. For instance in *Candida albicans* yeast- hypha

transition was seen in response to change in temperature, pH or the addition of serum in the medium (Shepherd *et al.*, 1979). While in *Mucor rouxii* presence of glucose and /or anaerobiosis favored yeast form (Peters and Sypherd, 1979). **Effect of different environmental conditions on glutamate dehydrogenase activities and its correlation with different morphological forms in *B. poitrasii* (Chapter III) was studied.** In *B. poitrasii*, the dimorphic transition with respect to the change in concentrations of glucose, temperature or pH has been studied earlier (Ghormade and Deshpande, 2000; Khale *et al.*, 1990). In this chapter the effect of these environmental triggers in semi-synthetic or complex media with respect to GDH activities and the morphological outcome has been studied. The peptones from different commercial suppliers have different levels of metal ions and other minor components (Chacko *et al.*, 1996). Therefore the effect of different peptones from different sources, specific metal ions like Zn^{++}, metal chelators on the GDH activities and Y-H transition was studied.

The effect of temperature of incubation, glucose, pH and metal ion content of medium was observed during yeast-hypha transition by monitoring germ tube formation. It has been studied earlier in *B. poitrasii* that the organic nitrogen source favored yeast formation while inorganic nitrogen source favored hypha formation (Khale *et al*, 1990). The similar studies on Y-H transition were carried out in a semi-synthetic medium (yeast nitrogen base). In the presence of >0.5 % glucose at 28 ˚C the yeast form was predominant after 48 h. The GDH activities were NAD-GDH, 0.120 ± 0.018 U/ mg and NADP- GDH 0.139 ± 0.020 U/ mg while low glucose (< 0.1 % glucose) favored hyphal form with NAD-GDH 0.930 ± 0.140 U/mg and

NADP-GDH 0.008 ± 0.001U/mg. The effect of temperature (22 ˚C-37 ˚C) on GDH activities and on Y-H transition was studied in a complex YP medium for 12 h. The percent germ tube formation was decreased from 22-37 ˚C, which was correlated with decreased NAD-GDH and increased NADP-GDH activities. It has been reported in *Candida albicans*, *Sporothrix schenckii, Mucor rouxii* and *Aureobasidium pullulans* that Y-H transition was affected due to the presence of Zn^{++} (Alsina and Rodriguez-Del Valle, 1984; Bartnicki-Garcia and Nickerson, 1962; Bedell and Soll, 1979; Reeslev *et al.*, 1993; Yamaguchi, 1975). In *B. poitrasii* Zn^{++} affected the relative proportion of the two enzymes NADP- and NAD- GDH and Y-H transition. The NAD-GDH activity decreased with increasing concentrations of Zn^{++}, while the NADP- GDH activity increased. The *in vitro* addition of Zn^{++} during assay affected GDH activities suggesting their correlation with the morphological outcome. Interestingly though one NAD-GDH was present in both the forms, the yeast NAD-GDH activity was affected due to the addition of Zn^{++}. This can be attributed to the different levels of phosphorylation in yeast and hyphal NAD-GDH.

One of the ways to understand dimorphism is the isolation of monomorphic mutants/non-dimorphic variants (Khale *et al.*, 1990). Therefore attempts were also made for the **isolation of mutants showing change in behavior in response to environmental triggers like temperature, pH and glucose (Chapter III B).**

Biochemical analysis of morphological mutants was carried out to distinguish alterations closely associated with the dimorphic transition from those, which have no correlation with morphogenesis. The effect of incubation temperature was studied on Y-5 mutant. At low temperature of incubation the Y-5 mutant

showed high NADP- GDH activities and low NAD-GDH activities as compared to wild type yeast form. It can be suggested that the yeast form mutant required higher GDH ratio to maintain its form under hypha- favoring condition. It was reported that yeast-form mutant reverted to hyphal form in the presence of 0.5 % (w /v) ethanol (Khale *et al.*, 1990). The effect of ethanol in the presence of Zn^{++} was studied to understand the role of GDHs in the reversion of yeast form mutant to the hyphal form. Attempts were made to isolate hyphal mutants using chemical mutagen to treat parent strain spores. However, the attempts were not successful which can be attributed to the fact that *B. poitrasii* has hyphal form as a natural form.

The Chitin and chitosan contents in the cell wall of the Y and H forms of *B. poitrasii* were also studied **(Chapter III C)**. In *B. poitrasii* the total hexosamine contents were 1.7 times higher in the hyphal form than the yeast form (Khale and Deshpande, 1992). Furthermore, the deacetylation of chitin to chitosan was higher in hyphal form than yeast form. It was interesting to correlate GDH and chitin deacetylase activities and chitosan contents in *B. poitrasii*. The results have been discussed. The chitosan contents were studied using solid state NMR.

Though the studies on dimorphic behavior of human pathogens are mainly with Y-H transition, in view of the importance of spores as the etiological agents the germination pattern under different conditions is important (Ghormade and Deshpande, 2000). Interestingly the sporangiospores and the zygospores were found to respond dimorphically during germination (Ghormade and Deshpande, 2000). Therefore the **glutamate dehydrogenase activities of sporangiospores and zygospores of *B. poitrasii* (Chapter III D)** were also studied. The NAD and NADP present in the whole cell and spore extract were quantified with a fluorimeter. The

yeast, hypha and the zygospore showed the presence of high levels of NAD and NADP (92, 45 and 38 AU (arbitrary units), respectively), while the sporangiospores contained low levels of NAD and NADP (15 AU) when measured under identical experimental conditions. Fluorimetric studies of the sporangiospores and the zygospores suggested that the nicotinamide coenzyme related GDH enzymes expressed in the actively growing yeast and hyphal cells could be possibly expressed in the spores as well. The native NADP-GDH activities were found to be higher in the sporangiospores (26.0 ± 1.0 U/mg) and the zygospores (46.0 ± 2.0 U/mg), as compared to the yeast (15.3 ± 2.1U/mg) and hypha (1.40 ± 0.2 U/mg). The NAD-GDH activities were 7.0 ± 1.0 U/mg in the sporangiospore and 213.0 ± 1.0 U/mg in the zygospore. After phosphorylation, only the hyphal NADP-GDH activities increased 4-folds and NAD-GDH activities increased by 1.8 fold. The NADP- and NAD-GDH enzyme activities, their relative proportion and phosphorylation status suggested the presence of the yeast-form NADP-GDH in the sporangiospore and zygospore that was also confirmed by activity staining.

In *B. poitrasii* one NAD-GDH and two isozymes of NADP-GDH were reported (Amin *et al.*, 2004). As NAD-GDH is common to the forms, **Purification and characterization of NAD-GDH from hyphal form of *B. poitrasii* (Chapter IV)** was carried out to understand its contribution to dimorphism, if any. The purification of NAD-GDH was carried out using ammonium sulfate fractionation, ion exchange chromatography on DEAE - Biogel A, Amicon ultra filtration (YM 10 membrane) and finally Affi-gel blue gel. The 63.92 fold purification was achieved. The enzyme was without any contamination of NADP-GDH activity. The

biochemical characterization and the possible role in dimorphism have been discussed.

In view of the need of antifungal agents, chitin metabolism in the cell wall synthesis is being studied (Deshpande, 1998). Glutamate dehydrogenase is one of the important enzymes involved in the initial stage of chitin synthesis (Deshpande, 1998). After studying the role of **glutamate dehydrogenase in yeast - hypha transition in *B. poitrasii,* its role in development of antifungal agents (Chapter V)** was also evaluated. Different microbial extracts were tested for their antifungal activity using *B. poitrasii* as a model. The effect of the potential GDH inhibitors was studied using Y-H transition and *in vitro* GDH activity inhibition studies. Isophthalic acid (5 mM), NAD-GDH inhibitor was used as a positive control (33.66 % inhibition). The culture filtrate of the *Pseudomonas* sp P18, *Bacillus* sp B14, *Bacillus* sp B15 and *Bacillus* sp B1 inhibited NAD-GDH activities in hyphal form by 21.47 %, 18.31 %, 25.02 % and 25.00 % respectively, which were also evident from the retardation in Y-H transition.

In *Mucor racemosus* the significant correlation of NAD-GDH with the Y-H transition was reported (Peters and Sypherd, 1979). In the present investigations, in **Conclusion (Chapter VI)** it was seen that NAD-GDH was an important component to decide relative significance of both NAD- and NADP-GDH activities on Y-H transition. Though one NAD-GDH was present in both the forms, the yeast NAD-GDH activity was affected due to the presence of Zn^{++}, this can be attributed to the different levels of phosphorylation of NAD-GDH in the hyphal and yeast form. The purification of NAD-GDH from the hyphal form was carried out. The biochemical

characterization of the NAD-GDH confirmed the presence of similar NAD-GDH in both the forms but their regulation was different. However, the present investigations suggested the possible significance of NAD-GDH as a target too. Further comparative evaluation with NADP-GDH will be useful to decide the target enzyme explicitly.

Chapter I: Introduction

1. Introduction

It is a conservative estimate that there are more than 1.5 million species of fungi, five times more the number of vascular plants and second only in diversity to the insects. The extreme diversity of physiological, biochemical and genetic forms in fungi has always been a source of inspiration for researchers.

Fungi are said to be 'modular organisms' as they have attributes, which are unique to them. They produce a variety of morphological structures in their life cycle: unicellular budding yeasts, hyphae, asexual and sexual spores. Therefore, the knowledge about the developmental studies of these morphological structures has been useful to understand the cell differentiation processes in higher eukaryotes.

The basic shape, form and structure of an organism (whether fungal, plant and animal) do not arise all at once. Rather, shape and form emerge as a result of a sequence of developmental adjustments. The whole process in which final organization and pattern of the organism is established is termed as 'morphogenesis'. This phenomenon contributes to the 'pattern formation', which characterize the 'body plan'.

Cell differentiation during development is a response to the external environmental signals (temperature, light, pH, oxygen) or the response to nutritional signals termed as 'inducers' or 'morphogens'. Yet the whole process of morphogenesis is controlled by intrinsic genetic programme (Moore, 1998).

1.1 Differentiation in fungi

Differentiation can be defined as a regulated series of events leading to changes from one stage to another in an organism. The vegetative growth in filamentous fungi includes: elongation of hyphae by hyphal tip growth, branching and septum formation. The regulation of cell polarity, kinetics of filamentous growth, the ultrastructural changes in the tip region, biochemical and molecular aspects of hyphal tip growth have been studied extensively by number of researchers (Bartnicki-Garcia, 1973; Gow, 1995; Grove, 1978; Prosser, 1995).

Septa occur in all actively growing vegetative mycelia of filamentous fungi as well as in yeasts except in the members of zygomycetes. However, septum formation can be observed during differentiation, e.g. conidium development in *Conidiobolus* sp., *Ballocephala verrucospora* and related zygomycetous fungi of Entomopthorales (Deshpande, 1992; Latge *et al*., 1989; Phadatare *et al*., 1991; Saikawa, 1989). In filamentous fungi septa usually serve to add rigidity to hyphae. The cell division cycle of yeasts is one of the simple morphogenetic pathways extensively studied from the biochemical and genetical perspectives in *Saccharomyces cerevisiae* (Cabib *et al*., 1989). Many fungi switch from apical extension to the generation of a round bud at an early stage of conidiation. The cells of pathogenic yeasts *Candida albicans* can grow in either yeast like form or as hyphae, reversibly depending on the environmental perturbations. In some fungi hyphae become interwoven to form small aggregates called sclerotia, which have the ability to resist adverse conditions (Willets, 1972). *Sclerotium rolfsii* was one of the most favorable models so far used to understand biochemical changes occurring

during differentiation (Cohen *et al*., 1986; Lachke and Deshpande, 1988; Rawn, 1991). Fungi show many variations in pattern of their sexual and asexual spores that are adapted for their particular roles in dispersal as well as survival (Cole, 1986). Asexual spores are of two main types, sporangiospores formed within sporangia and conidia formed externally on conidiophores. The fungal sexual spores show remarkable diversity e.g. zygospores (*Phycomyces*, *Mucor* and *Benjaminiella*), ascospores (*S. cerevisiae, Neurospora crassa*), and basidial development (*Schizophyllum commune*, *Ustilago maydis*) to name a few (Bolker *et al*., 1995; Ghormade *et al*., 2005; Staben, 1995). Among all the morphological transitions being studied to unravel the enigma of eukaryotic development, the study of fungal dimorphism stands out distinctly, primarily because of its reversible nature.

1.2 Dimorphism

Dimorphism can be defined as the ability of cells to grow either yeast-like (Y) or hypha - like (H) in a reversible manner in response to environmental conditions (Gow, 1995). Many shapes and forms are found in dimorphic fungi, employing two basic patterns of cell wall expansion, polarized regulated in hypha and non-polarized regulated in yeast.

Hyphae of dimorphic fungi may change to yeast form by either lateral and/ or by terminal budding and occasionally by arthrospore formation, while Y-H transition is by germ tube formation and further by hyphal tip elongation. In some cases, such as *Mucor rouxii* and *Histoplasma capsulatum* the filamentous form may comprise true, unconstricted branching hyphae. Whereas, in fungi such as *S. cerevisiae* mainly pseudohyphae consisting of chains of elongated yeast cells are

joined end to end (Gimeno *et al*., 1992). Hyphae or pseudohyphae may branch or give rise to lateral buds (sometimes called blastospores) at the junctions between the daughter cells. In many dimorphic fungi such as *C. albicans*, a range of cell types such as yeast cells, pseudohyphae (that can vary in the extent of bud elongation) and true unconstricted hyphae can be seen (Gow, 1995).

1.2.1 Significance of the study of dimorphism

Dimorphism is observed in fungi belonging to different taxonomic groups e.g. Zygomycetes like *Mucor* (Bartnicki-Garcia and Nickerson, 1962 a), *Mycotypha* (Schulz, 1974 a) and *Benjaminiella* (Khale, 1990), Ascomycetes such as *Candida* (Gow, 1995) and *Yarrowia* (Zinjarde *et al*., 1998) and Basidiomycetes like *Ustilago* and *Ophiostoma* (Brunton and Gadd, 1991), to name a few. Some of them are important pathogens of man and plants while others are known so far as saprophytes. In human pathogens including *C. albicans*, *H. capsulatum* the ability to switch between yeast and hypha is directly related to pathogenic behavior. The mutants defective in such growth mode transitions were reported to be non pathogenic (Lo *et al*., 1997). Hyphae were capable to breach the barriers in the host, whereas the yeast form was easily disseminated within the host (Hazan *et al*., 2002). Therefore, characterization of the conditions regulating dimorphism may provide important clues in efforts to design new antifungal agents.

An understanding of dimorphism in fungi is distinctly important for many reasons:

1. The yeast to hypha transition is the reversible growth response to change in environmental and nutritional signals. As it is reversible, it serves as a useful model to gain basic insights of morphogenesis and cellular differentiation in eukaryotes.

2. It is useful to understand evolutionary relatedness among the different taxonomic groups of fungi.

3. The pathogenic fungi have ability to alter their shapes in host tissues for survival and proliferation. Therefore, the understanding of dimorphism is useful to design novel antifungal agents, which possibly can arrest the morphological change.

4. It will be useful to explore role of dimorphic fungi in various biotechnological processes, e.g. *Mucor,* for heterologous protein production exploiting its dimorphic behavior (Wolff and Arnau, 2002). This approach aims the use of yeast form during the phase of biomass production while exploiting the secretory capacity of the filamentous form during the phase of protein production.

1.2.2 Pathogens

1.2.2.1 Human pathogens

Candida albicans

Candida albicans, is an opportunistic pathogen and causes candidiasis, an infection of epithelial and mucosal cell in humans. Both yeast and hyphal forms are seen in the infected tissue, elongating hypha penetrates into the host tissues while budding yeast cells are involved in tissue colonization. The various environmental

factors capable of influencing equilibrium between yeast and hyphal growth are mainly temperature, pH and serum (Gow, 1995).

Paracoccidioides brasiliensis

Paracoccidiodes brasiliensis causes paracoccidioidomycosis, a primary lung disease and secondary systemic disease, which have been reported to be endemic in Central and South America (Gooday and Adams, 1993; San-Blas, 1985). Temperature is the most critical environmental trigger and the yeast form produced at 37 ˚C is pathogenic in nature. Kanetsuna *et al*. (1972) reported that α- (1-3)-glucan was a single virulence factor present in the cell wall of yeast but absent in hyphal form which was reported to be non- pathogenic

Histoplasma capsulatum

Histoplasma capsulatum, the etiological agent of histoplasmosis is found worldwide. The hyphal form is found in the soil and the yeast form is the parasitic form, which multiplies in the lungs and within the reticuloendothelial cells of infected humans and animals. Maresca and Kobayashi (1989) extensively studied the dimorphism in *H. capsulatum*. The 25-37 ˚C temperature change is the key factor to trigger H-Y transition.

Scherr (1957) and later Pine and Peacock (1957) showed that in addition to a temperature of 37 °C, sulfhydryl containing compounds such as cysteine were necessary for the initiation of yeast-form development in *H. capsulatum*.

Sporothrix schenckii

Sporothrix schenckii, is a dimorphic fungus that occasionally infects humans to cause sporotrichosis, a chronic, usually benign disease involving cutaneous and

subcutaneous tissues, often associated with lymphangitis and lymph node enlargement. The free-living form of *S. schenckii* was hypha, while the parasitic yeast like cells were seen at the site of infection (Travassos, 1985). For the yeast form of *S. schenckii* temperature of incubation is generally 37 °C and an increased tension of CO_2 also favors yeast form.

Apart from these, the other pathogenic dimorphic fungi include *Blastomyces dermatitidis* (Domer, 1985), causative agent of blastomycosis an infection leading to lesions of skin and bones in humans, *Penicillium marneffei* (Kudeken *et al*., 1996) causative agent of penicilliosis, which is a chronic pulmonary and disseminated infection and *Wangiella dermatitidis* (Kwon-Chung and Bennett, 1992; Matsumoto *et al*., 1994; Nachman *et al*., 1996) associated with cutaneous and subcutaneous phaeohyphomycosis exhibit dimorphic (H-Y) switch at 25-37 ˚C.

Exophiala wernickii (Harris, 1985), etiological agent of tinea nigris an infection of skin, in which change in temperature from 25-37 ˚C triggers H-Y transition and the yeast-form prevalent at 37 ˚C was pathogenic. *Coccidiodes immitis* (Cole and Sun, 1985) the causative agent of coccidiodomycosis, caused infection of respiratory tract, skin and bones. The transition from hypha to yeast was prevalent when grown on glucose yeast-extract medium leading to formation of arthroconidia after 9-16 days. *Chrysosporium parvum,* a causative agent of adiaspiromycosis, a granuloma of human lungs, exhibits hypha to adiaspores transition when the temperature is changed from 30-37 ˚C (Cole and Sun, 1985).

Mucor

Mucor racemosus, is a dimorphic saprophytic zygomycetous fungus. However it can become pathogenic in a host compromised by immune deficiency. As expected, HIV patients and transplant patients are at high risk for *Mucor* infection. In *M. rouxii* the presence of high glucose in the medium favored yeast form while at low glucose concentration exclusively hyphal growth was seen (Bartnicki-Garcia, 1963; Sypherd *et al*., 1978). In *Mucor circinelloides* (syn. *racemosus*) high glucose concentrations and the presence of CO_2 triggered yeast cells while low glucose concentrations and presence of nitrogen favored hyphal form (Wolff *et al*., 2002). In *M. genevensis*, increased hexose concentration induced yeast development even under aerobic conditions (Rogers *et al*., 1974).

1.2.2.2 Plant pathogens

Ustilago maydis

Ustilago maydis is a worldwide smut pathogen of maize (*Zea mays* L). The haploid fungus grows in the form of saprophytic budding yeast. An obligate pathogenic dikaryotic hypha is produced after mating of compatible haploid cells. The hypha invades the plant and eventually forms diploid teliospores that fill the tumors induced in the infected plant. Dimorphism in *U. maydis* was reported to be governed by the *a* and *b* mating type loci. The *a* factor was necessary for conjugation tube formation and the *b* locus produced true hyphal growth (Banuett and Herskowitz, 1994). *In vitro* dimorphism in this organism was shown to be influenced by environmental factor like pH (Ruiz-Herrera and Martinez-Espinoza, 1998). Ruiz-Herrera *et al*. (1995) reported that cells grown at neutral or nearly

neutral pH displayed yeast like growth, whereas below pH 5.0 they displayed hyphal growth.

Ceratocystis ulmi

Ceratocystis ulmi the causative agent of Dutch Elm disease, colonized extensively in the yeast form in a tree. In *C. ulmi*, nitrogen controls dimorphism in defined liquid medium containing phosphate salts and either glucose or sucrose. The amino acid proline induced yeast morphology while ammonium, asparagine or arginine induced the hyphal morphology. The extent of colonization was found to be dependent on the vertical movement of the yeast cells in an infected xylem (Hornby *et al*., 2004; Kulkarni and Nickerson, 1981).

Some other plant pathogenic fungi like *Taphrina weisneri*, *Holleya sinecauda* also show dimorphic behaviour.

All *Taphrina* species are dimorphic and their hyphal forms are parasitic on vascular plants belonging to different families, where they cause diverse malformations of the infected tissue such as leaf curl, leaf blisters or spots, galls on stems or inflorescences and witches brooms. Economically important hosts include some fruit trees namely *Prunus* sp. (peach, plum, cherry) (Kramer, 1987; Mix, 1954).

Holleya sinecauda is a dimorphic plant pathogen, which was reported to be parasitic on oriental and yellow mustard (Schade *et al*., 2003).

1.2.3 Saprophytes

The literature studies showed that number of fungal species saprophytic in nature and with no known pathogenic behavior towards either humans or plants

exhibited dimorphism. This can be attributed to the stress induced changes for the survival.

Yarrowia lipolytica

A saprophytic fungus *Yarrowia lipolytica* shows morphological transition from yeast to hypha in response to nutritional factors like presence of casein, olive oil, *N*-acetylglucosamine (Novotny *et al*., 1994; Ota *et al*., 1984; Rodriguez and Dominguez 1984). In NCL, a marine isolate of *Y. lipolytica* is being studied extensively for the role of polyamines in dimorphism. Unlike most of the dimorphic fungi the strain uniquely exhibits Y-H transition under anaerobic conditions. The strain was isolated from oil polluted sea - water. The yeast form was found to degrade alkanes (Zinjarde *et al*., 1998).

Myrothecium verrucaria

Myrothecium verrucaria is a saprophytic deuteromycetous fungus, which exhibits the dimorphic transition from hypha to yeast when grown in a synthetic medium containing sucrose and ammonium sulphate at pH 4.0, while Y-H transition was observed in a medium containing $NaNO_3$ at pH 6.0. *Myrothecium verrucaria* produced extracellularly a complete complex of mycolytic enzymes *viz*. chitinase, chitosanase, β-1, 3-glucanase, mannanase and proteinase that significantly degraded hypha of *S. rolfsii* and *Fusarium* sp. (Deshpande, 1999; Deshpande, 2005; Vyas and Deshpande, 1989).

Aureobasidium pullulans

The dimorphic deuteromycetous fungus *Aureobasidium pullulans* grows commonly as a saprotroph on the surfaces of living or senescing leaves exploiting

the more readily utilizable nutrients. *A. pullulans* is an industrially important microorganism because it produces the polysaccharide pullulan and a wide range of enzymes, such as xylanase, glucoamylase and fructofuranosidase. The yeast form produces extracellular polysaccharide pullulan. The morphological transition in *A. pullulans* was triggered by change in pH or Zn^{++} ion concentration. The C and N limitations etc. affected transition (Jurgensen *et al*., 2001).

Benjaminiella poitrasii

Benjaminiella poitrasii is a saprophytic zygomycetous fungus. The factors triggering dimorphism in *B. poitrasii* are glucose, temperature and pH, which can be easily controlled under laboratory conditions. It shows rapid process of transition in either direction. The biochemical correlates of the morphological outcome have been studied extensively in *B. poitrasii* using stable monomorphic (yeast form) mutants (Deshpande, 1998 a).

The studies in *B. poitrasii* on sporangiospores and zygospore germination under different triggering conditions suggested that dimorphism is an intrinsic ability of the organism that is expressed throughout the life cycle (Ghormade and Deshpande, 2000).

1.3 Factors influencing dimorphism

Different dimorphic fungi respond differently to number of environmental signals such as temperature, pH, oxygen, light and nutritional signals such as glucose, nitrogen source, metal ions etc. in growth medium. Interestingly, all *Mucor* species do not respond to dimorphism triggering conditions similarly. For instance, oxygen depletion and the presence of CO_2 triggered yeast formation in *M.*

racemosus (Wolff *et al*., 2002) while in the presence of nitrogen hypha formation was prevalent. In case of *Y. lipolytica*, a marine isolate exhibited Y-H transition under anaerobic conditions (Zinzarde *et al*., 1998) while other reports suggest the influence of casein, olive oil, *N*-acetylglucosamine on the transition (Novotny *et al*., 1994; Ota *et al*., 1984; Rodriguez and Dominguez, 1984; Szabo, 1999).

The carbon dioxide concentration, which probably affects the oxidation – reduction potential, enhances the morphological conversion in *C. immitis*, *Y. lipolytica*, *M. rouxii* and *S. schenckii*. The pH, glucose concentration, the presence of serum etc., in the growth medium induced the morphological changes in most of these organisms singly or in combination (Barth and Gaillardin, 1997; Cole and Sun, 1985; Ruiz- Herrera, 1985; Travassos, 1985). The factors influencing dimorphism in different fungi are mentioned in the Table 1.1.

1.3.1 Biophysical factors

1.3.1.1 Temperature

Most of the human pathogenic fungi grow in the filamentous form at 25 ˚C and exist in a pathogenic yeast form at 37 ˚C, i.e. after they enter into the human body. *H. capsulatum*, the causative agent of histoplasmosis, grew in the hyphal form (saprophytic) at 28 ˚C and in a yeast (pathogenic) form at 37 ˚C. In *B. dermatitidis* (Cole and Sun, 1985), *S. schenckii* (Travassos, 1985), *P. brasiliensis* (San Blas and San Blas, 1985) and *C. immitis* (Domer, 1985), the elevation in the temperature caused the pathogenic yeast form to prevail. In case of *Candida,* the yeast and hyphal forms were triggered by temperature in conjunction with other factors such as blood serum and blood glucose (Shepherd *et al.*, 1979). *B. poitrasii,* a strain of

present investigations exhibits yeast form at 37 ˚C and hyphal form at 28 ˚C. However Khale *et al.* (1990) reported isolation of yeast monomorphic mutant, which exhibited yeast form under hypha favoring conditions too.

1.3.1.2 Oxygen

In dimorphic fungi anaerobiosis generally favored growth of the yeast form whereas presence of oxygen induced growth of the hyphae (Bartnicki –Garcia, 1963; Sypherd *et al*., 1978). For example in the cases of *M. rouxii* (Bartnicki-Garcia and Nickerson, 1962 a), *Mycotypha microspora* and *M. africana* (Schulz *et al.*, 1974 a) hyphal growth was observed under aerobic conditions while the yeast form was reported under anaerobiosis. A marine isolate of *Y. lipolytica* studied in NCL showed hypha formation under anaerobic conditions (Zinzarde *et al.*, 1998). Interestingly, in case of *Mucor* sp. merely absence of oxygen was not important but the presence of CO_2 or N_2 to create oxygen free atmosphere was significant (Bartnicki - Garcia and Nickerson, 1962 a).

1.3.1.3 pH

In *Mycotypha* the pH range 4.5 to 7.5 favored the yeast form, while the pH below or above this range pushed the fungus to grow in a hyphal form (Schulz *et al.*, 1974 a). Stewart *et al.* (1988) reported that in *C. albicans* when the pH of the medium was in the range of 6-8 germ tube formation was prevalent and in an acidic medium the yeast type growth was seen. It was suggested that cytoplasmic alkalinization accompanied germ tube formation (Kaur *et al*., 1988; Stewart *et al*., 1988). Changes in the pH of the medium induced a significant morphogenic response in *M. rouxii* (Bartnicki -Garcia and Nickerson, 1962 b) and *Y. lipolytica*

(Ruiz-Herrera and Sentandreu, 2002; Szabo, 1999). Generally the yeast form was favored in an acidic environment whereas hyphal growth was triggered due to increase of pH to ≥ 7. Unlike the above mentioned species, acidic pH triggered yeast to hypha transition in haploid cells of *U. maydis* (Martinez- Espinoza *et al*., 2004). In *B. poitrasii* the change in pH (from 4.0-8.0) of the growth medium triggered yeast to hypha transition irrespective of the glucose concentration of the medium and temperature of incubation (Ghormade and Deshpande, 2000 b).

1.3.2 Nutritional factors

1.3.2.1 Carbon source

In *M. rouxii* the presence of high glucose (> 8 %) in the medium favored yeast form while at low glucose concentration (< 0.5 %) exclusively hyphal growth was seen (Bartnicki-Garcia, 1963; Sypherd *et al*., 1978). The hexoses fructose, mannose and galactose triggered yeast development in *M. rouxii* in the decreasing order of effectiveness (Bartnicki-Garcia and Nickerson, 1962 a). In *M. circinelloides* (syn. *racemosus*) high glucose concentrations maintained yeast cells while at low concentrations filamentous form was favored (Wolff *et al*., 2002). In *M. genevensis*, increased hexose concentration induced yeast development even under aerobic conditions (Rogers *et al*., 1974). *B. poitrasii* also showed similar behavior as *Mucor* species (Khale *et al*., 1990). In *B. poitrasii* the presence of other sugars like xylose, mannose, sucrose, lactose in defined medium favored hyphal formation while in complex medium in presence of lactose and higher concentration of (>10 %) sucrose yeast form was favored (Khale *et al*., 1990).

Interestingly, in the presence of amino hexoses the morphological outcome was found to be different. For instance *N*- acetylglucosamine triggered hyphal growth in *C. albicans* (Mattia *et al*., 1982). The effect was attributed to the carbon and nitrogen contents of the sugar.

1.3.2.2 Nitrogen source

Complex nitrogen sources such as yeast extract and peptone or an amino acid mixture are required for the optimal growth of yeast cells, whereas rapid growth of hypha occurred with an inorganic ammonium salt as the sole nitrogen source. *S. cerevisiae* produced pseudohypha, chains of elongated yet completely separated cells when grown on solid media with limited amounts of certain nitrogen sources. This has been interpreted as a foraging strategy under limiting conditions (Mosch, 2000). Brown and Gow (1999) reported that nitrogen deprivation stimulated filamentation in *C. albicans.* Nitrogen sources were shown to have influence on the morphology of *Mucor* sp. (Orlowski, 1991) and *Y. lipolytica* (Szabo, 1999). In *Y. lipolytica* nitrogen source was also important for the yeast-hypha dimorphic transition in which filament formation was stimulated with increased concentration of ammonium sulfate, while glutamine and glutamate favored hyphal development (Ruiz-Herrera and Sentandreu, 2002; Szabo and Stofanikova, 2002). Ruiz-Herrera *et al*. (1995) reported that in *U. maydis* nitrogen source was an important factor for hyphal growth. In the presence of NH_4 NO_3 maximum hyphal growth with large branched hypha was observed while in the presence of KNO_3 the cells grew in yeast form.

1.3.2.3 Metal ions

The metal ions are involved in the regulation of different enzyme activities, which play role in cell wall metabolism. For example, during Y-H transition the presence of zinc affected germ tube formation in *H. capsulatum* (Pine and Peacock, 1957), *M. rouxii* (Bartnicki-Garcia and Nickerson, 1962 b) and *C. albicans* (Sabie and Gadd, 1990). In *Aspergillus parasiticus* it was reported that the dimorphic response depended on the presence of Mn^{++} ions in the medium (Garrison and Boyd, 1974). The presence of Mn^{++} upto 730 μM supported yeast form while higher manganese concentrations resulted in hyphal growth. In *S. cerevisiae* also polarized growth was enhanced when manganese salt was added in the medium (Asleson *et al.*, 2000). The effect of exogenous hemin (the oxidized form of heme, containing Fe^{++}) as an inducer of germination in *C. albicans* was observed along with the thermal shifting (37 °C) (Casanova *et al*., 1997).

The different environmental and nutritional factors affect morphogenesis leading in concomitant development of hypha and yeast like cells. In *M. rouxii* more than one trigger was found to induce dimorphism *viz*. incubation anaerobically under CO_2 always resulted in yeast like development but depending on the nitrogen source (glycine, serine and threonine) variable proportion of filaments might also be formed (Bartnicki-Garcia, 1962b).

The overriding effect of different environmental factors that use common signaling pathways was reported in *M. rouxii* (Bartnicki-Garcia, 1968). The Y-H morphogenesis in *M. rouxii* was shown to depend on the interplay of environmental factors such as glucose concentration of the medium and the presence of CO_2. When

the glucose concentration was decreased below 0.5 % (w / v), *M. rouxii* could develop hyphal growth at high CO_2 tensions. While in the presence of high glucose (8 %), the organism grew in yeast form even in an oxygen environment. It was suggested that nutritional stress overruled the yeast growth under anaerobic conditions. Bartnicki-Garcia (1968) further suggested that glucose prevented initiation of hyphal growth while CO_2 affected the biochemical process involved in hyphal growth. As the glucose affected the process of hyphal morphogenesis upstream of CO_2 it could produce a more pronounced effect than CO_2.

It was reported that in *C. albicans* hyphal growth was favored by presence of animal serum (horse, cattle or human). When incubated with serum at 37 ˚C and neutral pH, *C. albicans* undergoes a morphological change to form germ tubes and hyphae. But presence of high concentration of serum in the medium at acidic pH counteracted the pH effect and induced hypha formation even at acidic pH (Odds, 1988). The nature of the active compound from serum, which affected dimorphism in *C. albicans*, is still unknown.

The change in the pH of the medium was shown to induce strong morphogenetic response in *Y. lipolytica.* It grew in the yeast form in acidic environment whereas hyphal growth was observed with increase of pH to neutral. Organic nitrogen sources appear to be required for the morphogenetic effect of pH. Thus pH did not affect morphogenesis of *Y. lipolytica* directly via pH- dependent regulation of gene expression, but rather by interfering with the regulation of organic sources of nitrogen (Szabo and Stofanikova, 2002).

In *B. poitrasii,* the yeast form was favored by increase in glucose concentration, higher temperature and acidic pH (Chitnis and Deshpande, 2002; Ghormade and Deshpande, 2000; Khale *et al*., 1990). Ghormade *et al*. (2005) reported that the temperature had a more pronounced effect on the morphological outcome than glucose in *B. poitrasii*. During sequential exposures, the temperature exposure could reverse the glucose effect. The pronounced reversal of the glucose effect by the temperature exposure may be due to the immediate/ direct position of the Ca-CaM in comparison to cAMP in the proposed temperature and glucose dependent signal transduction pathway in *B.poitrasii*.

It has been well documented that the glucose signal is transduced through the cAMP dependent processes (Gadd, 1995; Rolland *et al.*, 2002). In case of *S. cerevisiae* the perturbations in temperature were shown to affect the level of Ca-calmodulin (Ca-CaM), a calcium binding protein (Sundberg *et al*., 1996).

1.3.2.4 Other factors

In *H. capsulatum* the formation of hypha was promoted by the presence of sulphydryl containing compounds like cysteine (Maresca and Kobayashi, 1989).

Serum and serum derivatives also promoted germ tube formation in *C. albicans* (Barlow *et al*., 1974), however the nature of triggering component in the serum is still unclear. Kim *et al*. (2000) reported that in *Y. lipolytica* the yeast to hypha transition was triggered in response to serum.

Table 1.1 Factors influencing dimorphic transition in fungi

Fungi	Factors influencing yeast to hypha transition	References
Benjaminiella poitrasii	Temperature (37→28 ˚C); glucose concentration (<1.0 %); pH (4→8)	Deshpande (1998 a), Ghormade and Deshpande (2000)
Candida albicans	Temperature (28→37 ˚C); Serum; *N*-acetylglucosamine (2.5mM); pH (4.5 →6.7).	Soll (1992), Gow (1995), Brown and Gow (1999)
Histoplasma capsulatum	Temperature (37 →25 ˚C); Cysteine or cystine (1mM) -Y form maintained transition retarded; Zinc (10 mg / 100 ml media)-Y form maintained;	Maresca and Kobayashi (1989), Scherr (1957), Maresca *et al*. (1994), Pine and Peacock (1957)
Mucor species	Carbon dioxide (<30 %) or $CO_2 \rightarrow N_2$ or air; Glucose concentration (<1.0 %); complex nitrogen source → ammonium salts; Inhibited by Acriflavin or chloramphenicol; phenethyl alcohol; sodium fluoride; Antimycin A and KCN; dibutyryl cAMP.	Stewart and Rogers (1978), Orlowski (1991), Gow (1995)
Paracoccidioides brasiliensis	Temperature (37 →25 ˚C)	San-Blas and San-Blas (1985)
Saccharomyces cerevisiae	Nitrogen starvation; Exogenous cAMP or dibutyryl cAMP.	Gow (1995)
Yarrowia lipolytica	Absence of oxygen	Zinjarde *et al*. (1998)
Mycotypha sp. (*M. microspora and M. africana*)	pH (5.8-6.5 → 4.5 or above 4.0); $CO_2 \rightarrow N_2$ or air; Glucose concentration (10 % w/v → 1.0 % w/v).	Schulz *et al*. (1974)

1.4 Second messengers

The signals from various environmental factors are transduced *via* second messengers such as Ca^{++}, cAMP, cGMP and inositol lipids and are translated into specific intracellular responses, important in growth, metabolism and differentiation (Gadd, 1995).

1.4.1 Calcium calmodulin

The role of Ca^{++} as a second messenger in fungal growth and differentiation has been investigated in several yeasts and fungi (Muthukumar *et al.,* 1987). Calcium can transduce external stimuli (such as environmental and nutritional) into specific intracellular effects. Many effects of calcium were mediated by calcium calmodulin (Ca- CaM), which affected the phosphorylation and dephosphorylation of enzymes including protein kinase, phospholipase A2, NAD^{+} kinase, adenylate cyclase and phosphodiesterase (Anraku *et al.*, 1991). Muthukumar and Nickerson (1984) reported that Ca^{++} - calmodulin interaction was necessary for hyphal growth in *C. ulmi* and its absence led to the yeast development. Using specific calcium calmodulin inhibitors like trifluoroperazine (TFP) it was showed that in *C. albicans* (Holmes *et al*., 1991; Paranjpe *et al*., 1990; Sabie and Gadd, 1989) and *S. schenckii* (Rivera –Rodriguez and Rodriguez-del Valle, 1992) Ca-CaM interaction was also found to be necessary for Y-H transition.

1.4.2 Cyclic AMP (cyclic adenosine monophosphate)

In fungi cAMP plays important role in a variety of morphological processes, such as conidiation, dimorphism, phototropism, hyphal branching, spore germination, etc. (Robson *et al.*, 1991). The presence of cAMP as well as its

lipophilic derivative, dibutyryl cAMP (dbcAMP) stimulated hyphal elongation and also conidiation in *N. crassa* (Terenzi *et al.*, 1976). In several species of *Mucor* cAMP was postulated to produce yeast like morphology (Orlowski, 1991). In other words, the endogenous levels of cAMP in the yeast form cells were more and also the exogenous addition of cAMP favoured H-Y transition. However, the relationship between cAMP and morphic state in another dimorphic fungus, *H. capsulatum* was reported to be opposite (Maresca and Kobayashi, 1989). In *H. capsulatum,* the level of cAMP was about five times higher in hyphal form than in the yeast form. Though the mechanism of action of cAMP is not clear, it can be suggested that it may function by activating a cAMP dependent protein kinase, a membrane receptor or polymerization of tubulin or may be by regulating cAMP phosphodiesterase activity.

In *B. poitrasii* the cAMP dependent phosphorylation of NAD- and NADP-dependent glutamate dehydrogenase was reported which influenced Y-H transition (Khale and Deshpande, 1993).

1.4.3 Cyclic GMP (cyclic guanosine monophosphate)

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

play an important role in dimorphism. In fungi, cGMP has been detected in only few organisms and has not been previously implicated in any particular function.

1.5 Biochemical correlates of dimorphism

The cell wall plays a major role in determining the morphology of the fungal cell. Harold (1997) suggested that the rigidity of the wall enable the fungi to assume a variety of forms such as penetrative ramifying hyphae, proliferating yeast cells and spores of many shapes and sizes.

1.5.1 Carbon metabolism

The requirement of hexoses for the yeast development explains the relationship between hexose catabolism and morphology. In an attempt to determine whether a change in the major pathways for dissimilation of glucose occurred as a consequence of morphogenesis in *Mucor,* the distribution of carbon catabolizing enzymes in yeast and hypha was studied. It was found that 14.0 % of glucose was catabolized *via* pentose phosphate pathway (PPP) in yeast cells, 28.0 % was processed *via* PPP in hyphal cells. Thus, a shift in the route by which glucose was catabolized occurred depending upon the morphological form of the organism (Inderlied and Sypherd, 1978). In view of these differences, major physiological differences on the expression of some key enzymes between the yeast and hyphal forms were thought to exist and three forms of pyruvate kinase were detected in *M. rouxii* (Friedenthal *et al*., 1973). Paznokas and Sypherd (1977) examined a variety of cultural conditions to determine if a relationship between the pyruvate kinase isozymes and morphological form occurred in *Mucor*. Their results indicated that pyruvate kinase did not appear to be involved in morphogenetic process.

The catabolism of glucose in *H. capsulatum* (Mahvi, 1965) and *P. brasiliensis* (Kanetsuna and Carbonell, 1966) routed through the Embden Meyerhof pathway in both yeast and hyphal forms. All the enzymes involved were present in both the forms of these fungi, although higher activities were found in the yeast form. The activity of glyceraldehydes-3-phosphate dehydrogenase showed a marked difference between the two forms, the activity in the hyphal form being 20 % of that in the yeast form. In *C. albicans* a greater percentage of glucose proceeded *via* the hexose monophosphate shunt in yeasts than in hyphal cells (Schwartz and Larsh, 1982).

The enzymes associated with carbon metabolism, which was also suggested to be involved in morphogenesis like phosphoglucose isomerase, phosphofructokinases were studied in both forms of *C.albicans* (Chattaway *et al.,* 1973).

1.5.2 Nitrogen metabolism

1.5.2.1 Glutamate dehydrogenase / glutamine synthetase and glutamate synthase

The enzymes involved in ammonia assimilation are: NAD-dependent glutamate dehydrogenase (E.C 1.2.1.2) and NADP-dependent glutamate dehydrogenase (EC 1.2.1.4), glutamate synthase (GOGAT, EC 1.4.1.13) and glutamine synthetase (GS, EC 6.3.1.2). Three distinct type of GDHs have been recognized on the basis of their coenzyme specificity: NAD^+ specific and $NADP^+$ specific encountered in bacteria, lower eukaryotes and plants and a third non

selective variety able to use both NAD^+ and $NADP^+$ (E.C 1.4.1.3) as cofactor was found in the animal kingdom (Smith *et al*., 1975).

In plants, the ammonia assimilation is mainly *via* the GS / GOGAT pathway and GDH probably catalyzes the deamination of glutamate (Srivastava and Singh, 1987; Stewart *et al*., 1995). In fungi such as *S. cerevisiae* (Avendano *et al*., 1997), *M. racemosus* (Peters and Sypherd, 1979) and *C. utilis* (Fergusson and Sims, 1974) the NADP-GDH was found to be the major route both in conditions of nitrogen excess and limitation while the GS / GOGAT pathway was relatively unimportant. However, in *C. tropicalis*, *C. parasilopsis* and *C. albicans* the GS/ GOGAT pathway was reported to be important for ammonia assimilation (Holmes *et al*., 1989). Lomnitz *et al*. (1987) reported that in *N. crassa* the NADP-GDH functioned in conditions of large supply of nitrogen while GS/ GOGAT pathway catalyzed ammonium assimilation when the concentration was low. In *Aspergillus nidulans* (Macheda *et al*., 1999) and *Agaricus bisporus* (Kersten *et al*., 1999) nitrogen assimilation was reported to follow the GS/ GOGAT pathway predominantly.

According to Le John and Stevenson, (1971) higher fungi Ascomycetes and Basidiomycetes possess two distinct GDHs one specific for NAD and the other for NADP whereas lower fungi possess a single NAD-GDH. The GDHs play a catalytic role in either oxidative deamination of glutamate or in reductive amination of α-ketoglutarate. GDHs lie at an important branch point in metabolism as they provide a link between amino acid metabolism and the tricarboxylic cycle, interlinking carbon and nitrogen metabolism.

The role and implication of the involvement of GDH in dimorphic transition is due to its involvement in the early circuitry of the metabolic pathway connecting the formation of chitin, a cell wall polymer and hence the morphology of cell (Khale *et al*., 1992).

The biochemical correlation of glutamate dehydrogenases with morphogenesis was reported in the dimorphic species of *Mucor*. Peters and Sypherd (1979) demonstrated the presence of two GDH's, one NAD-GDH and other NADP-GDH and correlated the CO_2 / N_2 induced yeast- hypha transition to the rise in the NAD-GDH activity. The level of NAD-GDH enzyme was 10 fold lower in yeast cells than in hypha.

In case of *B. poitrasii* studies on the activities of GDH, GS and GOGAT were performed as a function of temperature, nutritional conditions and the morphological form (Khale and Deshpande, 1993; Khale *et al*., 1992). The changes in the GS and GOGAT levels did not show any appreciable correlation with the morphological outcome. Since both NAD- and NADP- GDH were found in *B. poitrasii*, the quantitative relation between both enzymes was expressed as the NADP-/ NAD-GDH ratio evaluating its probable role in morphogenesis (Khale *et al*., 1992). An increase in GDH ratio led to a change in the morphology from hyphal to the yeast form.

Furthermore, it was also reported that two form specific NADP-GDHs, and one NAD-GDH contributed significantly in the morphological transition (Amin *et al*., 2004).

1.5.2.2 Ornithine decarboxylase

Polyamines are basic cationic micromolecules essential for the normal growth and development in bacteria, fungi, higher plants and mammals (Altman, 1989; Bachrach, 1973; Pegg and McCann, 1982; Smith, 1985; Stevens, 1981; Tabor and Tabor, 1984, 1985). Because of their net positive charge, it was considered that polyamines exert their multiple functions by binding to negatively charged molecules of the cell, mainly nucleic acids. Among the many roles played by polyamines, it was suggested that they protect DNA from enzymic degradation, X - ray irradiation, mechanical shearing and oxidative damage. They also stabilized RNA, prevented ribosome dissociation and *in vitro* they stimulated DNA and RNA synthesis and improved the fidelity of translation (Bachrach, 1973; Pohjanpelto and Knuutila, 1982; Tabor and Tabor, 1984).

A role for polyamines in cell differentiation was substantiated (Ruiz-Herrera, 1994; Slocum *et al*., 1984), although no clear-cut mechanism for this role was demonstrated. In fungi, changes in the polyamines, putrescine and spermidine, were associated with a variety of morphological changes (Stevens, 1981; Stevens and Winther, 1979). Usually the levels of putrescine and spermidine increased when morphogenesis was accompanied by a high rate of cell division, nucleic acid and protein synthesis. This phenomenon was observed with respect to germination of zoospores of *Blastocladiella emersonii* (Mennucci *et al*., 1975), *Achlya ambisexualis* (Wright *et al*., 1982), spherules of *Physarum polycephalum*, ascospores of yeasts and during morphogenesis of *M. racemosus* (Inderlied *et al.,* 1980).

The putrescine is produced from arginine and ornithine catalyzed by the rate limiting enzymes arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), respectively. Bacteria and higher plants have both the ADC and ODC pathways, while fungi are largely limited to the ODC pathway with occasional indications of a bio-degradative form of ADC. The ornithine decarboxylase (ODC, E.C 4.1.1.17) is reported to be the first and the most controlled enzyme in the biosynthetic route of the polyamines (putrescine, spermidine and spermine) (Ruiz-Herrera and Martinez-Espinoza, 1998). Transient increase in the levels of ODC activity and polyamines took place during the yeast to hyphae transition of the dimorphic fungi *M. racemosus* (Inderlied *et al*., 1980), *M. rouxii* and *M. bacilliformis* (Calvo-Mendez *et al*., 1987). The changes in both putrescine levels and ODC activity were consistent correlates of dimorphic transitions in *Mucor* species (Orlowski, 1991). In *M. bacilliformis,* ODC activity was found to be 3.5 times higher in the hyphal form than in the yeast form (Ruiz-Herrera *et al*., 1983).

In *U. maydis* it was observed that high levels of polyamines were associated with hyphal form while low polyamine levels were associated with yeast form (Reyna-Lopez and Ruiz-Herrera, 2004). San - Blas *et al.* (1996) reported that in *P. brasiliensis*, H-Y transition was accompanied by a high ODC activity.

1.5.3 Sulphur metabolism

In case of *H. capsulatum* temperature seems to affect the redox state of the -SH groups or the general redox potential in cells or both which would then determine the form of the organism.

The primary enzymes involved in the sulphur metabolism include cystine reductase, cysteine oxidase, sulfite reductase along with form specific proteins termed as protein P. Cystine reductase was a yeast form specific enzyme and played a role in hypha to yeast form transition by producing enough reducing equivalent in the form of cystine to trigger transition. Cysteine oxidase regulated the intracellular level of free cysteine or provided a metabolic product of oxidation of cysteine (cysteine sulfinilic acid), which was important for hypha to yeast transition (Kumar *et al*., 1983).

The differential regulation of cysteine oxidase, cystine reductase (a reduced nicotinamide adenine dinucleotide) dependent enzyme and sulfite reductase in yeast and hyphal form cells of *H. caspsulatum* illustrated the involvement of sulphur metabolism in dimorphic transition (Maresca and Kobayashi, 1989, 2000). **1.5.4 Cell wall metabolism**

It has been observed that 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 shown for *B. poitrasii* (Khale *et al.*, 1992), *M. rouxii*, (Orlowski, 1991), *C. albicans*, *S. schenckii*, *B. dermatitidis*, *H. capsulatum* and *P. brasiliensis* (San-Blas and San-Blas, 1983). The enzymes involved in cell wall synthesis and degradation, like chitin synthase,

1.2 Biochemical correlates in dimorphic fungi

Fungal system	Biochemical correlate of dimorphism	References
Benjaminiella poitrasii	(Y-H) Decrease in NADP- / NAD-GDH ratio; Decrease in endochitinase activity; Increase in native chitin synthase activity; Increase in *N*-acetylglucosaminidase activity.	Khale *et al.* (1992), Khale and Deshpande (1993), Deshpande *et al.* (1997), Ghormade *et al.* (2000)
Candida albicans	(Y-H) Changes in second messenger system including cAMP; calcium ions; calmodulin or inositol phosphate; Increase in cAMP level; Increase in native chitin synthase activity.	Soll (1985), Gow (1995), Brown and Gow (1999)
Histoplasma capsulatum	(Y-H) Expression of sulphite reductase; Increase in cAMP level; (H-Y) Initial uncoupling of oxidative phosphorylation followed by a drop in ATP levels and a decline in the rate of respiration; Expression of heat shock proteins; Expression of cysteine oxidase; Expression of cystine reductase.	Maresca and Kobayashi (1989), Maresca *et al.* (1994)
Mucor species	(Y-H) Increase in NAD –GDH activity; Decline in cAMP level; Increase in ornithine decarboxylase activity; Demethylation of DNA; Oxidative phosphorylation; (H-Y) Preference for fermentative carbon metabolism.	Stewart and Rogers (1978), Orlowski (1991)
Paracoccidioides brasiliensis	(Y-H) Change from α-glucan to β-glucan; (H-Y) Increase in levels of polyamines.	San-Blas and San-Blas (1983)
Saccharomyces cerevisiae	(Y-H) Increase in cAMP; Decreased amino acid uptake.	Niimi *et al.* (1980), Egidy *et al.* (1989), Sabie and Gadd (1992)

glucan synthetase, chitinase and *N*-acetyl glucosaminidase play important role in dimorphism. In a dimorphic fungus *M. rouxii* it was reported that chitin synthase (CS) in the yeast form was in less active zymogenic form and regulated by proteinases, while in the hyphal form CS was more active (Zou *et al.,* 1990). In *B. poitrasii*, in the yeast form cells CS was less active as compared to hyphal cells (Khale, 1990). It was further 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).

Glucan synthetase is also one of the important enzymes involved in dimorphism. In *P. brasiliensis*, glucan synthetase activity was more in the yeast form than the hyphal form (San-Blas, 1979). The other cell wall associated enzymes involved in dimorphism like endochitinase and *N*-acetyl glucosaminidase, were in *B. poitrasii* yeast and hyphal forms (Ghormade *et al.*, 2000). During the yeast - hypha transition, the *N*- acetylglucosaminidase activity increased steadily in *B. poitrasii*. The endochitinase activity during Y-H transition increased 12 fold between 6 and 12 h and thereafter remained unchanged up to 24 h. A reverse trend in the chitinolytic activities was observed during the hypha to yeast transition.

1.5.5 Heat shock proteins

Heat shock proteins are, induced very early during transition and may play a critical role in adaptation to the new environment in pathogenic dimorphic fungi. Heat shock proteins such as Hsp 60, Hsp 70 and Hsp 80 involved in morphogenesis were well studied in *H. capsulatum* (Maresca and Kobayashi, 1989), *B. dermatitidis* and *P. brasiliensis* (Da Silva, 1999).

Most of the studies of fungal morphogenesis have been dealt with biochemical changes, which occur prior to the dimorphic transition (Table 1.2). However, the correlation of biochemical changes with dimorphism is still not clear due to the strain dependent variations in stimuli inducing such transitions.

1.6 Genetic studies

Genes related to various functions in the cell have been extensively studied in different dimorphic systems as shown in Table 1.3. In *B. poitrasii* the presence of eight distinct chitin synthase (CHS) genes were reported (Chitnis *et al*., 2002). Two of these genes *viz. BpCHS 2* and *BpCHS 3* appear to be specific to the hyphal growth form (Chitnis *et al*., 2002). Genes encoding for cellular regulators like protein kinases (*STE 12*, *STE 20, PKC 1*), transcriptional activators (*TUP 1, EFG 1*), heat shock proteins (*HSP 70*) were thought to be playing an important role in the yeast to hypha transition of *S. cerevisiae* (Liu *et al*., 1994), *C. albicans* (Magee, 1997) and *H. capsulatum* (Maresca and Kobayashi, 1989), respectively. In *C. albicans,* northern analysis indicated that the expression for 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* (chitin synthase gene) were

Table 1.3 Key genes in the regulation of cellular processes suggested to be involved in morphogenesis

Genes encoding	*C. albicans*	*H. capsulatum*	*P. brasiliensis*	*U. maydis*	*S. cerevisiae*	*M. racemosus*	*Y. lipolyt*ica
Cellular regulators	*CST20, TUP1, ACPR, CPH1, EFG1, RBF1, SAP1-7, HST7, CZF1*	*HSP70, HSP83, HSP82*	*HSP70*		*BEM1, PKC1, CDC24, STE7, STE11, 12, 20, CUP*	*CUP*	*XPR2*
House-keeping genes					*CDC3, CDC30, CDC10-12*		
Cellular building blocks	*CHS1, CHS2*	*TUB1, TUB2*			*ACP2, MYO2, TPM1, PFY2, CHS2, 3, 5*		
Product unknown	*HYR1*	*YPS3, MS8*		*EG11*			

Function of genes

C. albicans - *CHS1, 2,* chitin synthase (Gow *et al*., 1993); *EFG1*, enhanced filamentous growth (Stoldt *et al*., 1997); *HYR*, hyphally regulated gene (Bailey *et al*., 1996); *HST7*, mitogen activated protein kinase components (Gow *et al*., 1995); *RBF1*, RPG box binding factor1 (Magee, 1997); *SAP1-7*, aspartyl proteinase gene (Hube *et al*., 1994); *TUP1*, transcriptional regulator (Magee, 1997).

H. capsulatum - *HSP 70* and *83*, heat shock protein; *TUB1*, α tubulin; *TUB2*, β tubulin; *YPS 3*, yeast phase specific gene (Maresca and Kobayashi, 1989); *MS8* – mold specific gene (Tian and Shearer, 2002).

P. brasiliensis - *HSP 70,* heat shock protein (Da Silva *et al.*, 1999).

U. maydis - *EG1*, filamentous growth gene (Bolker *et al*., 1995).

S. cerevisiae - *BEM*, bud emergence (Cabib *et al*., 1998); *CAP2*, capping protein; *CHS 2,3,5*, chitin synthase; *MYO2* myosin; *PFY2*, profiling (Harold, 1995); *STE 7,11,20*, kinases; *STE12* transcriptional factor (Liu *et al*., 1994); *TPM*, tropomyosin (Harold, 1995).

M. racemosus - *CUP*, copper metallothein gene (Cano-Canchola *et al*., 1992).

Y. lipolytica - *XPR 2*, alkaline protease (Madzak *et al*., 1999).

Adapted from Chitnis (2001), Ghormade (2000), Gow (1995), Maresca and Kobayashi (1989), Orlowski (1991)

preferentially expressed in the hyphal form. The genes that were expressed in the cells of only one form, such as *HYR1*, a hypha specific gene in *C. albicans* (Bailey *et al*., 1996) and *YPS3*, a yeast specific gene (Maresca and Kobayashi, 1989) and

MS8, mold specific gene (Tian and Shearer, 2002) in *H. capsulatum* were known but the information of their products are still unknown. However, there has been no gene linked constitutively to the dimorphic transition.

1.7 Ammonia assimilating enzymes

Generally in fungi, the carbon and nitrogen metabolisms are connected *via* ammonia assimilation and the participating enzymes are NAD-dependent glutamate dehydrogenase (NAD-GDH) and NADP-dependent glutamate dehydrogenase (NADP-GDH), glutamate synthase (GOGAT) and glutamine synthetase (GS) (Marzluf, 1981).

Earlier, in a dimorphic fungus, *B. poitrasii* the significance of the relative proportion of both NADP- and NAD- dependent glutamate dehydrogenase, measured as NADP- / NAD - GDH ratio in the yeast - hyphal transition was reported (Khale *et al*., 1992). Furthermore Khale-Kumar and Deshpande (1993) described the involvement of cAMP in the regulation of NADP-/ NAD –GDH ratio and in dimorphic transition of *B. poitrasii.* The presence of one NAD-GDH in both yeast and hyphal form cells and two active NADP-GDHs, one expressed in the hyphal form and other one in yeast form, in the parent strain of *B. poitrasii* were reported by Amin *et al*. (2004). The further studies showed that similar type of NADP-GDH is present in the parent strain yeast as well as in the mutant yeast (Amin *et al*., 2004).

Glutamine synthetase (GS)

Glutamine synthetase (L -glutamate: ammonium ligase, ADP forming) 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 (Baars *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.

In *N. crassa* under ammonium excess NADP-GDH and octameric glutamine synthetase formed by β-monomers participate in ammonium assimilation. In contrast in ammonium limiting conditions a tetrameric GS formed by α monomers and an NADH - dependent glutamate synthase are responsible for nitrogen assimilation.

$$\textbf{Glutamate + Ammonia + ATP} \xrightarrow{\textbf{GS}} \textbf{Glutamine + ADP + Pi}$$

Glutamate synthase (GOGAT)

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

$$\textbf{Glutamine + } \alpha\textbf{-ketoglutarate + NAD(P)H} \xrightarrow{\textbf{NAD(P)-GOGAT}} \textbf{2-Glutamate +NAD(P)}^{+}$$

Glutamate dehydrogenases

Glutamate dehydrogenases, fall into three distinct classes based on their coenzyme specificity and physiological function: NADP- GDHs are mainly involved in ammonium assimilation (glutamate production), NAD- GDHs are involved in

glutamate catabolism (or in α-ketoglutarate production) and GDHs that can use both the coenzymes NAD and NADP (E.C 1.4.1.3) (Ohshima and Soda, 1990).

$$\alpha\text{-ketoglutarate} + \text{Ammonia} + \text{NAD(P)H} \xleftrightarrow[\text{NAD-GDH}]{\text{NADP-GDH}} \text{Glutamate} + \text{NAD(P)}^{+}$$

By measurements of enzyme activity in *N. crassa* and yeasts a catabolic role has been assigned to the NAD-specific enzyme, whereas the NADP specific enzyme has been implicated in glutamate biosynthesis (Fergusson and Sims, 1971; Sanwal and Lata, 1961). This was confirmed by GDH minus mutants of *A. nidulans* (Arst *et al.*, 1975) and *S. cerevisiae* (Miller and Magasanik, 1990).

1.7.1 Localization of glutamate dehydrogenase

The glutamate dehydrogenases are located either in cytosol or in mitochondria. In fungi like *Blastocladiella*, *Achlya* and *Saprolegnia* most of the glutamate dehydrogenase activities were found in mitochondria (Le John *et al*., 1969; Le John, 1971). While the cytosolic GDHs were reported in case of *Neurospora* (Lomnitz *et al*., 1987), *Candida* (Hemmings, 1978) and *Saccharomyces* (Uno *et al*., 1984). In *B. poitrasii* major activities of NAD and NADP-GDH were observed in the cytosol (Amin *et al*., 2004).

1.7.2 Biochemical characteristics

1.7.2.1 Molecular weight

In the literature it was reported that the NAD - GDHs of fungi usually have a molecular weight ranging from 450 to 480 kDa and are composed of four identical subunits of about 116 kDa (Hemmings, 1980; Veronese *et al.*, 1974; Yang and LeJohn, 1994). The spores of the lower fungus *Phycomyces blakesleeanus* had a

dimeric NAD- GDH with a molecular weight of 98 kDa (Van Laere, 1988). The NADP- GDH from *N. crassa*, *C. utilis* and *S. cerevisiae* were found to be hexameric with molecular weight of 291, 270 and 270 kDa (Blumenthal and Smith, 1975; Gore, 1981; Neumann *et al*., 1976; Venard and Fourcade, 1972).

1.7.2.2 Kinetic studies of glutamate dehydrogenase

Hudson and Daniel, (1993) reported that the Km values for NAD^+ or $NADP^+$, α - ketoglutarate and NADPH are similar regardless of source, whereas the steady state values for glutamate, ammonia and NADH differ widely. Garnier *et al*. (1997) has reported in case of NAD-GDH from *Laccaria bicolor* the Km value for ammonium, α-ketoglutarate, glutamate was 37 mM, 1.35 mM and 3.6 mM respectively and for NADH and NAD it was 0.089 mM and 0.282 mM respectively. *L. bicolor* had lower Km values for glutamate and ammonia than most of the other fungi investigated. Similarly lower Km values have also been reported in *Dictyostelium discoideum* (Pamula and Wheldrake, 1991), *A. klebsiana* (Yang and LeJohn, 1994) and in *P. ostreatus* for ammonium and glutamate 3.3 and 0.18 mM respectively (Mikes *et al*., 1994) and *A. bisporus* for ammonium 6.3 mM (Baars *et al*., 1994).

1.7.2.3 Activators and inhibitors

In *Neurospora* both the enzymes NAD-GDH and NADP-GDH have been shown to be concurrently regulated by a repression -derepression type of mechanism. In the presence of glutamate or its nitrogenous precursors (urea, ammonia, alanine, aspartate, etc) the NADP -GDH was repressed and the NAD-GDH was simultaneously derepressed, while the NAD-GDH activity was inhibited

significantly by p-chloromercuribenzoate (PCMB), EDTA and glutaric acid. Haberland *et al*. (1980) have reported that the nucleotides guanosine triphosphate (GTP), guanosine monophosphate (GMP) and inosine monophosphate (IMP) at concentration of 10^{-3} M inhibited totally the activity of NAD-GDH. Sanwal (1961) reported in *Fusarium* that EDTA inhibited the activity of NADP-GDH while there was no effect on NAD-GDH and that PCMB inhibits activity of NADP-GDH while NAD-GDH was not inhibited. This reflected role of -SH groups in enzyme activity. The glutaric acid, the decarboxylated analog of glutamic acid inhibited the NAD-GDH and NADP-GDH by competing with the substrate for the active enzyme sites (Sanwal, 1961).

1.7.3 Regulation by phosphorylation and dephosphorylation

The NAD-GDH of the yeasts, *S. cerevisiae* and *C. utilis* are regulated by a phosphorylation - dephosphorylation system catalyzed by protein kinase (E.C 2.7.1.37) and alkaline phosphatase (E.C 3.1.3.1). In *S. cerevisiae* the conversion of active NAD-GDH to inactive form is regulated by the phosphorylation of the enzyme by both cAMP-dependent and cAMP-independent protein kinase. In *C. utilis*, phosphorylation of the active NAD-GDH was promoted by the starvation of glutamate, converted it into less active form and reaction is reversible *in vivo* and *in vitro* (Hemmings, 1980; Uno *et al.*, 1984).

1.7.4 Molecular characterization

The molecular characterization of the nitrogen metabolizing pathway in *S. cerevisiae* has shown that besides the NADP-GDH encoded by *GDH1* and GOGAT by *GLT1* there is a third route for glutamate biosynthesis, constituted by a NADP-

GDH1 isozyme (NADP-GDH 3) encoded by *GDH*3 (Avendano *et al.,* 1997; Cogoni *et al.*, 1995; Moye *et al.*, 1985). In the yeast *Kluyveromyces lactis* both NADP-GDH and GS-GOGAT activities were confirmed by null mutation studies (Romero *et al.*, 2000; Valenzuela *et al.*, 1995). In *N. crassa* anabolic role of NADP-GDH and catabolic role of NAD-GDH have been studied by characterization of these genes (Kapoor *et al.,* 1993; Kinniard and Fincham, 1983). Extensive studies on the complete nucleotide sequence of the *am* gene responsible for NADP-GDH enzyme in *N. crassa* have been carried out at the biochemical and molecular level (Fincham *et al.,* 2000; Kinniard and Fincham*,* 1983). GDH genes identified in different fungi are summarized in Table 1.4.

NADP-dependent glutamate dehydrogenase (encoded by *gdhA*) has been shown to play an important role in nitrogen metabolism and regulation in *A. nidulans* (Kinghorn and Pateman, 1973). In the presence of glucose and inorganic nitrogen source, *gdh A* is expressed which is reflected in terms of high levels of the enzyme activity (Hawkins *et al.*, 1989). The mutant of *A. nidulans*, lacking NADP-GDH activity was leaky and grew poorly than the parent strain, which was indicative of an alternative pathway. A double mutant wherein a *glt A* (glutamate synthase) and *gdh A* genes are inactivated was unable to utilize ammonium as nitrogen source (Macheda *et al.,* 1999). In another species *A. awamori*, widely used for the secretion of extracellular proteins, the *gdhA* encoding GDH responds to strong nitrogen source regulation at the transcriptional level (Cardoza *et al.*, 1998).

Table 1.4 Glutamate dehydrogenase genes from some fungi

Organism	Genes cloned	Enzyme Regulation	Reference
S. cerevisiae	NADP-GDH (*GDH1,3*) NAD-GDH (*GDH2*)	C /N limitation; Phosphorylation N limitation	Moye *et al*. (1985), Wilkinson *et al.* (1996), ter Schure *et al.* (1995)
K. lactis	NADP-GDH (*GDH1*)		Valenzuela *et al.* (1995)
N. crassa	NADP-GDH (*am* gene) NAD-GDH	C limitation	Kinniard and Fincham, (1983), Kapoor *et al.* (1993)
A. nidulans	NADP-GDH (*gdhA*)	C limitation	Hawkins *et al.* (1989)
A. awamori	NADP-GDH (*gdhA*)	N limitation, glutamic acid repression	Cardoza *et al.* (1998)
P. chrysogenum	NADP-GDH (*gdhA*)	---	Diez *et al.* (1999)
A. bisporus	NADP-GDH (*gdhA*) NAD-GDH (*gdhB*)		Schaap *et al.* (1996), Kirsten *et al.* (1999)

1.7.5 Role of glutamate dehydrogenases in evolution

The study of dimorphism is useful to understand evolutionary relatedness among the different taxonomic groups of fungi. More than forty species of lower fungi, Myxomycetes and Phycomycetes, were found to possess only an NAD-linked glutamate dehydrogenase. The higher fungi, Deuteromycetes, Ascomycetes and Basidiomycetes seem to produce two distinct forms of the enzyme one NAD-linked and the other NADP-linked. Among the lower fungi, oomycetes and hypochytridiomycetes have an unusual NAD- GDH, which can represent a

transitional form. This type of NAD- GDH is allosterically regulated by NADP and is kinetically similar to the NADP- GDH of higher fungi. The unique distribution of these two coenzyme specific forms makes it necessary to understand the mechanisms of enzyme regulation of the glutamate dehydrogenases operative in these fungi.

Glutamate dehydrogenase of the hexameric class is one of the best-known conserved proteins that has proved to be a potentially good tool for evolutionary studies (Wilson *et al.*, 1977). Benachenou-Lahfa *et al.* (1993) presented results on the multiple alignments of the 21-glutamate dehydrogenase sequences, which were deduced to the existence of two families of genes corresponding to paralogous proteins. The GDHs from Asparagus and tobacco plants were compared with the eubacterial and fungal GDHs (Pavesi *et al.*, 2000). It was reported that the plant GDHs have closer affinity to GDHs of thermophilic archaebacterial and eubacterial species than the fungi like *S. cerevisiae.* The plant GDHs have greater adaptation to heat stress conditions than the yeast enzyme.

Le John (1971) divided the NAD-linked enzyme of the Phycomycetes into three classes on the basis of their regulatory properties. All the Chytridiales and Mucorales possessed unregulated forms of glutamate dehydrogenase they form the type I enzyme. Type II enzyme was found in members of Blastocladiales, a large aquatic group and in *Absidia*, a genus of the Mucorales. These type II enzymes had a complex multivalent mode of regulation. Divalent metal ions such as Ca^{++} and Mn^{++} activated reductive amination reaction but inhibited the oxidative deamination reaction. The type III enzymes were found only in the Oomycetes and

Hypochytridiomycetes. These were the enzymes, which use NAD^+ as a substrate in catalysis, only interacting with $NADP^+$ when it functions as an allosteric modulator. When Oomycetes and Hypochytridiomycetes were grown in the presence of glucose or sucrose and limited amounts of amino acids, their glutamate dehydrogenase production was repressed (Le John, 1971).

Table 1.5 Different types of NAD-linked glutamate dehydrogenase found in Phycomycetes

Possible order of evolution	Enzyme type	Activators	Inhibitors	Glucose repression of synthesis
Chytridiales and most Mucorales	Type I	None	None	Negative
Blastocladiales	Type II	Ca^{++}, Mn^{++}, AMP	Citrate, ATP, FDP, EDTA, GTP	Negative
Few Mucorales	Type II	Ca^{++}, Mn^{++}, AMP	Citrate, ATP, FDP, EDTA, GTP	Negative
Hypochytridiales	Type III intermediate	$NADP^+$, NADPH, AMP, acetyl CoA derivatives, P-enolpyruvate	ATP, citrate GTP, Long chain acyl CoA derivatives, Ca^{++}, Mn^{++}	Positive
Saprolegniales Leptomitales Peronosporales	Type III	$NADP^+$, NADPH, AMP, acetyl CoA derivatives, P-enolpyruvate	ATP, citrate GTP, Long chain acyl CoA derivatives, Ca^{++}, Mn^{++}	Positive

Adapted from LeJohn (1971)

1.8 Relation between nitrogen metabolism and morphogenesis

The biochemical correlation of NAD-GDH with morphogenesis was reported in the dimorphic species of *Mucor*. Peters and Sypherd (1979) demonstrated the presence of two GDH's one NAD-GDH and other NADP- GDH and correlated the carbon dioxide and nitrogen induced yeast-hypha transition to the rise in the NAD-

GDH activity. In *Mucor bacilliformis* the hypha showed two fold more NAD-GDH than NADP-GDH (Ruiz-Herrera *et al*., 1983).

In *C. cinereus,* based on its kinetic characteristics, NAD-GDH is believed to perform both amination and deamination of glutamate, as the nutritional conditions require. A specific role has been postulated for NADP-GDH in the development of the fruit body of *C. cinereus*. NADP-GDH may act in cooperation with GS as an ammonium scavenging system. Microscopic studies of basidia involving activity staining demonstrated that the increase in activity of NADP-GDH was initiated as karyogamy became evident (Moore *et al.*, 1987). Furthermore, it was observed that ammonium, glutamine and their analogues inhibit basidium differentiation in *C. cinereus* (Chiu and Moore, 1990) and sporulation in *S. cerevisiae* (Delavier-Klutchko *et al.*, 1980).

In cap and stipes of the fruit bodies of *A. campestris* (*A. bisporus*) high activities of both NAD-GDH and NADP-GDH were found whereas, in C. *cinereus* high activities of only NADP- GDH were found (Table 1.6).

Table 1.6 Relation between nitrogen metabolism and morphogenesis

Organism	NAD- and NADP -GDH	Morphogenesis	References
Mucor racemosus	Increase in NAD-GDH	Y-H transition	Peters and Sypherd (1979)
Mucor bacilliformis	Increase in NAD-GDH	Y-H transition	Ruiz Herrera *et al*. (1983)
Coprinus cinereus	Increase in NADP-GDH	Fruit body formation	Moore *et al.* (1987)
Agaricus bisporus	High NAD-GDH and NADP-GDH	In stipes and fruit body formation	Moore and Al Gharawi (1976)

1.9 Antifungal agents

The incidence of fungal infections has increased tremendously during the last decade or so. As the number of cases suffering from cancer, AIDS and tuberculosis are increasing day by day and such patients having lowered immunity are highly proned for fungal infections (St. Georgiev, 1988). Even the infections like coccidioidomycosis and pseudomembranous candidiasis have been emerged as a significant threat to the larger population.

The resistance was developed to broad-spectrum antibiotics due to frequent use of antitumor and immunosuppressive agents, x-ray irradiation, steroids and oral contraceptives (Arai, 1974), which resulted in dramatic increase of fungal infections. This led to substantial need for effective antifungal drugs to combat fungal infections.

There are presently three major classes of antifungal agents which are clinically used namely azoles, allylamines and polyenes. Azoles such as fluconazole, itraconazole and miconazole inhibit biosynthesis of the membrane component ergosterol by blocking the action of lanosterol demethylase (encoded by *ERG11*). Allylamines such as terbinafine and morpholines such as fenpropimorph are also sterol biosynthesis inhibitors (SBIs) while polyenes such as amphotericin B directly interact with the cell membrane causing electrolytic leakage and subsequent cell death. Echinocandins such as caspofungin represent a new class of antifungal agents that target cell wall biosynthesis.

Table 1.7 Major classes of antifungal agents

Class and compound	Routes of administration	Mechanism of action
Polyenes		Interacts with ergosterol, Thereby disrupting the cytoplasmic membrane
Amphotericin B	Systemic	
Nystatin	Topical	
Azoles		Interact with cytochrome P-450 inhibit C-14 demethylation of lanosterol causing ergosterol depletion and accumulation of aberrant sterol in the membrane
Miconazole	Topical	
Ketoconazole	Systemic	
Fluconazole	Systemic	
Allylamines and thiocarbamates		Inhibit oxidosqualene cyclase thereby causing ergosterol depletion and accumulation of squalene oxides in the membrane.
Naftifine	Topical	
Terbinafine	Systemic	
Tolnaftate	Topical	
Morpholines		Inhibit sterol reductase and isomerase
Amorolfine	Topical	
Nucleoside analogs		Is deaminated to 5-fluorouracil (5 FU) causes miscoding and inhibit DNA synthesis
5-Fluorocytosine (5-FC)	Systemic	

Adapted from Ganguli *et al.,* (2001)

The fungal cell wall is made up of polymers such as mannan, chitin and α and β-glucans that are unique to the fungal kingdom, hence have been identified as possible targets (Fig. 1.1). Chitin synthase inhibitors such as nikkomycins have been extensively studied but not yet commercialized. Similarly, β-glucan inhibitors act as specific, non-competitive inhibitors for β- (1, 3)- glucan synthetase, involved in cell wall synthesis (Table 1.7).

The organo phosphorus fungicides like kitazin-P inhibited incorporation of ^{14}C -glucosamine into the fungal cell walls which may be due to inhibition of chitin

synthesis either directly by inhibiting chitin synthase or indirectly by altering membrane permeability (Bink *et al*., 1993).

Wenke *et al*. (1993) detected chitin synthase inhibiting activities in the culture filtrate of an osmophilic *Aspergillus fumigatus* strain HA 57-88. Two compounds, namely pseurotin A and 8-O-demethylpseurotin A have been identified which showed inhibition in *Coprinus cinereus* of solubilized and membrane bound chitin synthase activity.

Actinomycetes are the most favored sources of a variety of antibiotics. Sanglier *et al*. (1993) reported that more than 200 bioactive metabolites having antifungal and anti-yeast properties have been discovered from actinomycetes. Sakuda *et al*. (1987) reported the production of allosamidin, by *Streptomyces* species No.1713 which specifically inhibited chitinase. The allosamidin are pseudotrisaccharides consisting of a disaccharide of *N*-acetyl allosamine linked to an amino cyclitol derivative known as allosamizoline.

Of all the antibiotics produced by the genus *Streptomyces*, polyene antifungal antibiotics appeared to be the most common. Non-polyene antifungal antibiotics produced by actinomycetes *viz*. blasticidins, polyoxins, kasugamycin, endomycin and validamycin.

The polyoxins are characterized as a family of metabolites from *Streptomyces cacaoi* variety *asoensis* and the nikkomycins from *Streptomyces tendae*. Both of them are structurally similar to UDP-NAG, an active monomer of chitin. Since they are structurally similar, the antibiotic can mimic the action of UDP-NAG and bind to chitin synthase. This would block the active site of the

enzyme and thus make the enzyme unavailable to the monomer for polymerization. As these antibiotics are specifically targeted towards chitin synthase they do not show any significant toxicity to mammals and higher plants (Deshpande, 1998d).

Majority of antifungal antibiotics of bacterial origin have been derived from *Bacillus* and *Pseudomonas*. Amongst *Bacillus* species, *B. subtilis* seems to be a potential source of antifungal agents (mycobacillin, mycosubtilin and iturin). Tetaine, an antifungal antibiotic was isolated from *Bacillus pumilus* (Milewski *et al*., 1983). It is a dipeptide, which when transported into fungal cell *via* a peptide uptake system is hydrolysed by intracellular peptidases to alanine and its active portion anticapsin. It is a powerful inhibitor of glucosamine -6- phophate synthase acting as a substrate analogue of glutamine. In case of *C. albicans*, it has been seen that tetaine addition affected the yeast-hypha transition and showed fungicidal effect within few hours (Milewski *et al*., 1983).

Quite a good number of antifungal antibiotics have been reported from a variety of gram-negative, bacteria like *Gluconobacter* (Matsuhisa, 1990), *Erwinia* (Greiner and Winkelmann, 1991), *Serratia* (Shoji *et al*., 1989) and the gliding bacterium *Lysobacter gummous* (Meyers *et al*., 1985).

Fig. 1.1 Targets for antifungal agents (Modified from Groll *et al*., 1998)

In fungi, antibiotic production seems to be restricted to a few genera *viz*. *Aspergillus* and *Penicillium*. Griseofulvin is a non-polyene antifungal agent first isolated from a *Penicillium* spp. used to treat dermatophytosis caused by *Epidermophyton floccosum*, *Microsporum ausouinii*, *M. gypsum*, *M. canis*, *Trichophyton mentagrophytes*, *T. rubrum*, *T. interdigitale* and *T.verrucosum*. It inhibits fungal mitosis by disrupting the mitotic spindle through interaction with polymerized microtubules.

Nystatin is the most frequently used topical or oral polyene antibiotics. Nystatin preparations are available in form of suspension, tablets, cream and ointment formulations and are applied topically to the mucous membranes of the mouth, orpharynx, gut. It is toxic for parenteral administration and there is very little or no absorption when given orally (Henderson *et al*., 1968).

1.10 *Benjaminiella poitrasii* as a model system for screening of antifungal agents

In spite of the fairly large number of antifungal antibiotics there is a continuous interest in search for new antibiotics. Earlier the test introduced by Hutter *et al.* (1965) for the screening of antibiotics was based on morphological changes of the hyphae of a plant pathogen, *Botrytis cinerea*. However, the sensitivity of the test was limited to antibiotics with strong action against *B. cinerea*. Later on, same group reported the use of zygospore formation inhibition test using *Mucor hiemalis* to screen antifungal antibiotics (Kneifel *et al.*, 1974). Thrautomycin, a nucleoside antibiotic produced by *Streptomyces exfoliates*, was identified using this test. It was observed that (+) and (-) hyphae coming together for zygospore formation had different sensitivities towards thrautomycin.

As compared to fungal chitin synthases, insect chitin synthases are rarely reported; therefore, Brillinger (1979) used chitin synthase from *C. cinereus* as a model to mimick insect chitin synthases to screen insecticidal compounds. To evaluate the potential of dimilin, captan, kitazin P and parathion for insecticidal activity, *C. cinereus* chitin synthase was used. The chitin synthases from yeast and hyphal cells of *Mucor rouxii* with regard to morphogenesis were characterized (Orlowski, 1991). The stability of chitin synthase from yeast cells was greater than that from hyphal cells. Therefore the chitin synthase of yeast cells was tried as a target for the screening of inhibitors isolated from the fermentation broth of an actinomycetes isolate (Zou *et al*., 1990).

B. poitrasii grows in hyphal form (H form) at 28 ˚C and / or at low glucose concentration whereas development of the yeast form (Y form) occurs at 37 ˚C and / or at high glucose concentration in the medium (Khale, 1990; Khale *et al*., 1990). The studies on the chitin synthase activity from both the forms revealed that the amount of native activity is greater in hyphal cells than in yeast cells (Khale, 1990). This could be attributed to the activation state of the chitin synthase in yeast cells and /or the permeability of the cells to the antibiotic. As *B. poitrasii* is non-pathogenic and shows temperature dependent dimorphism similar to pathogens like *H. capsulatum*, *P. brasiliensis*, it can serve as the useful model to understand treatment and management of pathogenesis caused by these fungi and also can be used for the treatment of fungal infections (Deshpande, 1998 c).

Several pathogenic fungi require 24-48 h for the complete yeast-hypha transition (Domer, 1985). The transition of yeast cells into hypha in *P. brasiliensis*

was within 18 h. *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 is restricted. The transition in either direction in *B. poitrasii* is relatively rapid. Hence it is easier to pinpoint the correlation of biochemical events with morphological change (Deshpande, 1998 b; Khale *et al.*, 1990; Khale and Deshpande, 1992; Khale-Kumar and Deshpande, 1993). Stable, monomorphic (yeast-form; Y-2 and Y-5) mutants of *B. poitrasii* are also available and the biochemical correlates of morphological outcome for these mutants have also been studied. Recently, for the first time, it has been reported that in *B. poitrasii* the asexual and sexual spores also respond to the dimorphism triggering conditions as vegetative cells do (Ghormade and Deshpande, 2000). Therefore, *B. poitrasii* holds promise as a complete system to screen antifungal agents.

Previous studies with parent and yeast monomorphic mutant strains on the cell wall composition as well as on the enzymes involved in the nitrogen metabolism showed correlation in relative activities of the NADP- / NAD- glutamate dehydrogenase, chitin contents and morphological form in *B. poitrasii*. In the present studies the special emphasis has been given on the purification, characterization and regulation of the form specific NAD-glutamate dehydrogenases from *B. poitrasii.* This knowledge will be useful to understand the cause - effect relationship between the physiological changes inside the cell and subsequently the morphological outcome. Furthermore, the studies will be directed to screen

microbial sources for NAD- / NADP- glutamate dehydrogenase inhibitors for the development of novel antifungal agents.

1.11 Objectives of the present investigations:

1. To identify different environmental and nutritional factors *viz*. temperature, pH, glucose concentration, presence of metal ions, which affect dimorphism in *B. poitrasii*.

2. The isolation of morphological mutants of *B. poitrasii* and effect of environmental triggers e.g. temperature, pH and glucose.

3. To study biochemical correlates of dimorphism in *B. poitrasii* with special reference to the glutamate dehydrogenase enzymes.

4. Screening of antifungal agents using glutamate dehydrogenase of *B. poitrasii* as a target.

Chapter II: Materials and Methods

2.1 Materials

Table 2.1 Sources of chemicals

Chemicals	Supplier
α-Ketoglutarate	Sigma Chemical Co., St. Louis, USA
Affi gel blue gel	Biorad Laboratories Inc. Hercules, USA
Bacto-peptone	Difco Industries, Detroit, MI, USA
Chitosan	Sigma Chemical Co., St. Louis, USA
Chitin	Sigma Chemical Co., St. Louis, USA
DEAE fast flow Sepharose	Amersham biosciences, UK
Ethanol	Merck, Germany
Ethylenediamine tetraacetic acid	Sigma Chemical Co., St. Louis, USA
Ethyl methane sulfonate	Sigma Chemical Co., St. Louis, USA
Glucosamine	Sigma Chemical Co., St. Louis, USA
Nikkomycin	Sigma Chemical Co., St. Louis, USA
N N´- Methylene bis acrylamide	Sigma Chemical Co., St. Louis, USA
N-Methyl -N′-nitro -N nitrosoguanidine	Sigma Chemical Co., St. Louis, USA
Yeast extract	Difco Industries, Detroit, MI, USA

All other chemicals used were of analytical grade

Table 2.2 Conditions for the yeast - hypha transition in *B.poitrasii*

Inoculum	Transition
YPG 0.1 %, 28˚C	YPG 0.1% (22-37 °C)
YPG 0.1 %, 28˚C	YPG 0.1% (pH 4.0-8.0)
YPG 0.1 %, 28˚C	Yeast nitrogen base (YNB, 0.17 %), (Glucose 0.1-5 %)
YPG 0.1 %, 28˚C	YP, 28°C
YPG 0.1 %, 28˚C °C	YP (yeast extract, Difco; peptone, Hi-media), 28
YPG 0.1 %, 28˚C	YP (yeast extract, Difco; peptone, Sarabhai), 28 °C
YPG 0.1 %, 28˚C	YP (Zinc 0.1-1.0 ppm)

In all the media yeast extract (Difco) was used unless otherwise mentioned.

YPG - Yeast extract (0.3 %, Difco), Bacto- peptone (0.5 %, Difco), glucose (1 %).

The Difco YPG medium was used for transition studies and the modifications made in the medium were mentioned otherwise.

The inoculum used for the transition studies was 8×10^6 / 50 ml yeast cells.

Table 2.3 Conditions for the transition of Y-5 mutant of *B.poitrasii*

Inoculum	Transition
YPG 0.5 %, 28˚C	YPG 0.5 %, (22-37 °C)
YPG 0.5 %, 28˚C	YPG 0.5 %, (pH 4.0-8.0)
YPG 0.5 %, 28˚C	Yeast nitrogen base (YNB, 0.17 %), (Glucose 0.25-5 %)
YPG 0.5 %, 28˚C	YPG 0.5 %, (0.5 % ethanol), 28 ˚C
YPG 0.5 %, 28˚C myoinositol),	YPG 0.5 %, (0.5 % ethanol + 50 µg/ ml 28 ˚C
YPG 0.5 %, 28˚C ˚C	YPG 0.5 %, (0.5 % ethanol + Zn^{++} 0.5 ppm), 28
YPG 0.5 %, 28˚C myoinositol +	YPG 0.5 %, (0.5 % ethanol + 50 µg/ml Zn^{++} 0.5 ppm), 28 ˚C

In all the media yeast extract (Difco) was used unless otherwise mentioned.

YPG - Yeast extract (0.3 %, Difco), Bacto- peptone (0.5 %, Difco), glucose (0.5 %).

The Difco YPG medium was used for transition studies and the modifications made in the medium were mentioned otherwise.

The inoculum used for the transition studies was 8 x 10^6 / 50 ml Y-5 mutant yeast cells.

2.2 Methods

2.2.1 Reagents

All reagents and buffers were prepared in glass-distilled water. The reagents used for protein purification were prepared in deionized water. The stock solutions of the reagents required for different experiments were stored at 4˚C.

2.2.2 Organisms and culture conditions

The parent strain *Benjaminiella poitrasii*, a homothallic, zygomycetous dimorphic fungus and its monomorphic mutant (yeast-form, Y-5) were maintained and subcultured weekly on YPG (yeast extract, 0.3 %; peptone, 0.5 %; glucose 1 %; agar, 2 %) slants at 28 ˚C. The stable yeast-form mutant (Y-5) was isolated after mutagenesis of the spores of parent strain, using nitrosoguanidine (Khale *et al*., 1990). The growth in the liquid as well as on agar media was used for further studies after appropriate treatments as discussed in subsequent sections.

Yeast nitrogen base medium was used for transition studies by varying the carbon and nitrogen source as shown in Table 2.2.

The isolated bacterial cultures were maintained on nutrient agar medium (0.3 %, beef extract; 0.5 %, sodium chloride; 0.5 %, peptone; 2 %, agar) pH 6.8 at 28 ˚C. The actinomycete cultures isolated were maintained on Casein starch agar, CSA (1 %, starch; 0.03 %, casein; 0.2 %, KNO_3; 0.2 %, NaCl; 0.2 %, Na_2HPO_4; 0.05 %, $MgSO_4$; 0.02 %, $CaCO_3$; 0.01 %, $FeSO_4$; 2 %, agar) slants at 28 ˚C. The fungal cultures isolated were maintained on potato dextrose agar, PDA (20 %, potato; 2 %, dextrose; 2 %, agar) slants at 28 ˚C.

Stock culture of *C. albicans* was maintained on malt glucose yeast extract peptone medium, MGYP agar (0.3 %, malt extract; 1 %, glucose; 0.3 %, yeast extract; 0.5 %, peptone, 2 %, agar) at 28 ˚C. Stock cultures of *Mucor, Aspergillus niger* were maintained on Sabourauds dextrose agar, SDA (1 %, peptone; 2 %, glucose; 2 %, agar) pH 6.5 at 28 ˚C.

2.2.3 Spore suspension

The sporangiospores of *B. poitrasii* required developing either yeast or hyphal inoculum for yeast to hypha and reverse transition studies were obtained from 7 d old parent strain grown on YPG (1 %, glucose) agar at 28 ˚C.

Sporangiospores were harvested, washed and resuspended in sterile distilled water and count was taken using haemocytometer grid. The inoculum size used was 10^6 sporangiospores / 50 ml of culture medium. The sporangiospores and zygospores of *B. poitrasii* were obtained from 5 d old culture of the parent strain grown on YPG (1 %, glucose) agar.

To obtain sporangiospores free from yeast, mycelium and zygospores, the 5 d old slants were lightly scraped and examined microscopically as described earlier (Ghormade and Deshpande, 2000). To obtain zygospores free from other growth forms, the underlying hyphal bed was scrapped off and crushed with a mortar and pestle in distilled water. The suspension was filtered through muslin cloth and centrifuged at 500 g for 15 sec. To obtain clean suspensions of zygospore samples were repeatedly washed and centrifuged and examined microscopically.

Separation of forms and estimation of growth

Separation of the yeast and hyphal forms was carried out using glass fibre filter G1, Jensil, India. The filtrate gave yeast (Y) form cells and the hyphal (H) form remained on the filter. The morphological form and the purity were confirmed microscopically.

2.2.4 Yeast - hypha and reverse transition studies

The yeast form of *B. poitrasii* was obtained using YPG (1 %, glucose) medium with 8 x 10^6 spores / 50 ml as inoculum and incubated on rotary shaker at 28 ˚C for 24 h.

For the yeast to hypha transition, the yeast cells (8 x10^6 cells / 50 ml) were used to inoculate YPG (1 %, glucose) medium and incubated under shaking condition at 28 ˚C for 24 h. Morphological characterization was carried out as described earlier (Khale *et al*., 1992). Prior to harvesting, samples were examined microscopically on haemocytometer grid. In Y- to-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.

All other transition studies are mentioned in the chapters appropriately

2.2.5 Mutagenesis

To obtain morphological mutants using sporangiospores of *B. poitrasii* the following protocol was used.

The spores from 7 d old slant of YPG (1 %, glucose) were harvested in sterile distilled water and centrifuged. The aliquots containing 2 x10^2 spores/ ml were plated on plated on YPG agar containing 0.1 % glucose and modified Czapek

Dox agar (pH-8). Then exposed to ultraviolet irradiation (Philips UV germicidal lamp, 15 W) for different time intervals (3, 5, 10 and 15 min) and from different distance (15, 20 and 25 cm). Control kept was of plates unexposed to UV irradiation. Plates were incubated to obtain discrete colonies at 28 ˚C for 5-10 d.

The spores from 7 d old slant of YPG (1 %, glucose) were harvested in sterile distilled water and centrifuged. The aliquot containing 10^6 spores / ml was treated with 5.0 ml sterile 0.05 M citrate phosphate buffer of pH 6.6, containing MNNG 4.0 mg / ml. The mutagen action was stopped by using $Na_2S_2O_3$ as quenching agent. The mutagen treated spores were centrifuged at 2000 g for 10 min, washed 2-3 times with sterile distilled water and centrifuged after every washing at 2000 g for 10 min and plated on YPG agar containing 0.1 % glucose and modified Czapek Dox agar (pH-8). Plates were incubated to obtain discrete colonies at 28 ˚C for 5-10 d.

Survival curves were done with a constant concentration of MNNG (200µg / ml) and different times of exposure (1- 10 min).

The same protocol was used for the chemical mutagenesis using ethyl methane sulfonate (EMS) at 10- 40 mg /ml concentrations and the time of exposure was 3, 5, 10 and 15 min. Survival curves were done with a constant concentration of EMS (40 mg / ml) and different times of exposure (3- 15 min).

2.2.6 Isolation of different potential antifungal agents producing microorganisms

The serial dilution - agar-plating method was used for the isolation of potential antifungal agents producing microorganisms. Soil sample, 1g was

suspended in 10 ml sterile water. Serial dilutions were made and 1ml aliquot of the diluted sample was used for pour plate method. Nutrient agar for bacteria, casein starch agar for actinomycete and potato dextrose agar for fungi, supplemented with tetracycline, 10 µg / ml were used for isolation. The plates were incubated at 28 ˚C for 3-7 d.

2.2.7 Preparation of culture filtrates used for screening of antifungal agents

The microbial isolates, 32 bacteria, 22 actinomycete and 40 fungi were inoculated in the medium containing 1 %, soyabean meal; 1 %, yeast extract; 1.5 %, mannitol; 5 %, starch and incubated on rotary shaker at 28 (C for 96 h. The cell free supernatant obtained after centrifugation at 6800 g for 15 min were used for further testing.

2.2.8 Sporangiospore germination for antifungal testing

A spore suspension (8 x 104 sporangiospores / ml) was made by suspending sporangiospores from sporulating YPG (1 %, glucose) agar slants of B. poitrasii in distilled water. The suspension (50 µl) was spread in 10 cm plates. Small wells were made in the plates and the inhibitor solutions (culture filtrate) 100 µl concentrated 10 times were pipetted in them. About 50 spores / field were observed at 10 X.

2.2.9 Cell extract preparation

Cell extracts of yeast and hyphal form 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 ice cold water, followed by K- phosphate buffer of 5 mM, pH 7.2 containing 0.25 mM EDTA, 50 mM K2SO4. Cells were disrupted using Braun's homogenizer by treating them for 4 cycles of 15

s each. The samples were centrifuged at 12000 g for 15 min to obtain cell extract. DTT to a final concentration of 1.0 mM was added to the cell extract to maintain the desired enzyme activities.

2.2.10 Enzyme assays

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

The specific activity of intracellular NAD- and NADP-dependent GDH (E.C 1.4.1.2 and E.C 1.4.1.4, respectively) was measured using standard procedure, described by Khale et al. (1992). The reductive amination of (-ketoglutarate was measured by monitoring the decrease in the A_{340} of NAD(P)H. The reaction mixture (1ml) contained 50-100 µl of crude extract, 200 mM NH_4Cl and 30 mM α-ketoglutarate in 100 mM K-phosphate buffer (pH 8.0). The reaction was initiated by the addition of 125 µM NADH or NADPH to the sample cuvette. A control in which α-ketoglutarate was omitted from the reaction mixture was run for each assay.

2.2.10.2 Glutamate synthase (NADP-GOGAT, E.C 1.4.1.13 and NAD-GOGAT, 1.4.1.14)

The specific activity of intracellular glutamate synthase was measured using standard procedure described by Khale *et al*. (1992). GOGAT was measured by monitoring NADH and NADPH oxidation at 340 nm. The reaction mixture (1ml) contained 50 µl of crude extract, 10 mM α-ketoglutarate and 10 mM freshly prepared L-glutamine in 100 mM K-phosphate buffer (pH 7.8). The reaction was started by the addition of 0.2 mM NADH or NADPH to the sample cuvette. A

control in which L-glutamine was omitted from the reaction mixture was run for each assay.

2.2.10.3 Glutamine synthetase (GS, E.C 6.3.1.2)

The specific activity of intracellular GS was measured using standard procedure, described by Khale *et al*. (1992). A fresh concentrated assay mixture containing 18 mM hydroxylamine HCl, 0.27 mM $MnCl_2$, 25 mM sodium arsenate, 0.36 mM ADP and 135 mM tris - HCl buffer (pH 7.2) was prepared. The pH was adjusted to 7.2 at room temperature with 2.0 M NaOH or 1.0 M HCl. The reaction mixture (1ml) containing 0.4 ml of above assay mixture, 0.2 ml of cell extract and 0.3 ml of glass-distilled water was pre- incubated at 37˚C for 5 min. Then the reaction was initiated by the addition of 0.05 ml 0.2 M L-glutamine (final concentration, 20 mM). After incubation at 37 ˚C for 15 min, the reaction was terminated by the addition of 1ml of stop mixture (5.5 g $FeCl_3.6H_2O$, 2.0 g TCA and 2.1 ml concentrated HCl in a final volume of 100 ml). Samples were centrifuged to remove precipitate, and A_{540} of γ-glutamyl hydroxymate in the supernatant was read.

One unit (U) of GDH or GOGAT activity was defined as the amount of enzyme required to oxidize 1.0 nmol of NAD(P)H / min/ mg protein. One unit (U) of GS activity was defined as the amount of enzyme required to produce 1.0 nmol of γ-glutamyl hydroxymate / min/ mg protein.

2.2.10.4 Chitin deacetylase assay (CDA, E.C 3.5.1.41)

Chitin deacetylase assay activity estimated using ethylene glycol chitin as the substrate prepared according to the method of Nahar *et al*. (2004). Ethylene glycol chitosan (EGC, 40 mg) was treated with a mixture containing 400 mg

$NaHCO_3$ and 4.5 ml acetic anhydride and kept at 4 °C for 24 h. After addition of 200 µl acetic anhydride the mixture was allowed to stand further at 4 °C for 24 h. After dialysis, acetylated ethylene glycol chitosan (1mg / ml), was used as a substrate for the assay of CDA. The assay was carried out according to Kauss and Bausch (1988), with 100 µl of 50 mM sodium tetraborate buffer, pH 8.5, 100µl of 1mg/ml of EGC and 50 µl of enzyme incubated at 37 °C for 30 min. The reaction was terminated by the addition of 250 µl of 5 % (w/v) $KHSO_4$. For the color development, 250 µl of 5 % (w/v) $NaNO_2$ was added and allowed to stand for 15 min and 250 µl of 12.5 % (w / v) ammonium sulfamate ($N_2H_6SO_3$) was added. After 5 min, freshly prepared 250 µl 0.5 % (w / v) 3-methyl - 2- benzothiazoline hydrazone (MBTH) was added and the mixture was heated in a boiling water bath for 3 min. The tubes were cooled under tap water and 250 µl 0.5 % (w / v) $FeCl_3$ was added and estimated spectrophotometrically at 650 nm. One unit of enzyme released 1µ mol of acetate from ethylene glycol chitosan per min.

2.2.11 Protein estimation

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

Optical method

The protein estimation of the fractions, obtained from column chromatography during purification of NAD-GDH was carried out by optical method (Deshpande, 1981).

The protein concentration was measured by the absorbance (A) of the sample at 260 nm, 280 nm and 340 nm. The protein concentration (mg/ml) was calculated using the formula

4/7 [2.3(A 280 nm - A 340 nm) - (A 260 nm-A 340 nm)]

2.2.12 Estimation of reducing sugars

The reducing sugar glucosamine was measured by modified Elson-Morgan method using glucosamine as a standard (Reissig *et al*., 1955).

2.2.13 Enzyme purification

The crude cell extract of hyphal form of *B. poitrasii* was used for the isolation and purification of NAD-glutamate dehydrogenase by ammonium sulphate fractionation, DEAE column chromatography and affinity chromatography using Blue gel affi gel column. The preparative IEF was also carried out of cell extract to achieve single step purification of NAD-GDH.

All purification steps were carried out at 4 ˚C.

2.2.13.1 Isoelectric focusing

For the single step purification, the isoelectric focusing of cell extract was carried out using preparative isoelectric focusing unit on Horizontal Rotofer unit (60 ml) from Bio Rad, using ampholytes in the pH range 3-10. The 120 mg protein sample was mixed with 2 % ampholytes (pH 3-10) and run at 12 Watt where the current was 32 mA. The isoelectric focusing was carried out till the current observed reached a constant value 11 mA after 3 h. At the end of focusing, 20 fractions of 3 ml each were collected. The pH and the glutamate dehydrogenase activity of each

fraction were determined. The purity of the fraction containing maximum NAD-GDH activity was checked by native PAGE.

2.2.13.2 Ammonium sulphate fractionation

The precipitation of NAD-GDH from the cell extract of hyphal form of *B. poitrasii* was carried out as follows. The $(NH_4)_2SO_4$ was added up to 30 % saturation to the cell extract and mixture was stirred slowly for 45 min and the precipitate was separated by centrifugation at 4500 g at 4 ˚C. This is followed by addition of $(NH_4)_2SO_4$ to 50 % saturation to the supernatent. This mixture was stirred for 45 min and the precipitate was separated by centrifugation at 4500 g at 4 ˚C. The same process was repeated for 70 % and 90 % saturation. The precipitates thus obtained were dissolved in 5 mM potassium phosphate buffer (pH 7.2) containing 0.25 mM EDTA, 1 mM PMSF, 50 mM K_2SO_4 and 1 mM DTT and checked for NAD-GDH activity. The fraction having maximum activity was used for further purification.

2.2.13.3 DEAE ion exchange chromatography

DEAE - fast flow sepharose anion exchanger was used for purification of NAD-GDH of *B. poitrasii*. The ion exchanger was equilibrated with 30 mM K-phosphate buffer of pH 7.2 and a column (28 x 1.8 cm) was packed. Prior to loading, pH of the enzyme solution was adjusted to 7.2 and dialyzed it against 30 mM K-phosphate buffer of pH 7.2 containing 0.25 mM EDTA, 1 mM PMSF, 50 mM K_2SO_4, 1 mM DTT for 3-4 h. The column was washed with same buffer. The column was eluted with a 400 ml linear gradient of 0-0.6 M NaCl in 30 mM

potassium phosphate buffer of pH 7.2 containing, 0.25 mM EDTA, 1 mM PMSF, 50 mM K_2SO_4, 1 mM DTT. Fractions (3 ml) were collected at a flow rate 12 ml / h.

The different fractions collected after column chromatography was checked for NAD-GDH enzyme activity. The fractions having major specific activity were pooled. The pooled sample of enzymes was dialyzed using 30 mM potassium phosphate buffer of pH 7.2 containing 0.25 mM EDTA, 1 mM PMSF, 50 mM K_2SO_4, and 1mM DTT. The enzyme sample was concentrated by ultrafiltration using Amicon YM10 membrane.

2.2.13.4 Affinity chromatography

The fraction obtained after DEAE chromatography was loaded on a Blue gel affi gel (8.5 x 1.2 cm), equilibrated and washed with 30 mM potassium phosphate buffer (pH 7.2) containing 20 % glycerol, 0.25 mM EDTA, 1 mM PMSF, 50 mM K_2SO_4 and 1mM DTT. The column was eluted with a 200 ml linear gradient of 0-0.6 M NaCl in 30 mM potassium phosphate buffer of pH 7.2 containing, 0.25 mM EDTA, 1 mM PMSF, 50 mM K_2SO_4, and 1 mM DTT. Fractions (1.5 ml) were collected at a flow rate 6 ml / h.

The purity of the eluted enzyme sample was confirmed by native PAGE. The purified enzyme - NAD-GDH, thus obtained was used for further studies.

2.2.14 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was performed in 7.5 % (w/v) polyacrylamide slab gel at pH 8.3 as per Laemmli (1970) procedure. Protein staining was done using silver nitrate method of Blum *et al.* (1987).

2.2.15 Activity staining for NAD- and NADP - glutamate dehydrogenase

The activity staining was done according to the procedure of Amin *et al*. (2004). The intracellular proteins were separated on non-denaturing 7.5 % (w/v) polyacrylamide gels (pH 8.3). The staining mixture contained 100 mM L-glutamate, 0.06 mM phenazine methosulfate, 0.22 mM nitroblue tetrazolium and 0.026 mM NAD(P) in 100 mM K- phosphate buffer, pH 8.0. The gels were incubated in the staining solution until blue colored bands appeared. In order to eliminate the possibility of an artifact caused due to alcohol dehydrogenase, the staining was also carried out in the absence of glutamate (substrate). The gels were stored in methanol, acetic acid, water mixture (40: 10: 50).

2.2.16 Chitosan extraction

Chitosan extraction from *B. poitrasii* was carried out using method described by Chatterjee *et al.* (2005). The 24 h old yeast or hyphal cells suspension of *B. poitrasii* grown on YPG and YP medium, respectively were filtered using Whatman filter paper No.1 and washed with ice-cold distilled water. The washed biomass was homogenized in 1N NaOH using Braun's homogenizer for 4 cycles each of 15 sec. The homogenized mixture was further centrifuged and the cell wall pellet thus obtained was suspended in 1N NaOH in the ratio 1: 40, followed by autoclaving at 121 ˚C for 15 min to obtain a protein free fraction of the cell wall. The alkali insoluble mass thus obtained was washed thoroughly with distilled water. Then further washed with ethanol followed by centrifugation at 12000 g for 10 min. The residue was refluxed in 100 volumes of 2 % acetic acid at 95 ˚C for 24 h. The slurry was centrifuged at 12000 g at 4 ˚C for 45 min. Chitosan was precipitated out from

the supernatant when the pH was adjusted to 8.5 with 1N NaOH. The precipitate was washed several times with chilled water. The sample was then treated with acetone and dried in oven and stored at -20 ˚C.

2.2.17 Fluorimetric studies

The yeast or hyphal cells as well as sporangiospores and zygospores of *B. poitrasii* were homogenized in a Braun's homogenizer in cold potassium phosphate buffer (100 mM, pH 8.0). The cell extract thus obtained was centrifuged at 12,500 g for 15 min and the supernatant was used to determine the NAD and NADP contents by the fluorimetry at 340 nm excitation and 420 nm emission. NAD and NADP (SRL, India) were used to standardize the fluorimeter (LS 50 B, Perkin Elmer, USA).

2.2.18 NMR spectroscopy

The degree of deacetylation of chitosan of the hyphal and yeast forms of *B. poitrasii* was determined by NMR spectroscopy. All the NMR spectra were taken on a Bruker DR x 500 FT- NMR spectrophotometer operating at the Larmor frequency of 500 and 125 MHz for ^{1}H and ^{13}C, respectively.

^{13}C solid- state NMR experiments were carried out at ambient probe temperature (20 - 22 ˚C), using a Hartmann-Hahn cross - polarization scheme at an applied r. f field of 40 kHz, combined with Magic Angle Sample Spinning (CP/ MASS). The optimum mixing time of 1ms and a 5 s recycle delay were used. The spinning speed was kept at 10 kHz in order to minimize the interfering effects of spinning side bands. Free induction decays were collected in a 50 - kHz spectral window and were apodized with a 20 - Hz line broadening to increase the S / N. The

^{13}C *T*2 measurements were carried out under MASS by tailoring a spin - echo sequence to the CP sequence and the variable τ delay was synchronized to be an integer number of rotor revolutions. The decays were analyzed by online software. The chemical shifts were referenced to an external sample of spinning adamantine, with its high frequency signal taken as 37.8 ppm with respect to TMS. Quadrature phase cycling was incorporated in the pulse sequence to eliminate baseline and intensity artifacts.

Sample preparation for liquid state ^{1}H NMR

The solutions of chitosan were prepared by stirring at room temperature 10 mg of chitosan in a solution composed of 1.96 ml of D_2O and 0.04 ml of HCl (20 % in D_2O) and waiting about 30 min to ensure complete dissolution of the polymer. In these solutions, HCl is in excess compared with amino groups of chitosan so that the polymer is easily dissolved as described by Lavertu *et al*. (2003).

Method

The liquid - state ^{13}C spectra were collected using AV 400 operating at 400 MHz for proton. Samples were analyzed in a standard 5mm broadband probe at 70 ˚C, using D_2O lock. The experiments were run at 70 ˚C, temperature at which the solvent peak does not interfere with any of chitosan peaks. The ^{1}H spectra were collected with a spectral width of 6000 Hz, 30˚ flip angle and 5 sec relaxation delay. 200 transients were collected and processed with a line broadening of 0.3Hz pores to Fourier transformation.

$$DD = \frac{[I_{4.2\text{-}3.0} / 5] \times 100}{I_{2.0} / 3}$$

DD- Degree of deacetylation

I- Integral of peaks

The degree of deacetylation is calculated using above formula from the integral of the peaks in the region of 4.2 to 3.0 ppm, the area of peak at ~ 2.0 ppm.

Chapter III: Effect of different environmental conditions on glutamate dehydrogenase activities and its correlation with different morphological forms in *Benjaminiella poitrasii*

3.1.A Introduction

Dimorphism is the ability of the fungi to grow reversibly in two morphological forms *viz*, yeast and hypha. In general, studies on morphogenesis in fungi have been focused on the biochemical changes that occur during differentiation. In this regard enzymes involved in carbon and nitrogen metabolism and cell wall synthesis /degradation have been studied extensively (Deshpande, 1996).

In plants, for ammonia assimilation the GS/GOGAT pathway is predominant (Srivastava and Singh, 1987; Stewart *et al*., 1995). In fungi such as, *S. cerevisiae* (Avendano *et al*., 1997), *M. racemosus* (Peters and Sypherd, 1979), *C. boidinii* (Green and Large, 1984) and *C. utilis* (Fergusson and Sims, 1974) the NAD- and NADP-GDH play a major role as compared to the GS / GOGAT pathway. However in *C. tropicalis*, *C. parasilopsis* and *C. albicans* the GS / GOGAT pathway was reported to be significant for ammonia assimilation (Holmes *et al*., 1989). In *N. crassa* the NADP-GDH was found to be functioning in presence of high ammonium concentration, while the GS/ GOGAT pathway was significant when ammonium concentration was low (Lomnitz *et al*., 1987). In *A. nidulans* nitrogen assimilation was reported to follow the GS / GOGAT pathway predominantly (Macheda *et al*., 1999).

Both NAD^+ specific (E.C 1.4.1.2) and $NADP^+$ specific (E.C 1.4.1.4) of glutamate dehydrogenases were described in various fungi (Amin *et al*., 2004; Avendano *et al*., 1997; Perysinakis *et al*., 1995). A third type of enzyme, a GDH which used both NAD^+ and $NADP^+$ as cofactor (EC 1.4.1.3) was commonly reported from animal kingdom (Coleman and Foster, 1970).

Stewart and Moore (1974) suggested that in *Coprinus lagopus* NAD-GDH was normally involved in the hyphal growth while NADP-GDH being reserved for specific functions such as sporophore development.

In fungi, both NAD- and NADP- GDHs also occupy a key position in interlinking the carbon and nitrogen metabolism in chitin (a main cell wall polymer) synthesis. The morphology-associated expression of NAD-GDH was reported in a dimorphic fungus *M. racemosus* (Peters and Sypherd, 1979). In *B. poitrasii* the significance of the relative proportion of both NAD- and NADP- GDHs measured as NADP- / NAD-GDH ratio in the yeast-hypha transition was reported (Khale *et al*., 1992). Furthermore, Khale-Kumar and Deshpande (1993) described the involvement of cAMP dependent protein kinase in the regulation of NADP- and NAD-GDH activities and in dimorphic transition of *B. poitrasii*. Amin *et al.* (2004) reported the presence of one NAD-GDH in both yeast and hyphal form cells and two active NADP-GDHs, one expressed in hyphal form and other in yeast form, in the parent strain of *B. poitrasii*.

The present investigations aim to study the effect of temperature, pH of the growth medium, glucose, divalent metal ions like Zn^{++} and metal chelators on Y-H and reverse transition. The significance of nitrogen assimilating enzymes, in Y-H transition is also discussed.

Fig 3.1 Different phases in the life cycle of *B. poitrasii* a), yeast and hypha cells b) yeast-hypha transition c) hypha yeast transition, terminal budding d) hypha-yeast transition, lateral budding e) asexual sporangioles f) zygospore formation in: ladder like manner indicated by the arrow g) yeast like germination of sporangiospore and h) hyphal germination of sporangiospore

3.2.A Results

3.2.1.A Effect of temperature of incubation on Y-H transition in *B. poitrasii*

The Y-H transition in *B. poitrasii* was studied at different temperatures of incubation, ranging from 22 ˚C to 37 ˚C. The transition experiments were carried out as mentioned under Materials and Methods in YPG (0.1 %, glucose) for 12 h. It can be seen from the Table 3.1 that percent germ tube formation decreased from 80.0 % to 10.6 % as temperature increased from 22 - 37 ˚C.

Table 3.1 Effect of temperature of incubation on Y-H transition in *B. poitrasii*

Temperature of incubation (°C)	Germ tube (%)	NAD-GDH (U/mg)	NADP-GDH (U/mg)	NADP- / NAD-GDH ratio
22	80.0	1134.0 ± 71.0	6.8 ± 1.1	0.006
28	56.7	866.0 ± 55.0	10.0 ± 1.0	0.012
32	36.3	385.0 ± 30.0	16.9 ± 2.4	0.044
37	10.6	136.0 ± 22.0	21.0 ± 3.2	0.154

The NAD-GDH activity was decreased from 1134.0 ± 71.0 U/ mg at 22 ˚C to 136.0 ± 22.0 U/ mg at 37 ˚C, while NADP-GDH activity was increased from 22 ˚C (6.8 ± 1.1 U / mg) to (21.0 ± 3.2 U / mg) at 37 ˚C. The relative proportion of NADP-GDH and NAD-GDH activities increased from 0.006 to 0.154 from the hyphal form (80 % germ tube) to the yeast form (10.6 % germ tube) during Y-H transition (Table 3.1).

3.2.2.A Effect of medium pH on Y-H transition in *B. poitrasii*

The effect of pH on Y-H transition in *B. poitrasii* was studied at different initial pHs, of the YPG (0.1 %, glucose) medium ranging from 4.0 to 8.0. The transition experiments were carried out at 28 ˚C for 12 h.

Table 3.2 Effect of medium pH on Y-H transition in *B. poitrasii*

pH	Germ tube (%)	NAD-GDH (U/mg)	NADP-GDH (U/mg)	NADP- / NAD-GDH ratio
4	3.2	101.0 ± 82.0	44.0 ± 11.0	0.435
5	6.0	128.0 ± 68.0	32.0 ± 7.0	0.250
6	56.7	866.0 ± 69.0	10.0 ± 1.0	0.012
7	60.0	900.0 ± 55.0	9.0 ± 1.4	0.010
8	86.0	1200.0 ± 80.0	6.0 ± 0.7	0.005

As compared to initial pH 6.0, with initial acidic pHs the Y-H transition was retarded while it was increased with initial alkaline pH (Table 3.2). Both the enzyme activities and their relative proportions followed the trend, which can be correlated with the morphological outcome, *viz*. yeast or hyphal form.

3.2.3.A Effect of glucose in YNB medium on the morphological outcome in *B.poitrasii*

The effect of glucose concentration in medium containing organic nitrogen source (complex medium) on morphology and specific activities of ammonium assimilating enzymes in *B. poitrasii* was studied by Khale *et al*. (1992). This transition in complex organic (YPG) medium was under the influence of glucose

and organic nitrogen, which favored yeast-form in *B. poitrasii*. Therefore to study the effect of glucose on the morphological outcome the semi-synthetic medium (YNB) was used. The experiments were carried out using different glucose concentrations ranging from 0.1 to 5.0 % in YNB medium at 28 ˚C for 48 h, and in YPG (1 %, glucose) for comparison.

Table 3.3 Effect of glucose in YNB medium on the morphological outcome in *B. poitrasii*

Glucose concentration (%)	Form	NAD - GDH (U/mg)	NADP- GDH (U/mg)	NADP- / NAD-GDH ratio
0.10	H>Y	930.0 ± 140.0	12.0 ± 0.01	0.012
0.25	H<Y	224.0 ± 123.0	21.0 ± 0.02	0.093
0.50	H<Y	212.0 ± 46.0	27.0 ± 0.09	0.127
1.00	Y	120.0 ± 18.0	95.0 ± 0.14	0.788
5.00	Y	35.0 ± 5.0	139.0 ± 0.20	3.971
YPG (1%)	H<Y	84.02 ± 7.5	19.9 ± 0.03	0.236

As reported earlier by Khale *et al*. (1992), the NAD-GDH activity was repressed while NADP-GDH activity was induced as the glucose concentration was increased in the medium. The interesting observations were with 1 % glucose in both semi-synthetic and complex nitrogen media. As compared to YPG (1 %, glucose), the GDH ratio required to maintain Y form in a semi-synthetic medium was higher *viz*. 0.788 as compared to 0.236 (Table 3.3) observations were noted earlier in case of Y form mutants by Khale *et al*. (1992).

3.2.4.A Effect of different brands of peptone in growth medium on Y-H transition in *B. poitrasii*

Peptones from different commercial sources vary in their composition due to change in the source material and / or processing (Table 3.4). The concentrations of Zn^{++}, Mg^{++} and inorganic phosphate in Difco, Sarabhai and Hi-media were found to be different (Chacko *et al*., 1996). It was therefore, interesting to check the effect of different peptones used in the medium on Y-H transition and GDH, GS and GOGAT activities. The transition experiments were carried out in YP medium without glucose using yeast extract (Difco) and peptones from different commercial sources at 28 ˚C for 12 h. The concentrations of Zn^{++} were 0.01, 0.09 and 0.16 ppm in Difco, Sarabhai and in Hi-media peptone, respectively.

Table 3.4 Composition of peptones from different commercial sources

Component	Difco	Sarabhai chemicals	Hi - media
Mg^{++}	0.22	4.00	0.86
Mn^{++}	0.00	1.07	0.17
Fe^{++}	0.17	2.25	0.10
Zn^{++}	0.01	0.09	0.16
DNA	0.13	1.50	0.56
RNA	0.31	7.15	3.30
Pi	8.43	39.46	135.88

Adapted from Chacko *et al*. (1996)

The above values represent the effective concentrations (metal ions in ppm, DNA and RNA in mg, and inorganic phosphate (Pi) in mM) of each component when the respective peptones were used at a concentration of 5.0 g / L of the medium.

A significant change was noted in the specific activities of NAD-GDH and NADP-GDH and their relative proportion (GDH ratio) (Table 3.5). The germ tube formation was significantly affected in the presence of peptone from Himedia (17.64 %) as compared to the peptone used from Difco (61.50 %).

Table 3.5 Effect of different peptones on Y-H transition in *B. poitrasii*

Peptone	Germ tube (%)	NAD-GDH (U/mg)	NADP-GDH (U/mg)	NADP-/ NAD-GDH ratio
Difco	61.50	900.0 ± 45.0	9.0 ± 0.15	0.010
Sarabhai chemicals	27.15	244.0 ± 32.0	6.3 ± 0.12	0.026
Hi-Media	17.64	215.0 ± 31.0	8.1 ± 0.10	0.037

In the Difco medium NAD-GDH activity was 900.0 U/mg. The NAD-GDH activity was repressed in the presence of peptones from Sarabhai and Himedia, *viz*. 244.0 ± 32.0 and 215.0 ± 31.0 U/mg, respectively. The NADP-GDH activities were marginally affected .The GDH ratios showed correlation with morphological outcome i.e. lower ratio (0.010) in Difco while higher (0.037) in Himedia peptone medium (Table 3.5).

Table 3.6 Effect of different peptones in growth medium on glutamine synthetase and glutamate synthase activities in *B. poitrasii*

Peptone	GS activity (U/ mg)	NAD-GOGAT (U/ mg)	NADP-GOGAT (U/ mg)
Difco	7.68 ± 0.46	13.50 ± 1.0	4.80 ± 0.29
Sarabhai chemicals	4.63 ± 0.21	16.28 ± 1.5	3.81± 0.25
Hi-Media	6.01± 1.16	15.66 ± 1.4	3.94 ± 0.40

Though it has been reported earlier (Khale *et al*., 1992) and observed in the present studies that GDH activities have significant correlation with morphological outcome, the effect of peptones on GS and GOGAT activities was also studied (Table 3.6). There was no significant correlation between composition of peptone (mainly Zn^{++}) and GS, NAD-GOGAT and NADP-GOGAT activities.

3.2.5.A Effect of Zn^{++} on Y-H transition in *B. poitrasii*

The effect of addition of Zn^{++} in Difco medium on Y-H transition was studied. As glucose favors yeast formation in *B. poitrasii,* the experiment was carried out in YP medium as described under Materials and Methods at 28 ˚C for 12 h.

Table 3.7 Effect of Zn^{++} on Y-H transition in *B. poitrasii*

Addition of Zn^{++} (ppm)	Germ tube (%)	NAD-GDH (U/mg)	NADP- GDH (U/mg)	NADP-/ NAD-GDH ratio
No addition	61.50	952.0 ± 45.0	9.22 ± 0.8	0.009
0.10	33.75	357.0 ± 36.0	17.71 ± 0.5	0.049
0.15	29.39	215.0 ± 30.0	18.10 ± 0.3	0.084
0.50	23.79	204.0 ± 30.0	19.00 ± 0.2	0.093

It can be seen from Table 3.7 that NAD-GDH activity was repressed as the Zn^{++} concentration was increased from 0.01 ppm (Difco with no addition) to 0.51 ppm (Difco with 0.5 ppm addition). While in case of NADP-GDH activity the effect of Zn^{++} addition over 0.1 ppm was marginal. The GDH ratio showed the biochemical correlation with morphological outcome *viz*. low ratio (0.009) when

germ tube percent was high (61.50 %) and high ratio (0.093) when Y-H transition was retarded to a greater extent (23.79 %).

3.2.6.A Effect of addition of EDTA in the growth medium on the Y-H transition in *B. poitrasii*

In the earlier section (3.1.2.5) the effect of Zn^{++} addition in Difco medium was studied. The Table 3.8 describes the effect of an addition of EDTA (20 ppm) in Hi-media YP medium. It can be seen from the Table 3.8, germ tube formation increased from 17.64 % to 48.80 %, in YP and YP + EDTA medium, respectively. Difco medium was used as a control for the minimum level of Zn^{++} (0.01 ppm).

Table 3.8 Effect of addition of EDTA in the growth medium on the Y-H transition in *B.poitrasii*

Medium	Germ tube (%)	NAD-GDH (U/mg)	NADP-GDH (U/mg)	NADP- / NAD-GDH ratio
YP (Difco)	61.50	952.0 ± 45.0	9.22 ± 0.8	0.009
YP (Hi-media)	17.64	285.0 ± 31.0	28.1 ± 1.0	0.098
YP (Hi-media + EDTA 20 ppm)	48.80	576.0 ± 35.0	16.8 ± 0.5	0.029

It further indicated that addition of higher concentration of EDTA could be required to achieve same level of Y-H transition, measured as (%) germ tube formation in 12 h. The GDH activities showed biochemical correlation with germ tube formation.

3.2.7.A Effect of *in vitro* addition of Zn^{++} in assay mixture on glutamate dehydrogenase activity of *B. poitrasii*

The effect of Zn^{++} (0.1 and 0.5 ppm) in a reaction mixture on the specific activities of NAD- and NADP-GDH of yeast and hyphal form was studied. The *B. poitrasii* yeast cells were grown in YPG (1 %, glucose) medium at 37 ˚C and the hyphal cell in YP medium at 28 ˚C for 24 h, and the crude cell extract was obtained as mentioned under Materials and Methods.

Table 3.9 Effect of *in vitro* addition of zinc in assay mixture on glutamate dehydrogenase activities of *B.poitrasii*

Addition of Zn^{++} (ppm)	Yeast		Hypha	
	NAD-GDH (U / mg)	NADP- GDH (U / mg)	NAD - GDH (U / mg)	NADP- GDH (U / mg)
No addition	92.0 ± 7.5	15.3 ± 2.1	724.0 ± 22.0	1.40 ± 0.20
Zn^{++} 0.10 ppm	50.0 ± 6.0	23.0 ± 2.4	861.0 ± 24.0	2.20 ± 0.23
Zn^{++} 0.50 ppm	43.0 ± 5.0	33.2 ± 3.2	807.0 ± 24.0	1.60 ± 0.21

The NAD-GDH activity of Y form decreased by Zn^{++} (0.1 ppm) addition while NADP-GDH of Y-form and NAD- and NADP-GDH of H-form were increased (Table 3.9). It can be seen from the Table 3.9, there was no proportional effect of enzyme activation or inhibition when concentration was increased from 0.1 to 0.5 ppm.

3.3.A Discussion

In most of the dimorphic fungi, glucose and temperature changes affect Y-H or *vice versa* transition (Deshpande, 1996). The temperature and glucose concentration in the medium both affected germ tube and budding in *B. poitrasii* (Ghormade *et al*., 2005; Khale *et al*., 1992). The studies on the effect of different

incubation temperatures on the Y-H transition and on GDH activities suggested that temperature had more pronounced effect on NAD-GDH activity than the NADP-GDH activity in *B. poitrasii* (Table 3.1).

The increase in the temperature is known to cause a transient dissipation of the cytochemical pH gradient across the plasma membrane leading to decrease in the internal pH of the cell (Coote *et al*., 1991). Intracellular acidification is also known to be effected by glucose utilization that in turn stimulates cAMP synthesis (Gadd, 1995). The change in internal pH has been shown to regulate many processes of cellular differentiation. For example, in case of *C. albicans* the switch from budding yeast to germ tube formation was associated with the alkaline cytoplasmic pH (Robson *et al*., 1996). The internal pH is influenced by pH of the medium in which the cells are suspended. In case of *B. poitrasii* acidic pH (4.0) favoured the yeast form while the alkaline pH (8.0) promoted the hyphal growth, as evident by the percent germ tube formation (Table 3.2).

In *B. poitrasii* effect of glucose on the transition in the organic N medium and on its GDH activities was studied extensively (Khale *et al.*, 1992). Subsequently, Ghormade *et al*. (2005) reported the overriding effect of temperature signal over glucose. Recently, the studies on the overriding effect of the dimorphism triggering factors, temperature and glucose were reported (Ghormade, *et al*., 2005). During sequential exposures, the temperature exposure reversed the glucose effect in a complex organic N medium. This was attributed to the sharing of the common signaling pathway by both, temperature and glucose triggers (Ghormade *et al*., 2005). The effect of glucose in the semi-synthetic medium on Y-H transition was

studied (Table 3.3). Interestingly, the GDH ratio required to maintain yeast form was relatively higher than the cells growing in the YPG (1 %, glucose) medium (Table 3.3).

The effect of peptones from different commercial sources was mainly correlated with the Zn^{++} concentration (Table 3.5). The presence of Zn^{++} affected morphological outcome in *C. albicans* (Bedell and Soll, 1979; Yamaguchi, 1975), *M. rouxii* (Bartnicki -Garcia and Nickerson, 1962b), *H. capsulatum* (Maresca and Kobayashi, 2000) and *A. pullulans* (Krogh *et al*., 1998). The increased levels of Zn^{++} always favored yeast morphology in these fungi. In case of *B. poitrasii* the addition of Zn^{++} in Difco medium retarded germ tube formation (Table 3.7). Alternatively, the EDTA addition in Himedia (with high Zn^{++} concentration) favored germ tube formation (Table 3.8). The studies with bovine glutamate dehydrogenase (Coleman and Foster, 1970), explained the functions of Zn^{++} as an allosteric inhibitor. Bedell and Soll, (1979) reported in the case of *C. albicans* that hypha formation took place by zinc sensitive and zinc resistant pathways. It was also observed in case of *C. albicans* that in the presence of Zn^{++} ions, RNA synthesis was ceased which in turn inhibited the germ tube formation and thus favored yeast form (Yamaguchi, 1975). In case of *M. rouxii* (Bartnicki-Garcia and Nickerson, 1962 b) and *S. cerevisiae* (Asleson *et al*., 2000) the chelation of metal ions by EDTA was reported to affect morphogenesis. In the present investigations, Zn^{++} (0.1 ppm) was found to repress NAD-GDH activity while NADP-GDH activity was increased (Table 3.7). However, further increase in the Zn^{++} concentration affected NAD-GDH marginally. The reverse trends in the activities were seen when EDTA was added during growth

(Table 3.8). In the *in vitro* experiments Zn^{++} (0.1 ppm) was found to inhibit NAD-GDH of yeast form while NADP-GDH of yeast form and NAD- and NADP-GDH activities of hyphal form were found to be increased (Table 3.9). However, further increase in the Zn^{++} concentration to 0.5 ppm did not show any significant effect. Thus, the investigations suggested that the effect of Zn^{++} on glutamate dehydrogenase activities could also be one of the factors involved in controlling Y-H transition in *B.poitrasii*.

B Isolation of morphological mutants of *B. poitrasii*

3.1.B Introduction

Most of the studies on fungal morphogenesis are carried out to understand biochemical correlates of the differentiation (Martinez-Pacheco *et al*., 1989). The correlation in biochemical changes associated with morphogenesis is not yet clear due to variations in response to stimuli inducing transitions. One of the ways to understand dimorphism is the isolation of monomorphic mutants or the non-dimorphic variants. Ruiz-Herrera *et al*. (1983) studied monomorphic mutants of *M. bacilliformis* to distinguish alterations associated with morphogenesis. Cannon (1986) and Gil *et al*. (1990) isolated monomorphic mutants of *Candida* in order to understand the regulatory mechanism involved in dimorphism. The efforts were also made for isolation of stable yeast form mutants of *C. albicans* after mutagenesis with ethyl methane sulfonate (EMS) (Elorza *et al*., 1994).

Khale *et al.* (1990) reported the isolation of yeast form mutants Y-2 and Y-5 as well as slow growing hyphal mutant M-1 of *B. poitrasii* after chemical mutagenesis using *N*-Methyl-*N*′-nitro-*N*- nitrosoguanidine (MNNG) treatment of the

parent strain spores. As the complex nitrogen favored yeast-form growth, the initial yeast growth though lesser than the parent strain was observed in case of M1 mutant. In the true sense, the M1 morphological mutant was not monomorphic hyphal mutant. Ghormade (2000) reported that on the synthetic media, *B. poitrasii* sporangiospores germinate directly into hyphal form. The present investigations report the attempts made to isolate different morphological mutants either yeast or hyphal form, on the complex nitrogen source and synthetic medium and their evaluation with respect to glutamate dehydrogenase activities along with parent and Y-5 mutant.

3.2.B Results

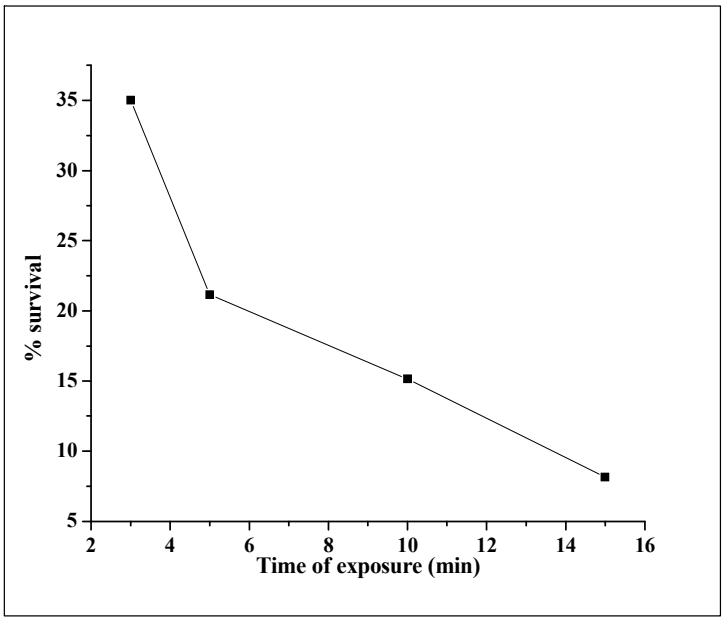
3.2.1.B UV mutagenesis

Attempts were made for the isolation of morphological mutants using physical mutagen such as ultraviolet light. The sporangiospores obtained from 7 d old slant of *B. poitrasii*, were spread on modified Czapek Dox medium (pH 8) and YPG (1 %, glucose). As reported earlier (Ghormade, 2000) during germination, the sporangiospore initially enlarged and produced bud like structure in presence of organic nitrogen *viz*. peptone and yeast extract that subsequently produced yeast colony or hyphal form in response to the environmental conditions. In the synthetic modified Czapek Dox medium containing $NaNO_3$ and glucose (0.1 %) and pH 8.0, the sporangiospore germinated by producing germ tube at 28 ˚C in 72 h.

The plates spread with a suspension of sporangiospores of *B. poitrasii* were placed at various distances (15, 20, 25 cm) from the UV light source (Philips UV germicidal lamp, 15W) and exposed for different time intervals (3, 5, 10, 15 min). The growth was examined by monitoring colony characteristics and observing the

morphology microscopically upto 120 h at an interval of 24 h. No growth was observed on modified Czapek Dox and YPG (1 %, glucose) medium plates kept at a distance of 15 and 20 cm away from the UV source and exposed for 3, 5, 10 and 15 min.

Fig. 3.2 Survival % of sporangiospores of *B. poitrasii* after exposure to UV light on YPG (1 %, glucose medium)



The percent survival on YPG (1 %, glucose) from 25 cm distance exposed for 3, 5, 10, 15 min depleted in Fig. 3.2. The plates showing less than 10 % survival after the exposure for 15 min were examined for their morphological and cultural characteristics.

Table 3.10 Colony characteristics of the isolates obtained after UV mutagenesis (YPG, 1 % glucose medium)

Isolate	Size (mm)	Shape	Colour	Margin	Elevation	Consistency
uv 1a	1-2	Circular	Off-white	Entire	Flat	Moist
uv2a	3	Circular	Off-white	Serrate	Flat	Moist
uv3a	2	Circular	Off-white	Entire	Flat	Moist
uv4a	3	Circular	White	Irregular	Raised	Dry
uv5a	3-4	Circular	White	Irregular	Raised	dry
uv6a	3	Circular	White	Irregular	Raised	dry
uv7a	2	Circular	Off-white	Entire	Flat	moist
uv8a	3	Circular	White	Irregular	Raised	dry
uv9a	3-4	Circular	White	Irregular	Raised	dry
uv10a	3	Circular	Off-white	Irregular	Flat	moist
uv11a	4	Circular	White	Irregular	Raised	dry
uv12a	5	Circular	White	Irregular	Raised	dry
uv13a	4-5	Circular	White	Irregular	Raised	dry
uv14a	7	Circular	White	Irregular	Raised	dry
uv15a	4-5	Circular	White	Irregular	Raised	dry
uv 16a	5	Circular	White	Irregular	Raised	dry
uv17a	4	Circular	White	Irregular	Raised	dry
uv18a	5	Circular	White	Irregular	Raised	dry
uv19a	4	Circular	White	Irregular	Raised	dry

The isolate on YPG (1 %, glucose) named uv1a to uv3a and uv7a showed 2-3 mm, off white, circular, opaque colonies with entire margin and moist consistency while uv4a - uv 19a colonies except uv7a showed irregular margin indicating presence of germ tube forming cells at the periphery under the microscope (Table 3 .10).

Table 3.11 Colony characteristics of isolates obtained after UV mutagenesis (Modified Czapek Dox medium)

Isolate	Size (mm)	Shape	Colour	Margin	Elevation	Consistency
uv1b	2	Irregular	White	Irregular	Raised	Dry
uv2b	3	Irregular	White	Irregular	Raised	Dry
uv3b	2-3	Irregular	White	Irregular	Raised	Dry
uv4b	3	Irregular	White	Irregular	Raised	Dry
uv5b	3	Irregular	Greyish	Irregular	Raised	Dry
uv6b	3	Irregular	White	Irregular	Raised	Dry
uv7b	2-3	Irregular	White	Irregular	Raised	Dry
uv8b	2	Irregular	White	Irregular	Raised	Dry
uv9b	3	Irregular	Greyish	Irregular	Raised	Dry
uv10b	3	Irregular	Greyish	Irregular	Raised	Dry
uv11b	3	Irregular	White	Irregular	Raised	Dry
uv12b	3	Irregular	White	Irregular	Raised	Dry

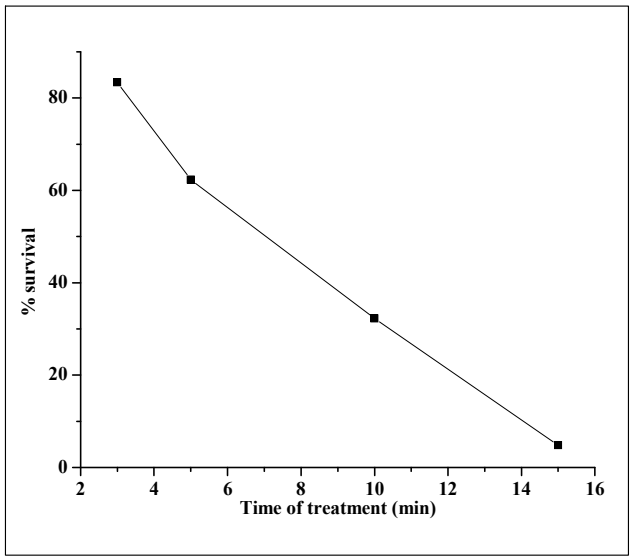
The morphological and cultural characteristics of the 12 isolates on modified Czapek Dox medium were also noted which were 2-3 mm in size, white, with a dry consistency and with an irregular margin. But none of the mutants showed exclusive yeast or hyphal growth. The difference in the colony characteristics can be attributed to the different percent of yeast or hyphal cells and the growth rate (Table 3.11).

3.2.2.B Chemical mutagenesis

a). Ethyl methane sulfonate (EMS)

The sporangiospore suspension (2 x 10^4 spores / ml) of *B. poitrasii* was treated with 10-40 mg / ml of EMS for 3, 5, 10, 15 min. After addition of 10 % $Na_2S_2O_3$ as quenching agent, 2-3 washes with sterile distilled water were given to the suspension by centrifugation at 2000g for 10 min

Fig 3.3 Survival % of sporangiospores of *B. poitrasii* after treatment with EMS on YPG (1 %, glucose) medium



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The spore suspension was then plated on YPG (1 %, glucose; pH 6.5) and modified Czapek Dox medium (pH 8) at 28 ˚C, which were observed after an interval of 24 h upto 5 days. When the suspension of sporangiospore was treated with EMS 10, 20, 30 mg / ml survival was > 90 % as compared to the untreated sporangiospores plated on YPG (1 %, glucose) and Czapek Dox medium. Therefore, for isolation of morphological mutant the EMS concentration used was 40 mg / ml and the % survival for 3, 5, 10, 15 min was 83.37, 62.36, 32.25 and 4.83 % respectively on YPG (1 %, glucose) medium (Fig 3.3).

The plates showing less than 10 % survival after exposure for 15 min were examined for their morphological and cultural characteristics. The isolates EMS 1a to EMS4a were circular, off white coloured with entire margin and moist consistency (Table 3.12). The isolates EMS5a to EMS9a were white, circular in shape, with an irregular margin, raised and dry consistency.

Table 3.12 Colony characteristics of isolates obtained after chemical mutagenesis using EMS (YPG, 1% glucose medium)

Isolate	Size (mm)	Shape	Colour	Margin	Elevation	Consistency
EMS1a	3	Circular	Off-white	Entire	Flat	Moist
EMS2a	3	Circular	White	Entire	Flat	Moist
EMS3a	2-3	Circular	Off-white	Entire	Flat	Moist
EMS4a	3	Circular	White	Entire	Flat	Moist
EMS5a	3-4	Circular	White	Irregular	Raised	Dry
EMS6a	4	Circular	White	Irregular	Raised	Dry
EMS7a	3	Circular	White	Irregular	Raised	Dry
EMS8a	3	Circular	White	Irregular	Raised	Dry
EMS9a	3	Circular	White	Irregular	Raised	Dry

The morphological and cultural characteristics of the 5 isolates on modified Czapek Dox medium were also noted which were 2-3 mm in size, white, with a dry consistency and an irregular margin except for EMS 2b and EMS 3b which were found to be greyish in colour (Table 3.13).

Table 3.13 Colony characteristics of isolates obtained after chemical mutagenesis using EMS (Modified Czapek Dox medium)

Isolate	Size (mm)	Shape	Colour	Margin	Elevation	Consistency
EMS1b	2	Irregular	White	Filamentous	Raised	Dry
EMS2b	2-3	Irregular	Greyish	Filamentous	Raised	Dry
EMS3b	3	Irregular	Greyish	Filamentous	Raised	Dry
EMS4b	3	Irregular	White	Filamentous	Raised	Dry
EMS5b	2	Irregular	White	Filamentous	Raised	Dry

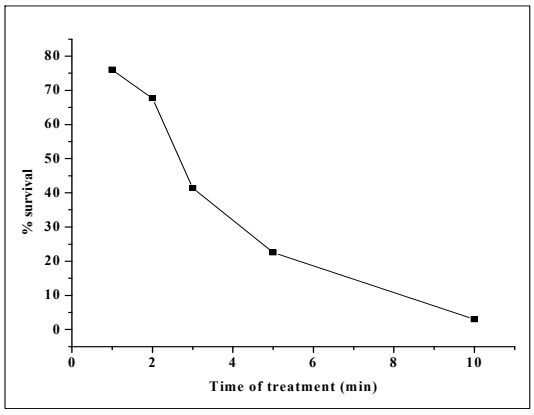
b). N-Methyl-N'-nitro-N- nitrosoguanidine (MNNG)

The sporangiospore suspension (2 x 10^4 spores / ml) of *B. poitrasii* were treated with 200- 4000 µg / ml of MNNG for 1, 2, 3, 5, 10 min. After addition of equal volume of 1 % $Na_2S_2O_3$ as a quenching agent. The spore suspension was then

plated on YPG (1 %, glucose) and Czapek Dox medium (pH 8) at 28 ˚C observed after an interval of 24 h upto 120 h.

The sporangiospore suspension could not survive at MNNG concentrations of 4000, 2000, 1000, 400µg / ml. Therefore, further experiments were carried out using MNNG at 200 µg / ml and the sporangiospores were treated for 1, 2, 3, 5 and 10 min (Fig 3.4).

Fig 3.4 Survival % of sporangiospores of *B. poitrasii* after treatment with MNNG on YPG (1 %, glucose) medium



The YPG (1 %, glucose) plates showing less than 3 % survival after exposure for 10 min were examined for their morphological and cultural characteristics and described in Table 3.14. The isolate MNNG 1a obtained after MNNG treatment was off white in colour, circular in shape, with entire margin and moist consistency. The other isolates MNNG 2a, MNNG 3a and MNNG 4a were circular, white, with serrated margin, raised and moist consistency. On Czapek Dox medium, however, colonies were not seen.

Table 3.14 Colony characteristics of isolates obtained after chemical mutagenesis using MNNG (YPG, 1% glucose medium)

Isolate	Size (mm)	Shape	Colour	Margin	Elevation	Consistency
MNNG1a	2	Circular	Off white	Entire	Flat	Moist
MNNG2a	2-3	Circular	White	Entire	Raised	Moist
MNNG3a	3	Circular	White	Entire	Raised	Moist
MNNG4a	2-3	Circular	White	Entire	Raised	Moist

Less than 10 % mutants, obtained after UV, EMS and MNNG treatments, which survived, were slow growing and also showed delayed sporulation as well as zygospore formation (4-6 d) at 28 ˚C when incubated under hypha favoring conditions. Furthermore, when these germinated colonies were inoculated on YPG (1 %, glucose) medium at 37 ˚C for 48 h i.e. under yeast favoring conditions the yeast cells were developed. As we were interested in the isolation of yeast or hyphal form monomorphic mutants which survived in one form under dimorphism triggering conditions. The isolated mutants did not fulfill the requirement of the present investigations. Therefore, the investigations were carried out using Y-5 mutant, which was isolated earlier in the group (Khale *et al*., 1990).

3.2.3.B Effect of temperature of incubation on Y-5 mutant of *B. poitrasii*

Effect of temperature of incubation on Y-5 mutant of *B. poitrasii* was studied from 22 ˚C to 37 ˚C. The experiments were carried out in YPG (0.5 %, glucose) for 48 h as mentioned under Materials and Methods.

Table 3.15 Effect of temperature of incubation on Y-5 mutant of *B. poitrasii*

Temperature of incubation (˚C)	NAD-GDH (U/mg)	NADP-GDH (U/mg)	NADP- / NAD- GDH ratio
22	44.0 ± 3.0	18.0 ± 1.3	0.619
28	49.0 ± 4.1	28.0 ± 1.8	0.571
32	110.0 ± 18.4	32.0 ± 2.1	0.290
37	169.0 ± 20.0	36.0 ± 2.3	0.213

It can be seen from the Table 3.15 that, the NAD-GDH (44.0 ± 3.0 to 169.0 ± 20.0 U / mg) and NADP-GDH (18.0 ± 1.3 to 36.0 ± 2.3 U / mg) activities were increased as temperature of incubation increased from 22 ˚C to 37 ˚C. The relative proportion of NADP-GDH and NAD-GDH activities were also decreased from 0.619 to 0.213. The NADP- / NAD- GDH ratio was more at 22 ˚C than at 37 ˚C, this may be due to maintenance of Y-5 mutants in the yeast form under hypha favoring conditions (22 ˚C).

3.2.4.B Effect of medium pH on Y-5 mutant of *B. poitrasii*

The effect of pH on Y-5 mutant of *B. poitrasii* was studied at different initial pHs, ranging from 4.0 to 8.0 of the YPG (0.5 %, glucose) medium. The experiments were carried out at 28 ˚C for 48 h under similar conditions as mentioned under Materials and Methods.

Table 3.16 Effect of medium pH on Y-5 mutant of *B.poitrasii*

Medium pH	NAD-GDH (U/mg)	NADP-GDH (U/mg)	NADP- / NAD-GDH ratio
4	163.0 ± 9.2	34.0 ± 2.1	0.208
5	92.1 ± 4.3	31.0 ± 1.9	0.344
6	49.0 ± 2.1	28.0 ± 1.8	0.571
7	41.0 ± 2.0	24.4 ± 1.3	0.585
8	32.00 ± 1.3	20.00 ± 0.9	0.625

It can be seen from the Table 3.16 that the morphological mutant Y-5 maintained the yeast form at all the pHs studied and the NADP- / NAD-GDH ratio was increased (0.208 to 0.625) with increase in the medium pH (4 to 8). The NADP- / NAD- GDH ratio was more at pH 8.0, this can be attributed to the maintenance of Y-5 mutants in the yeast form under hypha favoring conditions (pH 8.0).

3.2.5.B Effect of glucose in growth medium on Y-5 mutant of *B.poitrasii*

The effect of glucose (ranging from 0.5 % to 5.0 %) in the semisynthetic (YNB) growth medium on Y-5 mutant cells of *B. poitrasii* at 28 ˚C for 48 h under similar conditions as mentioned under Materials and Methods was seen.

Table 3.17 Effect of glucose in growth medium on Y-5 mutant of *B. poitrasii*

Glucose concentration (%)	NAD-GDH (U/mg)	NADP - GDH (U/mg)	NADP- / NAD-GDH ratio	
			Semisynthetic medium	YPG medium
0.5	49.0 ± 2.1	28.0 ± 1.8	0.57	0.57
1.0	10.43± 1.2	24.0 ± 1.6	2.30	2.7
5.0	1.56± 0.8	15.0 ± 1.4	9.58	13.3

As glucose concentrations in the medium increased from 0.5 % (0.515) to 5.0 % (9.58) the relative proportion of NADP-GDH and NAD-GDH was increased (Table 3.17), this may be due to maintenance of Y form of Y-5 mutant. When these findings were correlated with the temperature and pH studies of Y-5 mutant wherein the higher NADP- / NAD-GDH ratio was prevalent at hypha favoring conditions (22 ˚C and at alkaline pH), a decrease in the relative proportion of NADP- and NAD-GDH activities was expected at higher glucose concentration in both the media.

3.2.6.B Effect of Zn^{++} in growth medium on Y-5 mutant of *B. poitrasii*

The effect of addition of Zn^{++} in growth medium on Y-5 mutant was studied in YPG (0.5 %, glucose) medium at 28 ˚C for 48 h as described under Materials and Methods.

Table 3.18 Effect of Zn^{++} in growth medium on Y-5 mutant of *B. poitrasii*

Addition of Zn^{++}	NAD-GDH (U/mg)	NADP-GDH (U/mg)	NADP-/ NAD-GDH ratio
No addition	49.0 ± 6.1	28.0 ± 2.8	0.571
Zn^{++} 0.5 ppm	43.8 ± 3.3	42.0 ± 2.5	0.864

In the presence of 0.5 ppm Zn^{++} the Y-5 mutant maintained its yeast form. The GDH ratio was found to be increased in the presence of Zn^{++}. It can be seen from Table 3.18 that NAD-GDH activity decreased while NADP-GDH activity was increased in the presence of Zn^{++} (0.5 ppm).

3.2.7.B Effect of ethanol and myo - inositol on Y-5 mutant and parent strain of *B. poitrasii*

Effect of ethanol (0.5 %, w/v), myo- inositol (50 μg / ml) and zinc (0.5 ppm) on morphological change in parent as well as Y-5 mutant of *B. poitrasii* was studied in YPG (0.5 %, glucose) medium at 28 ˚C for 48 h under similar conditions as mentioned under Materials and Methods.

Table 3.19 a. Effect of ethanol, Zn^{++} and myo-inositol on Y-5 mutant of *B.poitrasii*

Medium	NAD-GDH (U/mg)	NADP-GDH (U/mg)	NADP-/ NAD-GDH ratio
Difco (0.5 % Ethanol, w/ v)	128.6 ± 6.8	15.8 ± 0.9	0.122
Difco (0.5 % Ethanol + Zn^{++} 0.5 ppm)	80.1 ± 3.9	29.6 ± 2.4	0.369
Difco (0.5 % Ethanol + 50 μg / ml myo-inositol)	76.0 ± 4.2	27.3 ±1.5	0.359
Difco (0.5 % Ethanol + 50 μg / ml myo-inositol + Zn^{++} 0.5 ppm)	74.0 ± 2.9	29.9 ± 2.4	0.404

The Y- 5 mutant grown in 0.5 % (w/v) ethanol-containing medium showed reversion to hyphal form, which was expressed in percent germ tube formation (40 %, **Figure 3.5). The NADP- / NAD-GDH ratio of Y-5 mutant grown in ethanol** containing medium was 0.122 and further in presence of zinc (0.5 ppm) it was increased to 0.369, with a decrease in percent germ tube formation from 40 % to 13.58 % (Table 3.19a). Similar trend was noted in parent strain of *B. poitrasii* (Table 3.19 b).

Table 3.19 b Effect of ethanol, Zn^{++} and myo-inositol on parent strain of *B. poitrasii*

Medium	Form	NAD-GDH (U/mg)	NADP-GDH (U/mg)	NADP- / NAD - GDH ratio
Difco	H	952.0 ± 45.0	9.22 ± 1.4	0.009
Difco (0.5 % Ethanol, w/v)	H	1249.0 ± 72.0	6.00 ± 3.0	0.004
Difco (Zn^{++} 0.5 ppm)	H<Y	204.0 ± 51.0	18.97 ± 2.0	0.093
Difco (0.5 % Ethanol + Zn^{++} 0.5 ppm)	H>Y	750.0 ± 24.0	10.12 ± 1.4	0.013
Difco (0.5 % Ethanol + 50 µg/ml myo-inositol)	H>Y	789.0 ± 32.0	9.46 ± 2.0	0.012
Difco (0.5 % Ethanol + 50 µg/ml myo-inositol + Zn^{++} 0.5 ppm)	H>Y	759.0 ± 37.0	4.20 ± 0.5	0.017

When myo-inositol (50 µg / ml) was added to the ethanol-containing medium, percent germ tube formation was decreased from 40 % to 18.87 % and NADP-/ NAD- GDH ratio was increased from 0.122 to 0.359 further in presence of Zn^{++} (0.5 ppm) no significant change was observed in percent germ tube formation as well as in NADP- / NAD –GDH ratio (Table 3.19a). Similar trend was noted in parent strain of *B. poitrasii* (Table 3.19 b).

Fig 3.2 Ethanol induced transition in yeast monomorphic mutant (Y-5) of *B.poitrasii*. a) yeast mutant (Y-5) of *B. poitrasii* b) ethanol induced hypha formation in Y-5 yeast mutant of *B. poitrasii*

3.2.8.B Effect of *in vitro* addition of Zn^{++} on glutamate dehydrogenase activities in Y-5 mutant of *B. poitrasii*

The effect of Zn^{++} on the specific activities of NAD- and NADP-GDH of Y-5 mutant was studied in the presence of 0.1 and 0.5 ppm concentrations of zinc in assay mixture. The Y-5 yeast mutant cells of *B. poitrasii* were grown in YPG (0.5 %, glucose) medium at 28 ˚C for 48 h and the crude enzyme extract was obtained as mentioned in Materials and Methods.

Table 3.20 Effect of *in vitro* addition of Zn^{++} on GDH activities of Y-5 mutant of *B. poitrasii*

Addition of Zn^{++}	NAD - GDH (U/ mg)	NADP- GDH (U/ mg)
No addition	49.0 ± 3.2	28.0 ± 1.8
Zn^{++} 0.10 ppm	55.9 ± 2.6	32.7 ± 2.3
Zn^{++} 0.50 ppm	58.8 ± 2.2	36.6 ± 1.4

When the concentration of Zn^{++} in assay mixture increased from 0.1 ppm to 0.5 ppm the NAD-GDH as well as NADP-GDH activities were increased from 17.0 to 18.8 U / mg and from 6.4 to 10.6 U / mg respectively (Table 3.20).

3.3.B Discussion

Temperature or nutritional factors or both are usually the causative factors for triggering dimorphic change in *B. poitrasii*. One of the ways to understand the mechanism of Y-H transition of *B. poitrasii* and its correlation with the biochemical changes was to use monomorphic mutants, which maintain one morphological form, either yeast or hypha, under dimorphism triggering conditions. Earlier it has been reported that morphological mutant Y-5 of *B. poitrasii* exhibited Y form irrespective of growth temperature or glucose concentration (Khale *et al*., 1990). The attempts

were made to isolate hyphal form monomorphic mutant of *B. poitrasii*. However, most of the isolates obtained after mutagenesis showed both the forms with different levels of yeast and hyphal cells under the experimental conditions (Table 3.10 – Table 3.14).

Therefore, in the present investigations the yeast- form (Y-5) mutant was used. In Y-5 mutant of *B. poitrasii* it was observed that the yeast form was maintained at all the temperatures (22 ˚C to 37 ˚C) and pHs (4 to 8) studied. The high NADP- / NAD -GDH ratio was prevalent in Y-5 mutant at all the incubation temperatures, which may be necessary to maintain the yeast form even at hypha favoring condition *viz*. 22 ˚C or pH 8.0.

In Y-5 mutant of *B. poitrasii* it was observed that the yeast form was maintained at all glucose concentrations (0.5 % to 5.0 %) studied and NADP-/ NAD-GDH ratio was increased. When these findings were correlated with the temperature and pH studies of Y-5 mutant wherein the higher NADP- / NAD-GDH ratio was prevalent at hypha favoring conditions (22 ˚C and at alkaline pH), a decrease in the relative proportion of NADP- and NAD-GDH activities was expected at higher glucose concentration. Since, the present studies were carried out at 28 ˚C and not at 22 ˚C the expected trend was not observed. This can possibly be attributed to the overriding effect of temperature signal over the glucose as described earlier (Ghormade *et al*., 2005).

In *C. tropicalis* (Tani *et al.,* 1980) and in *A. pullulans* (Sevilla *et al*., 1983) the ability of ethanol in bringing about the morphological transition was attributed to the interaction between ethanol and the cell membrane. In case of *B. poitrasii* a

similar mechanism may be operative since the organism did not require ethanol for its growth (Khale *et al*., 1990). As the mutation in *B. poitrasii* is not lethal, it is possible that observed transitions were caused by a protein morphic factor which in turn was controlled / regulated by the presence of ethanol or myo-inositol. Manning and Mitchell, (1980) reported similar effect of ethanol and myo-inositol in *C. albicans*.

In the present studies, in the ethanol containing medium the decrease in the NADP- / NAD-GDH ratio and an increase in percent germ tube formation was observed thus resulting in initiation of reversion of Y-5 mutant. Furthermore, the addition of Zn^{++} in the growth medium retarded the germ tube formation and increased the NADP- / NAD-GDH ratio.

In *C. tropicalis* it was seen that phosphatidyl inositol metabolism was enhanced by ethanol and triggered hyphal growth while myo-inositol plays an important role in morphogenesis through the regulation of the phospholipid signaling pathway (Omi and Kamihara, 1989). The present studies in *B. poitrasii* revealed that the addition of Zn^{++} and myo-inositol to the transition medium containing ethanol further decreased germ tube formation with increase in the GDH ratio. This can be attributed to the different signaling pathways for the ethanol and Zn^{++} effect on the Y-H transition in *B. poitrasii*.

C Isolation of chitin and chitosan from *B. poitrasii*

3.1.C Introduction

Cell walls of the zygomycetous group of fungi contain chitosan, a deacetylated form of chitin (Bartnicki-Garcia, 1968). Earlier studies on the chemical composition of *B. poitrasii* cell wall indicated the presence of more hexosamine contents in the hyphal form than the yeast form (Khale and Deshpande, 1992). However, the proportion of deacetylation was found to be more in the yeast form than the hyphal form. In other words, the chitosan: chitin ratio in the yeast cell walls was 5.40 while in the hyphal form cell walls it was 2.95.

Therefore, the present investigations have been undertaken to understand the possible correlation between GDH and chitin deacetylase activities and the chitosan contents if any. The possible implications in the dimorphism, *per se* and the biotechnological significance of these studies are discussed.

3.2.C Results

3.2.1.C Isolation of chitosan from H and Y cells of *B. poitrasii*

The cell wall chitosan contents along with degree of deacetylation from hypha and yeast form of *B. poitrasii* were measured. The hyphal cells were grown in YP medium at 28 ˚C for 24 h and yeast cells were grown at 37 ˚C for 24 h at similar conditions mentioned under Materials and Methods. Chitosan extraction from *B. poitrasii* was carried out using method described by Chatterjee *et al.* (2005). The chitosan was extracted from different fungi *viz*. *G. butleri, R. stolonifer* and *B. poitrasii* (hypha and yeast form) and compared with crab shell chitin and chitosan (Sigma). The acetoamide peak observed in the crab shell chitin (Sigma) sample was

absent in the chitosan samples (Sigma) as well as in the fungal cultures studied. The C = O and CH_3 signals at 35 and 175 ppm were found to be low in chitosan samples from the fungal cultures and the chitosan from crab shell (Sigma) as compared to the chitin (Sigma). The details are given in the Table 3.23. The solution state ^{1}H NMR and the solid-state ^{13}C CP/ MAS NMR spectroscopy revealed that the chitosan isolated from the hyphal and yeast form of *B. poitrasii* have similar degree of deacetylation (97.57 %).

3.2.2.C Possible correlation between glutamate dehydrogenase and chitin deacetylase activities and chitosan contents of *B. poitrasii*

The hyphal cells and yeast cells were grown in YPG (1 %, glucose) medium at 28 ˚C for 24 h at similar conditions mentioned under Materials and Methods. The two forms (yeast and hypha) were separated and the glutamate dehydrogenase and chitin deacetylase activities were estimated.

Table 3.21 Correlation between glutamate dehydrogenase and chitin deacetylase in *B. poitrasii*

	NAD-GDH (U/mg)	NADP-GDH (U/mg)	NADP-/ NAD-GDH ratio	Chitin deacetylase (U/mg) x 10^{-3}	Chitin deacetylase (U/ g biomass) x 10^{-3}	Chitosan* contents (%)
Hypha	70.4 ± 5.2	8.0 ± 1.3	0.11	0.904 ± 0.010	7.59	26.6
Yeast	18.4 ± 2.5	15.1 ± 1.8	0.82	0.261 ± 0.007	10.70	17.3

*Khale and Deshpande (1990)

It can be seen from the Table 3.21 that the specific NAD-GDH and CDA activities were more than 3 times higher in the hyphal form than the yeast - form (Table 3.21). However, total CDA activity in the yeast form is higher than the

hyphal, which in conformity with the higher chitosan to chitin ratio in the yeast form.

In the hyphal form of *B. poitrasii* higher NAD-GDH activity (70.4 ± 5.2 U/mg) and increase in chitin deacetylase activity (0.904 ± 0.010 x 10^{-3} U/mg) was observed with an increase in chitosan content. While low NAD-GDH activity and low chitin deacetylase activity (0.261 ± 0.007 x 10^{-3} U/mg) was observed in the yeast cells with a decrease in chitosan content (Table 3.21).

3.2.3.C Effect of different carbon sources to obtain specific morphology of *B. poitrasii*

As reported earlier that the hyphal form of *B. poitrasii* contains higher amount of chitosan than the yeast form, studies were undertaken to optimize growth parameters, which would support hyphal form. The effect on morphology and biomass production of different carbon sources such as maltose, sucrose, mannitol and sorbitol (0.1, 1.0 and 5.0 %) in the YP medium was studied at 28 ˚C for 48 h under similar conditions as mentioned under Materials and Methods.

It can be seen from the Table 3.22 that in the presence of maltose (1 %) maximum biomass was obtained, which showed higher % of hyphal cells too. Though mannitol supported hyphal form growth of *B. poitrasii* the biomass was low.

Table 3.22 Effect of different carbon sources on morphology of *B.poitrasii*

Carbon source	Concentration (%)	Biomass wet weight (g)	Form
Maltose	0.1	3.30	H
Maltose	1.0	6.60	H> Y
Maltose	5.0	5.60	H< Y
Mannitol	0.1	0.36	H
Mannitol	1.0	0.34	H
Mannitol	5.0	2.06	H
Sucrose	0.1	0.32	H
Sucrose	1.0	0.51	H<Y
Sucrose	5.0	0.60	Y
Sorbitol	0.1	0.35	H
Sorbitol	1.0	0.42	Y
Sorbitol	5.0	0.46	Y
Glucose	1.0	2.00	Y>H

The biomass was obtained from 100 ml of growth medium

3.2.4.C Comparison of chitosan from zygomycetous fungi

Amongst the several zygomycetous fungi *Rhizopus nigricans* and *R. arrhizus* (Andrade *et al*., 2000) and *Gongronella butleri* (Tan *et al*., 1996) have been studied for their chitosan contents. As compared to other zygomycetous fungi studied the chitosan obtained *B. poitrasii* has higher degree of deacetylation (Table 3.23, Fig 3.3).

Table 3.23 Characterization of chitosan from different sources

Source of chitosan	Amount of chitosan* (g)	Degree of deacetylation (%)
Crab shell chitosan (Sigma)	-	88.00
Gongronella butleri	0.888	87.49
Rhizopus stolonifer	0.760	87.49
Benjaminiella poitrasii (Hypha)	0.936	97.57
Benjaminiella poitrasii (Yeast)	0.830	97.57

*Amount of chitosan extracted from 10 g dry weight of biomass of respective fungus

3.3.C Discussion

In addition to understand the role of GDHs in the dimorphic transition in *B. poitrasii* the present investigations were undertaken to evaluate *B. poitrasii* as a potential source for chitosan production. Earlier the hypha of various fungi including *Phycomyces blakesleeanus*, *M. rouxii*, *Colletotrichum lindemuthianum* and *Absidia coerulea* have been reported as an alternative sources of chitin and chitosan (Knorr and Klein, 1986).

Chitosan, a derivative of chitin, is formed by the complex action of chitin synthase and chitin deacetylase. The chitosan of different molecular weights (MW) were produced by zygomycetous fungi when they were grown on media comprising different pH and composition. Fungi were also easily cultured using relatively simple nutrients.

Fig 3.6 Determination of degree of deacetylation of chitosan from *B. poitrasii* using ^{13}C CP/ MASS NMR spectroscopy

The only critical factor in chitosan production from fungi was the time of harvesting which affects the yield (Tan *et al*., 1996).

It has been reported in *B. poitrasii* that the chitin content was three times higher while the chitosan was 1.5 times more in the hypha than in the yeast form cells (Khale and Deshpande, 1992). In the present studies attempts were made to extract chitosan from *G. butleri*, *R. stolonifer* and *B. poitrasii*, hypha and yeast form and it was further characterized by determining degree of deacetylation using solution state ^{1}H NMR and the solid- state ^{13}C CP/ MASS NMR spectroscopy.

The manipulations of the growth parameter to obtain the hyphal form are influenced mainly by the relative proportion of GDHs and CDA activities. The influence of metal ions for instance, was studied in *S. cerevisiae* (Martinou *et al*., 2002) and *Absidia orchidis* (Jaworska and Konieczna, 2001) on chitin deacetylase. In *B. poitrasii* as reported in section 3.2.5.A, presence of Zn^{++} increased NADP-GDH and decreased NAD-GDH activity and the yeast form was prevalent. As seen in Table 3.21 the yeast form has higher CDA activity than the hyphal form. This increase in CDA activity in yeast form can be correlated with a Zn^{++} effect.

D Glutamate dehydrogenase activities of sporangiospores and zygospores of *B. poitrasii*

3.1.D Introduction

Until now, the studies on dimorphic fungi were limited to the vegetative form i.e. yeast to hypha transition (Gow, 1995). Ghormade and Deshpande, (2000) reported the studies on sporangiospore and zygospore germination in *B. poitrasii* under dimorphism triggering conditions.

In the present investigations the correlation between GDH activities and the germination of sporangiospores and zygospores in *B. poitrasii* has been studied.

3.2.D Results

3.2.1.D Role of NAD- and NADP- glutamate dehydrogenases in the germination of sporangiospores of *B. poitrasii*

The cofactors NADP and NAD present in the yeast and hyphal cells and sporangiospores and zygospores were quantified with a fluorimeter at 340 nm excitation and 420 nm emission as mentioned under Materials and Methods.

Table 3.24 Role of NAD- and NADP- glutamate dehydrogenases in the germination of spores of *B. poitrasii*

Morphological forms	NADP and NAD (AU)	NAD-GDH (U/mg)	NADP-GDH (U/mg)	NADP- / NAD - GDH ratio
Yeast	92	92.0 ± 7.5	15.3 ± 2.1	0.166
Hypha	45	724.0 ± 22.0	1.40 ± 0.2	0.001
Sporangiospore	15	7.0 ± 1.0	26.0 ± 1.0	3.714
Zygospore	38	213.0 ± 1.0	46.0 ± 2.0	0.215

High levels of NAD and NADP were observed in yeast, hypha as well zygospore extracts while the sporangiospore extract showed low levels of NAD and NADP (15AU) (Table 3.24). The NAD- and NADP- GDH enzymes were actively expressed in yeast and hyphal cells as well as in the spores. The higher ratio of the NADP- / NAD-GDH activities was noted in the sporangiospores, zygospore and yeast form of *B. poitrasii*. The higher ratio of the NADP-/ NAD-GDH activities

suggested the presence of yeast-form NADP-GDH in the sporangiospores and zygospore.

3.2.2.D Activity staining of NADP-glutamate dehydrogenase in *B. poitrasii*

In *B. poitrasii*, the NADP-GDH was reported to be differentially expressed in the yeast and hyphal forms (Amin *et al*., 2004). The activity staining of NADP-GDH of the yeast and hypha forms showed the differential expression of NADP-GDH (Fig 3.6). The relative mobility for NADP-GDH of yeast, sporangiospores and zygospore was same (R_f 0.33) while for NADP-GDH of hypha R_f was 0.13.

The NADP- and NAD-GDH enzyme activities and their relative proportion suggested the presence of the yeast form NADP-GDH in the sporangiospores and the zygospore that was confirmed by activity staining (Fig 3.6). As a single NAD-GDH

enzyme was present in the yeast and hyphal form but were quantitatively different the activity staining for NAD-GDH was not done.

3.3.D Discussion

Ghormade and Deshpande (2000) reported autofluorescence of yeast, hypha, sporangiospores and zygospore of *B. poitrasii* by using epifluorescence microscope with an excitation at 340 nm and emission at 420 nm. At this range of excitation wavelength, NADH and flavoproteins were reported to be the main contributors for the fluorescence (Franke *et al*., 1980). The actively growing yeast and hyphal cells of *B. poitrasii* showed green/ blue fluorescence, which could be attributed to the presence of NAD and NADP or FAD in the cells. The zygospore showed a yellow

Fig 3.4 Activity staining of NADP-glutamtae dehydrogenase of hypha, yeast, sporangioles and zygospores of *B.poitrasii*. Lane 1: hypha, Lane 2: yeast, Lane 3: sporangioles, Lane 4:zygopsore

fluorescence while the autofluorescence for sporangiospores was not detected this might be due to the thick spore wall (Ghormade *et al.*, 2005).

Autofluorescence and fluorimetric studies in *B. poitrasii* sporangiospores and the zygospores suggested that the nicotinamide coenzyme related GDH enzymes expressed in the actively growing yeast and hyphal cells could be possibly expressed in the spores also. The NADP- and NAD-GDH enzyme activities, their relative proportion suggested the presence of yeast form specific NADP-GDH in the sporangiospores and zygospore. These investigations suggest that *B. poitrasii* exhibits dimorphism as an intrinsic ability, where its sexual, asexual and vegetative phase responded to the dimorphism triggering conditions. This makes dimorphism a commitment of the organism (*B. poitrasii*) during the entire life cycle rather than restricting it to a particular phase.

Chapter IV: Purification and characterization of NAD - glutamate dehydrogenase from hyphal form of *B.poitrasii*

4.1 Introduction

Growth in the yeast form of *B. poitrasii* demands the obligate presence not only of a hexose as a carbon and energy source but also of an aminated organic compound as the nitrogen source, whereas an ammonium salt alone is sufficient to support growth of hyphae. These observations suggested that metabolic enzymes may be differently expressed in the two forms of *B. poitrasii* (Khale *et al*., 1992).

The three enzymes involved in ammonia assimilation are reported to be glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH). Higher fungi ascomycetes and basidiomycetes possess two distinct GDHs one specific for NAD-GDH (E.C 1.4.1.2) and the other NADP-GDH (E.C 1.4.1.4) whereas lower fungi possess a single NAD-GDH (Le John and Stevenson, 1971). The GDHs play a catalytic role in either oxidative deamination of glutamate or in reductive amination of α-ketoglutarate. Glutamate dehydrogenases (GDHs) comprise an important group of enzymes, occupying a critical position interlinking carbon and nitrogen metabolism, catalyzing the assimilation and dissimilation of ammonia. The enzymes are recognized to be important because of the pivotal position in metabolism occupied by glutamate and α-ketoglutarate and their ability to enter into many types of pathways.

In fungi such as *S. cerevisiae* (Avendano *et al*., 1997), *M. racemosus* (Peters and Sypherd, 1979) and *C. utilis* (Fergusson and Sims, 1974) the NADP-GDH is the major route both in conditions of nitrogen excess and limitation while the GS/ GOGAT pathway is relatively unimportant. However, in *C. tropicalis*, *C. parasilopsis* and *C. albicans* the GS/ GOGAT pathway was found to be important

for ammonia assimilation (Holmes *et al*., 1989). In *N. crassa* the NADP-GDH functions in conditions of large supply of nitrogen while GS/ GOGAT pathway catalyzes ammonium assimilation when the concentration is low (Lomnitz *et al*., 1987). In *A. nidulans* (Macheda *et al*., 1999) and *A. bisporus* (Kersten *et al*., 1999), nitrogen assimilation was reported to follow the GS/ GOGAT pathway predominantly.

Usually GDHs were reported to be either hexamer or tetramer with a subunit molecular mass 48-55kDa (Kapoor *et al*., 1993; Yang and Le John, 1994).

The biochemical correlation of glutamate dehydrogenases with morphogenesis was reported in the dimorphic species of *Mucor* (Bartnicki-Garcia and Nickerson, 1962 b). Peters and Sypherd (1979) demonstrated the presence of two GDHs, one NAD-GDH and other NADP-GDH and correlated the CO_2 / N_2 induced yeast-hypha transition to the rise in the NAD-GDH activity. Among the different biochemical processes involved in the morphological changes, the relative proportion of NAD- and NADP-GDH activities was correlated with the yeast-hypha transition in *B. poitrasii* (Khale *et al.,* 1992). Furthermore, Amin *et al*, (2004) reported the presence of two NADP-GDHs, one hypha specific and other one yeast-form specific while NAD-GDH was common to both the forms and contributed significantly in the morphological outcome.

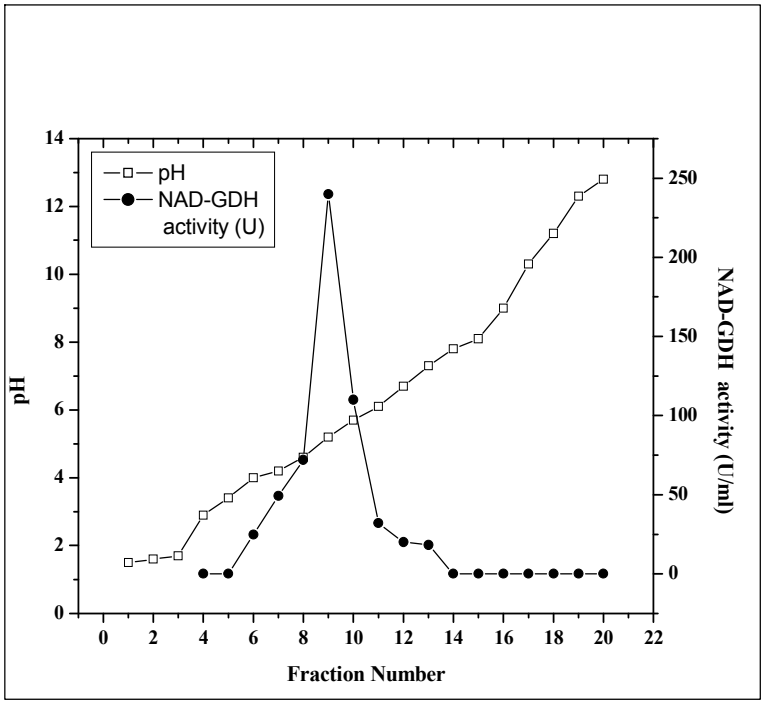
In the present investigations the attempts were made to purify NAD-GDH from the hyphal form cells and to understand its role in the dimorphism of *B. poitrasii*.

4.2 Results

4.2.1 Single step purification of NAD-glutamate dehydrogenase from *B. poitrasii* by isoelectric focusing

Isoelectric focusing (pH 3-10) was attempted for the single step purification of the NAD-GDH from hyphal form of *B. poitrasii*. Though all the NAD-GDH activity was seen as a single peak around pH 5.2 it was not a purified protein (Fig 4.1). In the protein staining after native PAGE it showed 8-10 bands. Therefore, the conventional purification protocols were followed.

Fig 4.1 Purification of NAD-glutamate dehydrogenase from *B.poitrasii* by isoelectric focusing



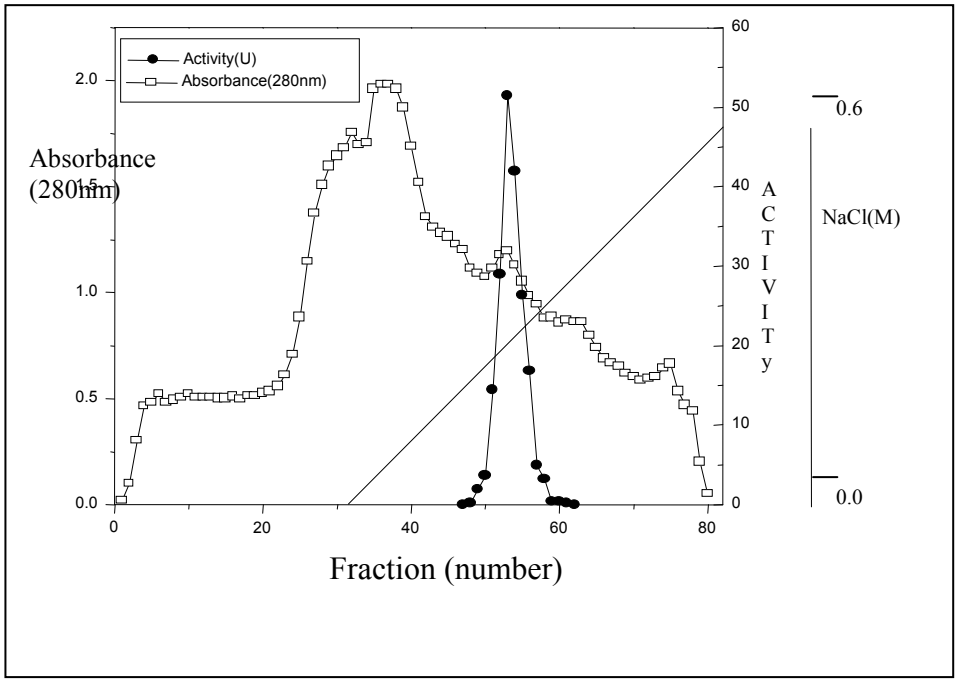
4.2.2 Purificaton of NAD-glutamate dehydrogenase from *B. poitrasii*

The sequential steps used to purify NAD-GDH from the crude cell extract of *B. poitrasii* hyphal form consist of ammonium sulphate fractionation, anion

exchange chromatography using DEAE fast flow sepharose, and affinity chromatography using Affi -Gel Blue Gel (Table 4.1).

In ammonium sulfate fractionation the maximum NAD-GDH activity appeared in the fraction, 30-50 %. The 30-50 % saturated fraction with 80.18 % activity was collected which was dialysed and then concentrated by Amicon ultrafiltration using 10,000 Da cut off and then loaded on anion exchange chromatography column. The enzyme elution was carried out using 0 - 0.6 M NaCl in 30 mM K-phosphate buffer, pH 7.2.

Fig 4.2 Protein elution pattern from DEAE fast flow sepharose and related NAD-glutamate dehydrogenase activity

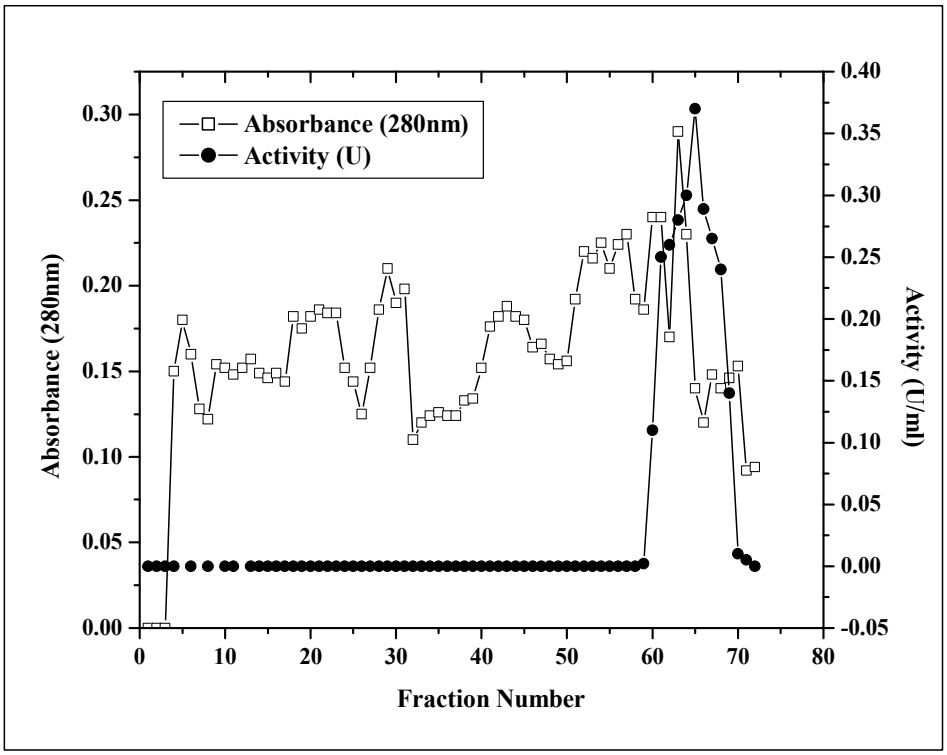


Out of 80 fractions collected (2.5 ml each), the fractions (52-54 number) showing maximum activity were pooled and were was used for further purification

(Fig 4.2). After dialysis it was then concentrated using Amicon ultrafiltration. The anion exchange chromatography showed 45.95 % recovery of NAD- GDH activity (Table 4.1).

Subsequent affinity chromatography using Affi gel blue gel elution of protein was carried out using 0-0.6 M NaCl (Fig 4.3). Native PAGE of the peak activity fraction (65 number) revealed a single major band when the gel was stained using silver nitrate according to method developed by Blum *et al*., (1975) (Fig 4.4).

Fig 4.3 Protein elution pattern from Affi-gel blue gel and related NAD-glutamate dehydrogenase activity



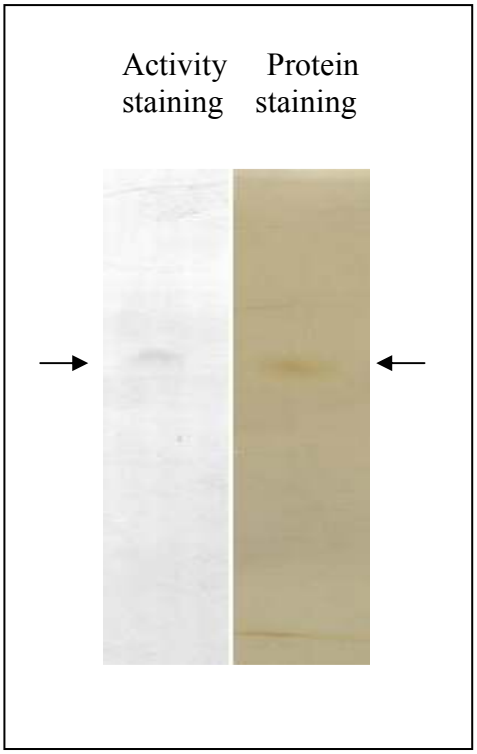
The NAD-GDH which was free of NADP-GDH activity was purified 73.00 fold with 3.14 % of the initial total activity and the specific enzyme activity was 57.53 U / mg of protein.

Table 4.1 Purification of NAD-glutamate dehydrogenase from hyphal form *B. poitrasii*

Purification step	Total activity (Unit) $x10^3$	Total protein (mg)	Specific activity (U/mg) $x10^3$	Enzyme yield (%)	Fold purification
Crude extract	1023	1298.22	0.788	100	----
$(NH_4)_2SO_4$ (0-30 %)	42.31	589	0.071	4.13	0.090
$(NH_4)_2SO_4$ (30-50 %)	820.24	482.49	1.70	80.18	2.157
$(NH_4)_2SO_4$ (50-90 %)	26.66	217	0.122	2.606	0.154
Amicon ultrafiltration (10,000Da, cut off)	658.58	430.22	1.530	64.37	1.941
DEAE fast flow sepharose	470.07	13.45	34.93	45.95	44.32
Amicon ultrafiltration (10,000Da, cut off)	170.16	4.56	37.31	16.63	47.34
Affi-Gel Blue gel	32.21	0.56	57.53	3.14	73.00

Fig 4.4 Analytical polyacrylamide gel electrophoresis of NAD-glutamate dehydrogenase

(Native PAGE: 7. 5 % (w/ v) polyacrylamide gel)

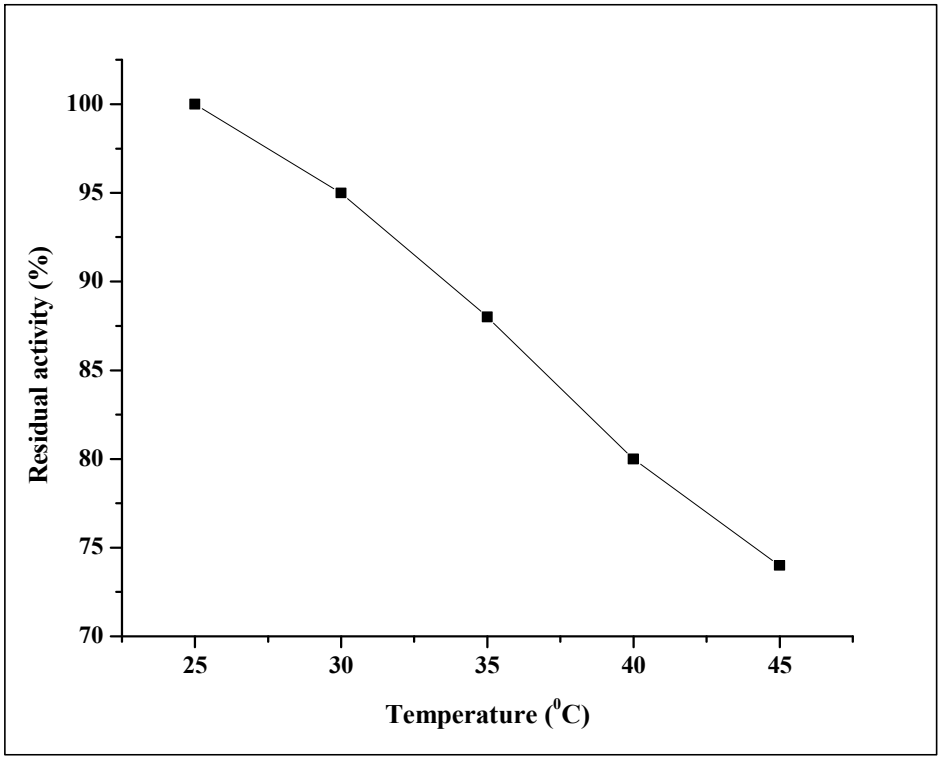


4.2.3 Biochemical characterization of purified glutamate dehydrogenase

4.2.3.1 Effect of temperature on enzyme activity and stability

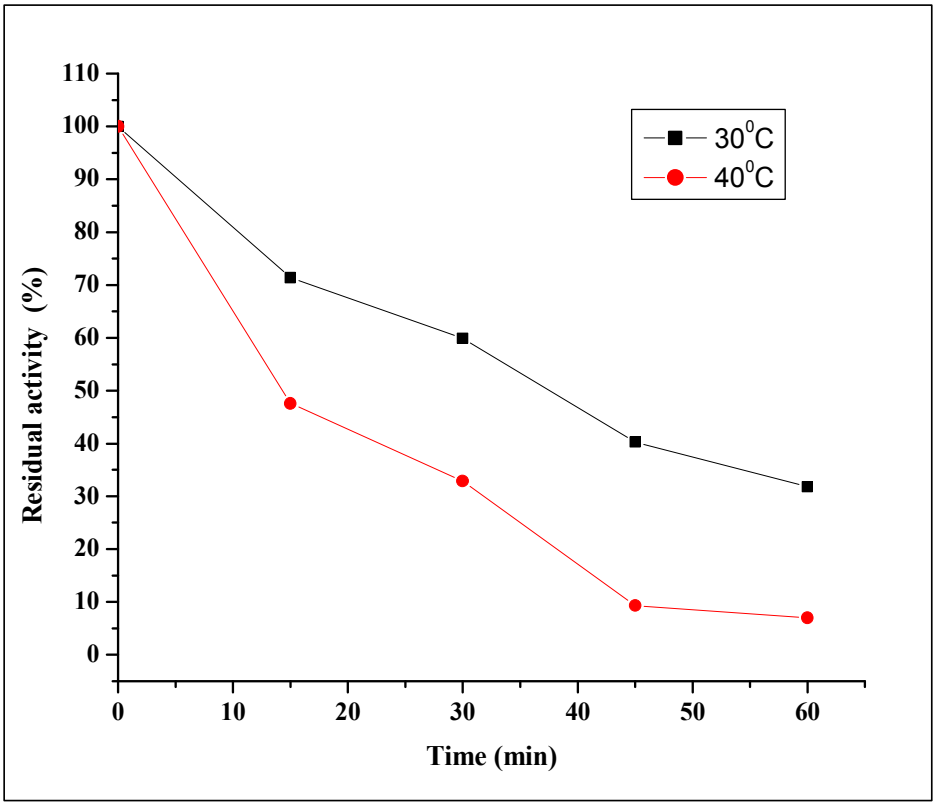
Effect of temperature on activity of purified NAD-GDH from hyphal form of *B. poitrasii* was determined using standard assay system. The enzyme activities at varied temperatures ranging from 20-45 ˚C were determined. The optimum temperature for purified preparation it was 25 ˚C (Table 4.2, Fig 4.5).

Fig 4.5 Optimum temperature of NAD-glutamate dehydrogenase from hyphal form of *B. poitrasii*



The temperature stability of the enzyme was detected by incubating enzyme at 30-60 ˚C and pH 8.0 for different time intervals. The temperature stability curves of NAD-GDH are presented in Fig 4.6. The enzyme showed maximum activity at 30 ˚C and retained 80 % of the maximum activity at 40 ˚C. The enzyme was not stable above 50 ˚C, while it retained its 50 % activity for 40 and 25 min at 30 and 40 ˚C. The literature survey indicated that the temperature optimum of NAD-GDH of *Laccaria bicolor* was estimated to be 30 ˚C (Garnier *et al*., 1997).

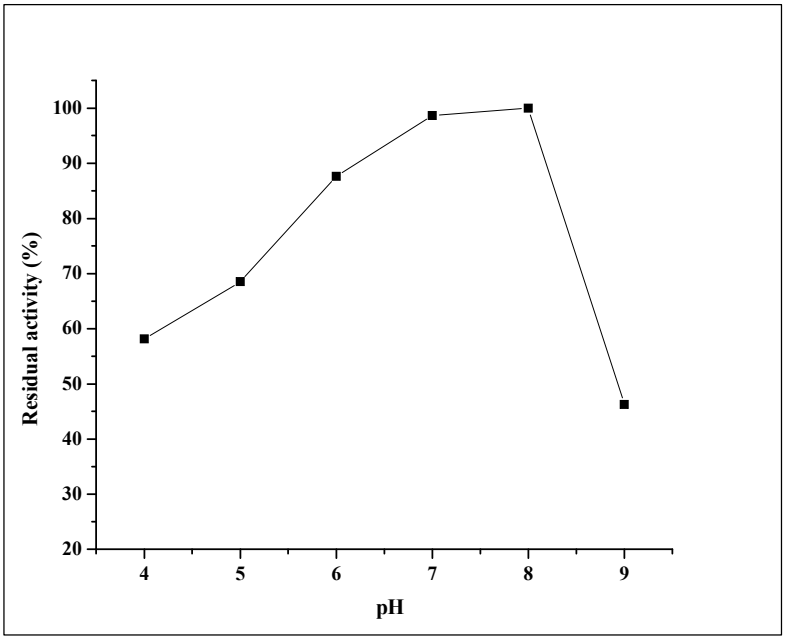
Fig 4.6 Stability of NAD-glutamate dehydrogenase from hyphal form of *B. poitrasii* at 30 ˚C and at 40 ˚C



4.2.3.2 Effect of pH on enzyme activity and stability

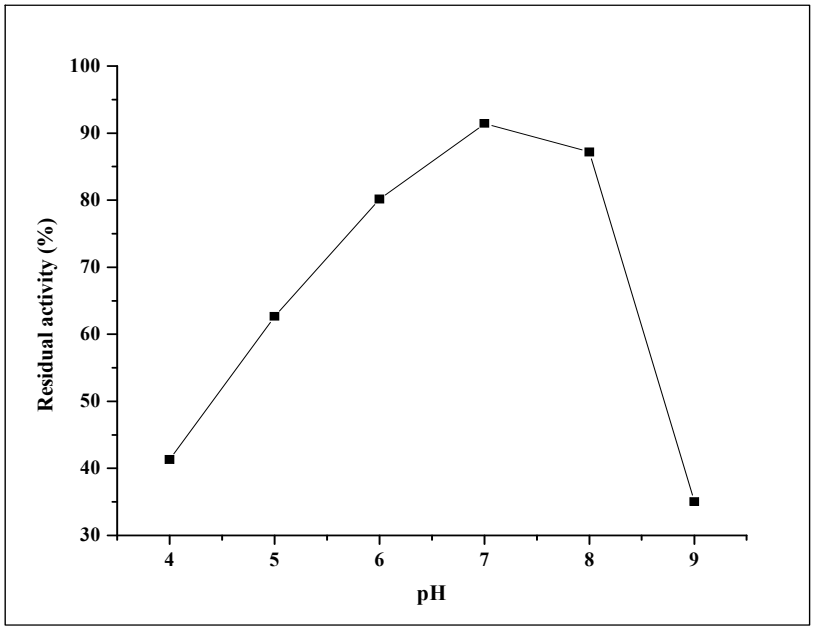
The amination activity of purified NAD-GDH of hyphal form of *B. poitrasii* was determined at varied pH ranging from 4-9. The purified enzyme NAD-GDH was active in a broad pH range of 4 to 9 with pH optimum at 8.0 (Fig 4.7).

Fig 4.7 Optimum pH of NAD-glutamate dehydrogenase from hyphal form of *B. poitrasii*



The pH stabilities of the enzyme were determined by incubating it in respective buffers of different pHs for 2 h. The enzyme was stable in alkaline pH range of 7-8 (Fig 4.8). The NAD-GDH retains around 90 % activity after incubation for 2 h at pH 7.0 while 85 % residual activity was detected at pH 8.0. The 50 % of the enzyme at pH 7.0 was seen after 4 h.

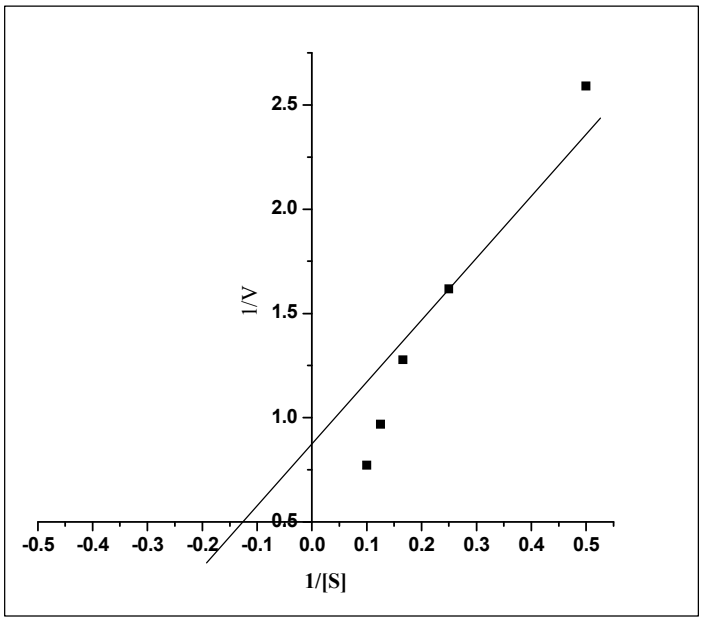
Fig 4.8 The effect of pH on the stability of NAD-glutamate dehydrogenase from hyphal form of *B. poitrasii*



4.2.2.3 Km and Vmax

The assay parameters *viz.* the substrates like α-ketoglutarate (ranging from 10-50 mM), ammonium chloride (50-300 mM) and NADH (ranging from 0.075 mM -1.75 mM) were optimized. The measurement of activity of NAD-GDH at different substrate concentrations yielded a linear Lineweaver Burk plot.

Fig 4.9 Line weaver-Burk plots for the determination of Km of NAD-glutamate dehydrogenase using α-ketoglutarate



The Michaelis constants (Km) with α-ketoglutarate and for ammonium chloride were 7.81 mM and 294 mM concentration and Vmax was 1.602 U/mg (Fig 4.9, 4.10) and the Vmax was 2.049 U/mg. The biochemical characteristics of NAD-GDH are given in Table 4.2.

Fig 4.10 Lineweaver-Burk plots for the determination of Km of NAD-glutamate dehydrogenase using ammonium chloride

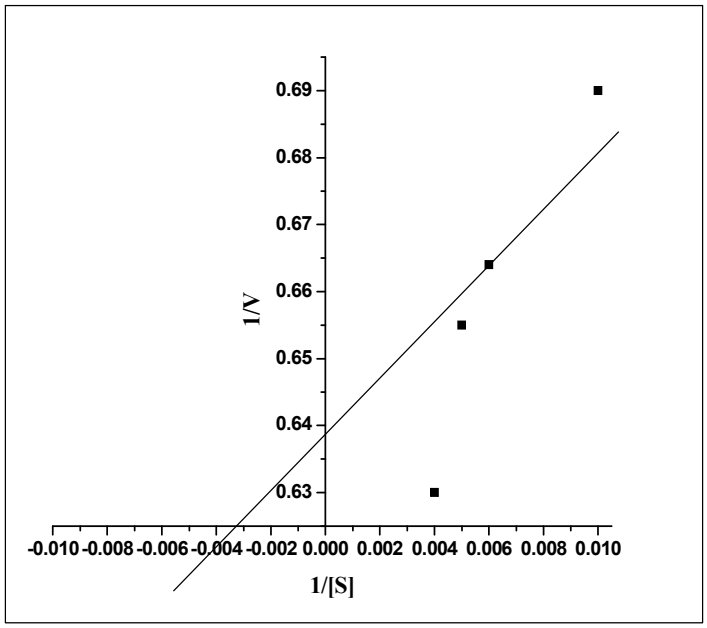


Table 4.2 Biochemical characterization of purified NAD-glutamate dehydrogenase

Parameter	NAD - GDH
	Hypha
Optimum temperature (˚C)	25
Optimum pH	8.0
Km (α-ketoglutarate) (mM)	7.81
Vmax (U/mg)	2.049
Km (ammonium chloride) (mM)	294

4.3 Discussion

The main aim to purify glutamate dehydrogenase(s) from *B. poitrasii* to understand their immunological relatedness with each other and the correlation between enzyme nature and the morphology. However, the purification of GDHs from *B. poitrasii* was found to be difficult due to the less stability of both the GDHs from the either form. As only one NAD-GDH is present in both the forms and NAD-GDH was reported to be important in the evolution of fungi (Le John, 1971), the purification of it was attempted.

The purification of NAD-GDH was reported from *L. bicolor, Streptomyces fradiae* and *N. crassa* and other organisms. In *L. bicolor* the NAD-GDH was reported to be extremely thermolabile (Garnier *et al.,* 1997). While in case of *S. fradiae* NAD-GDH 70 % loss of activity was seen even at -20 ˚C in 24 h (Nguyen *et al.,* 1997). In case of *N. crassa* NAD-GDH, the activity was not stable at the -20 ˚C (Veronese *et al*., 1974).

The purification of NAD-GDH from *L. bicolor, A. nidulans* and *N. crassa* to homogeneity required various steps such as ammonium sulphate fractionation upto 30-55 % saturation, DEAE ion exchange chromatography and gel filtration chromatography (Garnier *et al*., 1997; Stevens *et al*., 1989; Veronese *et al*., 1974). Some researchers demonstrated use of as alumina Cγ gel chromatography and Affigel - blue agarose for the purification of GDHs from *N. crassa* (Stachow and Sanwal, 1967) and *C. utilis* (Hemmings, 1980). The most of the purification protocols resulted in purification of homogenous glutamate dehydrogenase however yield obtained was very low. After the last step of purification, in case of *B.*

poitrasii the NAD-GDH enzyme was extremely unstable even in the presence of several protectants such as glycerol, dithiothreitol used at higher concentrations. This is in agreement with the high thermolability of this enzyme already reported for a number of basidiomycetous fungi including *A. bisporus* (Baars *et al*., 1994) and *Pleurotus ostreatus* (Mikes *et al*., 1994). Similar instability was also found in lower fungi.

The pH optimum for NAD-GDH from *A. nidulans* was pH 8.0 (Stevens *et al*., 1989). Similarly, the *B. poitrasii* NAD-GDH has optimum pH 8.0 (Table 4.2). Only one NAD-GDH is reported from *B. poitrasii* and hyphal and yeast form. As the hyphal form is favoured at alkaline pH in *B. poitrasii* and the optimum pH of the purified NAD-GDH was found to be pH 8.0. The optimum pH indicates its possible role to favor hyphal form in *B. poitrasii*.

NAD-GDH from *L. bicolor* (Garnier *et al*., 1997) exhibited Km value 37 mM and 1.35 mM for ammonium and α-ketoglutarate, respectively. Similar Km values were reported for the enzymes from *Dictyostelium discoideum* (Pamula and Wheldrake, 1991) and from *Achlya klebsiana* (Yang and LeJohn, 1994). However lower Km values were found in *P. ostreatus* for ammonium and glutamate as 3.3 and 0.18 mM, respectively (Mikes *et al*., 1994) and *A. bisporus* for ammonium as 6.3 mM (Baars *et al*., 1994). Though the purification of the NAD-GDH was successfully achieved for further in vitro biochemical characterization, with respect to the amino acids of active site regulation of activity by phosphorylation-dephosphorylation immunological relatedness with other GDHs from *B. poitrasii* and its evolutionary significance large quantity of the purified enzyme are necessary

Chapter V: Glutamate dehydrogenase in yeast - hypha transition of *B. poitrasii* and its role in development of antifungal agents

5.1 Introduction

For the past two decades, fungal infections have increased dramatically, their occurrence being more frequent in people with suppressed immune system (Diamond, 1991). Thus fungal infections are now important causes of morbidity and mortality of hospitalized patients.

The majority of fungi are saprophytic in nature and only those that can adapt to survive in host tissue become pathogenic. These fungi change their normal vegetative developmental stages in the host tissue. The morphological change in pathogenic fungi is triggered by host body temperature, blood sugar level, serum and the nature of host tissue. The resulting morphological forms are usually budding yeasts, hyphae, spores or sclerotic bodies. A variety of biochemical correlates are known for morphological changes and for pathogenesis. The morphological changes *viz*. hypha to yeast transition or *vice versa*, formation of sclerotic bodies are sufficient to provoke a protective immune response in animals and therefore, are of potential therapeutic interest.

Three major groups of antifungals used clinically include: the polyene antibiotics, azole derivatives and the allylamines (Ghannoum and Rice, 1999; Gooday, 1995). The resistance developed to azole drugs and toxic effects of polyene antibiotic Amphotericin B, were reported to be some of the limitations associated with the current antifungal agents (Hunter, 1995), This necessitates a search for new antifungals, which will prove to be effective in halting the spread of fungal infections.

The main emphasis is on prevention of morphological transition, *viz*. saprophytic to an invasive form. In addition to immunological studies with respect to the host susceptibility to fungal pathogens, the analysis of vital cellular functions in the pathogens is important to identify potential target for its control. However, it should be noted that fungi are eukaryotes like mammalian cells and therefore, the agents that inhibit fungal cellular processes are likely to affect the same in the patient, producing toxic side effects. In this regard, morphological change can be used as a selective event to visualize their potential as an ideal target for antifungal drugs.

Earlier cell wall biosynthesis polymers such as chitin, glucan, mannan, mannoproteins were studied as a potential target for development of antifungal drugs. Antifungals developed against these targets were papulacandin, aculeacin A, echinocandin B, pradimicin and benanomycin (Wood, 1998; Gooday, 1995). The enzyme which is not studied as an antifungal target is glutamate dehydrogenase. In fungi two distinct, NAD- and NADP- dependent GDH enzymes have been reported (Amin *et al*., 2004). The human GDH has been reported to be a homo hexamer with six identical subunits consisting of 505 amino acids each with dual specificity using either NAD or NADP as a cofactor (Zaganas and Plaitakis, 2002).

For the screening of potential antifungal compounds and / or microorganisms that produce such compounds different tests were carried out such as, hyphal tip bursting (Chitnis, 2001; Ghormade 2000; Patil *et al*., 2001; Zhu and Gooday, 1992), inhibition of yeast to hypha transition (Davila *et al.*, 1986; Frost, 1995; Ghormade, 2000), inhibition of spore germination (Ghormade, 2000;Gooday *et al.*, 1976; Zhu

and Gooday, 1992), retardation in hyphal tip elongation (Davila *et al*., 1986; Gooday *et al*., 1976; Smith *et al.*, 1990; Tariq and Devlin, 1996; Watanabe *et al*., 1997; Zhu and Gooday, 1992), *in vitro* inhibition of chitin synthase (Brillinger,1979; Kobinata *et al*.,1980; Sakurai,1999), glucan synthase (Hawser and Islam ,1999) and chitinase activities (Sakuda ,1996).

Some of the biochemical correlates for Y-H or reversible transition reported so far include: chitin synthase, chitinase, ornithine decarboxylase and glutamate dehydrogenase (Deshpande, 1996). The antifungal agents using chitin synthase and chitinase as a target have been developed (Wood, 1998; Gooday, 1995) and inhibitors of ornithine decarboxylase are in use against plant pathogenic fungi (Rajam *et al*., 1985). No significant attempts have been made to develop antifungal agents, which inhibit GDH. The studies in the previous sections on *B. poitrasii* suggested the role of GDH during different stages of life cycle of *B. poitrasii* i.e. in yeast, hypha, sporangiospores and in zygospores. Therefore, it was thought worthwhile to screen different chemical compounds as well as microbial culture filtrates, as potential GDH inhibitors.

In the present investigations *B. poitrasii* was used to screen actinomycete, bacterial and fungal cultures, which produce potential inhibitors of GDH enzymes. For the testing of the efficacy of the antifungal agents two stages / phases of the life cycle *viz.* sporangiospore germination and yeast-hypha transition were used. Additionally *in vitro* inhibition of GDH activities of *B. poitrasii* was also studied.

5.2 Results

5.2.1 Screening of microorganisms as potential glutamate dehydrogenase inhibitor producers

For screening of potential GDH inhibitors the bacterial, actinomycetes and fungal cultures used were isolated from soil samples from different locations. The soil samples were collected from rhizosphere from the various vegetables and peanut plants from different locations in and around Pune (geographic latitude 18.31˚N, longitude 73.55 ˚E) Maharashtra, India. The soil samples were serially diluted in sterile saline and 50 µl of each of the sample was spread on different media such as casein starch agar, nutrient agar and minimal media plates for the isolation of actinomycete, bacterial and fungal cultures, respectively and incubated at 28 ˚C upto 96 h. The total 94 isolates were further purified. The predominant genera of bacteria isolated were: *Bacillus* sp (22), *Pseudomonas* sp (7) and *Serratia* sp (3). A total 40 fungal cultures were isolated using minimal medium and 22 actinomycete cultures were isolated using casein starch agar as mentioned earlier in Materials and Methods. The culture filtrates of these cultures grown in inhibitor production medium as under section 2.2.7 at 28 ˚C for 96 h were used for further screening. The culture filtrates used for plate assay were concentrated 10 times.

A sporangiospore suspension (8 x 10^3 spores / plate) of *B. poitrasii* was spread on the YPG (1 %, glucose) agar plate and the 200 µl inhibitor culture filtrate were placed in wells bored in the plates and incubated at 28 ˚C for 48 h.

Table 5.1 Screening of bacterial cultures as potential inhibitor producers

Inhibitors	*B.poitrasii* zone of inhibition (mm)
Bacillus sp B 1	13
Bacillus sp B 2	8-9
Bacillus sp B14	11
Bacillus sp B15	15
Bacillus sp B17	9
Bacillus sp B 21	8
Bacillus sp B 22	12
Pseudomonas sp P 8	6-8
Streptomyces sp	6-8
Volutella sp	9
Synthetic organic compounds	
S-1 (100 µg)	9
S-3 (100 µg)	8
S-10 (100 µg)	5-6

Out of 94 cultures isolated only 8 bacterial, 1 actinomycete and 1 fungal culture filtrates showed inhibitory activity against *B. poitrasii*. The cultures showing zone of inhibition above 5 mm were used for screening of potential glutamate dehydrogenase inhibitors (Table 5.1). The maximum zones of inhibition were shown by the culture filtrates of *Bacillus* sp B15 and *Bacillus* spB1 (Table 5.1).

5.2.2 Effect of crude mixture of inhibitor(s) on the yeast- hypha transition in *B. poitrasii*

Effect of different bacterial culture filtrates on the yeast- hypha transition of *B. poitrasii* was studied by measuring inhibition of germ tube formation. *B. poitrasii* yeast cells grown in YPG (1 % glucose) medium at 28 ˚C for 24 h were used as an inoculum. Using potential isolates identified (Table 5.1), the transition experiments were carried out in YP medium at 28 ˚C for 12 h. The percent inhibition of germ tube formation for *Bacillus* sp B 17 culture filtrate was 60.57 % and for *Bacillus* sp B15 it was 68.11 % (Table 5.2). Among the 3 synthetic organic compounds tested, compound S-1 (5 μg/ml) showed 70.25 % inhibition whereas isophthalic acid (2.5mM) a known inhibitor of NAD-GDH showed 73.00 % inhibition (Table 5.2).

Table 5.2 Effect of inhibitor producing bacterial culture filtrates on the yeast-hypha transition in *B. poitrasii*

Inhibitor	Germ tube, 12 h (%)	Inhibition (%)
Control	61.50	-
Bacterial culture filtrates		
Bacillus sp B1	33.88	44.90
Bacillus sp B 2	46.82	23.86
Bacillus sp B14	35.39	42.45
Bacillus sp B15	19.61	68.11
Bacillus sp B17	24.24	60.57
Bacillus sp B21	37.93	38.32
Bacillus sp B22	35.54	42.20
Pseudomonas sp P18	39.52	35.73
Streptomyces sp	53.36	13.23
Volutella sp	47.15	23.33
Synthetic organic compounds		
S-1 (5 µg / ml)	18.30	70.25
S-3 (5 µg / ml)	47.20	23.26
S-10 (5 µg / ml)	43.80	28.79
Isophthalic acid (2.5 mM)	16.60	73.00

5.2.3 *In vitro* inhibition of NAD- and NADP- glutamate dehydrogenase activities of *B. poitrasii*

The NAD- and NADP- GDH activities of the crude enzyme extract obtained from hyphal- and yeast-form cells of *B. poitrasii* were determined with and without (control) the bacterial culture filtrates and with synthetic organic compounds (5 ug / ml) along with isophthalic acid (2.5 mM) as a positive control.

Table 5.3 *In vitro* inhibitions of NAD- and NADP- glutamate dehydrogenase activities from yeast and hyphal form of *B. poitrasii*

Inhibitor	Hypha		Yeast	
	NAD-GDH (U/mg)	NADP-GDH (U/mg)	NAD-GDH (U/mg)	NADP-GDH (U/mg)
Control	724.00 ± 22.00	1.400 ± 0.20	92.00 ± 1.5	15.3 ± 2.1
Bacterial and fungal cultures				
Bacillus sp B 1	324.78 ± 4.40 (55.14)	0.742 ± 0.08 (47.06)	52.53 ± 0.54 (42.90)	6.36 ± 0.55 (58.39)
Bacillus sp B 2	587.07 ± 15.45 (18.92)	1.076 ± 0.22 (23.12)	84.00 ± 0.9 (8.54)	3.72 ± 0.42 (75.73)
Bacillus sp B14	591.32 ± 16.00 (18.31)	1.005 ± 0.14 (28.20)	56.94 ± 0.62 (38.10)	4.17 ± 0.44 (27.28)
Bacillus sp B15	562.54 ± 15.42 (22.30)	0.210 ± 0.03 (85.30)	78.00 ± 0.8 (14.64)	6.56 ± 0.61 (42.9)
Inhibitor	Hypha		Yeast	
	NAD-GDH (U/mg)	NADP-GDH (U/mg)	NAD-GDH (U/mg)	NADP-GDH (U/mg)
Bacillus sp B 21	520.70 ± 13.10	0.949 ± 0.10	62.61 ± 0.65	3.89 ± 0.41

	(28.08)	(32.15)	(31.94)	(25.45)
Bacillus sp B 22	543.00 ± 13.00 (25.00)	0.989 ± 0.11 (29.42)	80.00 ± 0.8 (12.33)	15.3 ± 2.1 (ND)
Pseudomonas sp P18	568.16 ± 15.50 (21.47)	0.906 ± 0.10 (35.30)	92.00 ± 1.5 (ND)	4.99 ± 0.44 (67.44)
Streptomyces sp	589.48 ± 15.64 (18.58)	1.113 ± 0.09 (20.47)	58.75 ± 0.54 (36.14)	15.3 ± 2.1 (ND)
Volutella sp	603.45 ± 14.40 (16.65)	1.141± 0.09 (18.45)	57.22 ± 0.55 (37.80)	11.88 ± 0.82 (22.34)
Synthetic organic compounds				
S-1 (2.5 µg / ml)	59.58 ± 1.02 (91.77)	1.400 ± 0.20 (ND)	55.73 ± 0.58 (39.42)	8.74 ± 0.62 (42.85)
S-3 (2.5 µg / ml)	510.99 ± 9.20 (29.42)	1.018 ± 0.22 (27.28)	92.00 ± 1.5 (ND)	15.3 ± 2.1 (ND)
S-10 (2.5 µg / ml)	681.42 ± 17.01 (5.88)	0.890 ± 0.09 (36.36)	62.61 ± 0.7 (31.94)	15.3 ± 2.1 (ND)
Isophthalic acid (2.5 mM)	189.61 ± 4.33 (73.81)	0.759 ± 0.08 (45.74)	29.44 ± 0.26 (68.00)	8.782 ± 0.66 (42.60)

The % inhibition of enzyme activities is indicated in the parentheses

The NAD-GDH activity in the presence of culture filtrates tested showed the percent inhibition of NAD-GDH activities from 18.31 to 35.14 %. Amongst the cultures tested *Bacillus* sp B1 showed highest percent inhibition of NAD-GDH activity from hyphal form by 55.14 % (Table 5.3). Out of the synthetic organic compounds tested S-1 compound showed 91.77 % inhibition of NAD-GDH activity whereas the isophthalic acid showed 73.81 % inhibition. The NADP-GDH activity

from hyphal form was inhibited by 85.30 % by the culture filtrate of *Bacillus* sp B 15 whereas isophthalic acid showed 45.74 % inhibition. The NAD-GDH from the yeast form was inhibited to 42.90 % by the culture filtrate of *Bacillus* sp B1 and by isophthalic acid by 68.00 %. While the NADP-GDH from the yeast form of *B. poitrasii* was inhibited by 75.73 % by the culture filtrate of *Bacillus* sp B2 and using isophthalic acid inhibition was 42.60 % (Table 5.3).

5.2.4 Effect of potential glutamate dehydrogenase inhibitors on pathogenic fungi

As the culture filtrates of the isolated bacterial and fungal cultures along with the organic compounds showed inhibition of glutamate dehydrogenase activity in *B. poitrasii* and also inhibited germ tube formation, these were used for further evaluation as potential inhibitors of growth in selected human pathogenic fungi. Inhibition of growth of pathogenic fungus *C. albicans* was found to be maximum by culture filtrate of *Bacillus* sp B15, inhibition of *Mucor* was by *Bacillus* sp B2 and S-1 compound and Bacillus sp B15 and organic compound S-10 were found to be effective for *Aspergillus niger*.

Table 5.4 Effect of potential glutamate dehydrogenase inhibitor producer microbial culture filtrates on pathogenic fungi

Inhibitors	Zone of inhibition (mm)		
	Candida albicans	*Mucor* sp.	*Aspergillus niger*
Bacillus sp B 1	6-7	9	8-9
Bacillus sp B 2	7	12	8
Bacillus sp B14	6	3-4	4
Bacillus sp B15	11	11	13
Bacillus sp B17	5	3-4	4
Bacillus sp B 21	4-5	4	2
Bacillus sp B 22	4	4	2
Pseudomonas sp P18	2-3	8	8
Streptomyces sp	4	2	3-4
Volutella sp	4	3	3-4
Synthetic organic compounds			
S-1 (5 µg)	5	12	6-8
S-3 (5 µg)	7	10	6
S-10 (5 µg)	6	10	9

5.3 Discussion

Several inhibitors of biochemical correlates of morphogenesis were studied in detail using different screening tests and model organisms. Davila *et al.* (1986) reported that papulacandin B, inhibited glucan synthase in *P. brasiliensis*. In this

case hyphal growth and the yeast-hypha transition were inhibited but no effect was observed on yeast growth. Inhibitors of ornithine and arginine decarboxylases reduced the growth of the fungus *B. cinerea* grown on Czapek Dox medium (Smith *et al.*, 1990). Zhu and Gooday (1992) studied the effect of nikkomycin and echinocandin on differentiated and undifferentiated hypha of *C. cinereus* and for *M. rouxii* on spore germination and hyphal growth. 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 solubilized forms of chitin synthase. Frost (1995) used whole cell of *C. albicans* to identify inhibitors towards cell wall synthesis and assembly. The authors used efficacy of sorbitol protection and inhibition of cell morphogenesis in the presence of these inhibitors as indicators of novel antifungal agents. Tariq and Devlin (1996) reported that nikkomycin showed selective toxicity against chitinous fungi that were parasitic on the host organisms.

Similar types of biochemical changes are reflected during morphological change in *B. poitrasii*. Therefore, it could be used as a test system to evaluate the *in vivo* and *in vitro* effect of inhibitors. It has been also reported that relative proportion of both NADP- and NAD -GDH ratio plays an important role in morphogenetic process in *B. poitrasii* (Khale *et al*., 1992). Therefore, GDH enzyme was studied as a potential target for developing new antifungal agents.

Among all the culture filtrates tested as potential inhibitor producers the *Bacillus* sp B1 and *Bacillus* sp B15 showed maximum zone of inhibition of spore germination and Y-H transition was inhibited significantly by the culture filtrates

from *Bacillus* sp B17, *Bacillus* sp B15 and synthetic organic compound S1 and isophthalic acid (Tables 5.1 and 5.2).

The *Bacillus* sp B1 culture filtrate inhibited mainly NAD-GDH activity of hyphal form while *Bacillus* sp B15 inhibited NADP-GDH activity. The *Bacillus* sp B2 culture filtrate effectively inhibited NADP-GDH of the yeast form of *B. poitrasii*. Further purification of these compounds and their validation with plant and human pathogenic fungi are necessary initial steps towards their effective application of the GDH as a target in agriculture and human health care.

Chapter VI: Conclusion

Conclusion

From the literature it is evident that different biochemical correlates of the morphological outcome have been studied in different fungal strains (Deshpande, 1996). As cell wall is the key determinant of the morphology, the enzymes involved in cell wall metabolism are the favored correlates identified for the studies. In this regard, other than chitin synthases and chitinases, enzymes involved in carbon and nitrogen metabolism have also been studied significantly (Chitnis, 2001; Deshpande, 1996; Ghormade, 2000; Ghormade *et al*., 2005).

Peters and Sypherd (1979) in *Mucor racemosus* correlated the expression of NAD-GDH with the morphological outcome. Khale *et al*. (1992) reported that the relative proportion of the two ammonia-assimilating enzymes expressed, as NADP-/ NAD- GDH ratio was important in deciding the morphological outcome in the dimorphic fungus *B. poitrasii*. Futhermore, it has also been reported that the expression of NADP-GDH is form specific, while the NAD-GDH is common in the two forms i.e. yeast and hypha (Amin *et al.*, 2004). Interestingly, the NAD-GDH native enzyme activity levels are 10 fold lower in the yeast form cells than the hyphal form cells.

Khale *et al*. (1992) studied the effect of glucose in a complex medium on the GDH activity and its correlation with Y-H transition in *B. poitrasii*. In the present studies the effect of temperature, pH, glucose in the synthetic medium and different brand peptones with different Zn^{++} and on NAD-GDH and NADP-GDH activities on Y- H transition has been observed.

Most interesting observation was the effect of different brand peptones as nitrogen source on the Y-H transition in *B. poitrasii.* For instance, NAD-GDH activity in the cells grown in Difco peptone was high and the germ tube formation was greater (60 %) but in Himedia peptone it was decreased (17.64 %). This was attributed to the difference in metal ion concentration in the different peptones. As reported in *C. albicans*, *H. capsulatum* and *M. rouxii* the presence of metal ion Zn^{++} inhibited the germ tube formation. Further the effect of Zn^{++} on morphological form was studied. The effect of different concentrations of Zn^{++}, metal chelators like EDTA confirmed the role of Zn^{++} in the Y-H transition in *B. poitrasii* which is effected through the regulation of GDH activities. In this regard, the studies with the monomorphic mutant (Y-5) supported the findings with the parent strain. Though the membrane fluidity plays an important role in the yeast or hyphal growth in fungi as suggested by Harold (1997), our data indicates that Zn^{++} had more pronounced effect than the ethanol on the Y-H transition in Y-5 mutant of *B. poitrasii*. Indeed it could have been conclusive if the true hypha form monomorphic mutant could have been isolated.

The relationship between NAD-GDH and chitin deacetylase with respect to the effect of Zn^{++} and the chitosan contents of the yeast and hyphal form in *B. poitrasii* indirectly supported the findings on GDHs and their role in dimorphism in *B. poitrasii*. These observations can be further used for the commercial chitosan production using *B. poitrasii*. The *in vitro* study of purified GDHs was one of the approaches used to understand its explicit role in dimorphism of *B. poitrasii.*

As NAD-GDH shows biochemical correlation with Y-H transition in *B. poitrasii,* it was considered as new target for the development of antifungal agents. Screening for NAD-GDH inhibitors was carried out using three tests 1) spore germination, 2) observation of germ tube formation during Y-H transition and 3) measurement of NAD-GDH activity from the yeast and hyphal form of *B. poitrasii.* The antifungal metabolites from *Bacillus* sp B1 and *Bacillus* sp B15 which inhibit NAD-GDH activity, Y-H transition, indicate the possible role of NAD-GDH in the dimorphism of *B. poitrasii*.

Thus, during the course of this work the significant role of NAD-GDH as biochemical correlate in the Y-H transition of *B. poitrasii* was established. The purification of the enzyme was carried out to through light on its biochemical characteristics. The evaluation of NAD-GDH, the biochemical correlate of dimorphism in *B. poitrasii* as potential antifungal target was carried out.

Chapter VII: References

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List of Publications

1. Vandana Ghormade, Sudhakar Sainkar, Chetan Joshi, **Namita Doiphode** and Mukund Deshpande. (2005) Dimorphism in *Benjaminiella poitrasii*: Light, Fluorescence and Scanning Electron Microscopy studies of the vegetative and reproductive forms with special reference to the glutamate dehydrogenase, a novel fungicidal target. Journal of Mycology and Plant Pathology. **35:** 1-11.
2. **Namita Doiphode**, Chetan Joshi, Vandana Ghormade and Mukund Deshpande. Effect of zinc on yeast – hypha transition in *Benjaminiella poitrasii* Mycopathologia. (Communicated)

Papers presented in Symposia/Conferences/Meetings, etc.

1. **Namita Doiphode**, G.J. Kaur, F. Shirazi, U.Manimaran and M.V.Deshpande. Chitin metabolizing enzymes: novel targets for antifungal agents. Presented in poster session at Yeast 2005: An International Meeting on yeast biology at IISc, Bangalore from September 27-29, 2005.
2. **Namita Doiphode**, C.Joshi, N.Srinivas, M.Kulkarni, R.Meti, V.Ghormade and M.V. Deshpande. Fungal dimorphism and antifungal strategies. Presented at research student meet at NCL, Pune on February 26, 2005.
3. Vandana Ghormade, Sudhakar Sainkar, Chetan Joshi, **Namita Doiphode** Mukund Deshpande. Dimorphism in *Benjaminiella poitrasii*: Light, Fluorescence and Scanning Electron Microscopy studies of the vegetative and reproductive forms with special reference to the glutamate dehydrogenase, a novel fungicidal target. Journal of mycology and plant pathology. Udaipur. October 15-17, 2004.

4. **Namita Doiphode**, Chetan Joshi, Trupti Joshi and Mukund Deshpande. Role of NAD- and NADP-dependent glutamate dehydrogenase in yeast-hypha transition of *Benjaminiella poitrasii*. Presented at research student meet at NCL, Pune on February 27, 2003.
5. Trupti Joshi, Chetan Joshi, **Namita Doiphode** and Mukund Deshpande. Effect of different organic nitrogen sources on the yeast -hypha transition in *Benjaminiella poitrasii*. Presented in poster session at Yeast 2003:An International Meeting on yeast biology at IMTECH, Chandigarh from February 20 -22, 2003.
6. **Namita Doiphode**, Chetan Joshi, Trupti Joshi and Mukund Deshpande. Role of NAD- and NADP-dependent glutamate dehydrogenase in yeast-hypha transition of *Benjaminiella poitrasii*. Presented in poster session at Yeast 2003:An International meeting on yeast biology at IMTECH, Chandigarh from February 20-22, 2003.