CHAPTER II

MATERIALS AND METHODS
### 2.1 Materials

#### Table 2.1 Sources of chemicals

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>Sisco research laboratory, India</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>2-Ketoglutarate</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Blue sepharose</td>
<td>Amersham biosciences, UK</td>
</tr>
<tr>
<td>Bacto-peptone</td>
<td>Difco Industries, Detroit, MI, USA</td>
</tr>
<tr>
<td>Cyclohexylamine</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>DEAE fast flow Sepharose</td>
<td>Amersham biosciences, UK</td>
</tr>
<tr>
<td>Diethyl pyrocarbonate</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>Merck, Germany</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Merck, Germany</td>
</tr>
<tr>
<td>Ethylenediamine tetraacetic acid</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
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<tr>
<td>N,N'-Methylene bis acrylamide</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
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<tr>
<td>Fluorescein isothiocyanate</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
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<td>L-ornithine</td>
<td>SD-fine chemicals India</td>
</tr>
<tr>
<td>pGEM-Teasy vector</td>
<td>Promega (USA)</td>
</tr>
<tr>
<td>Primers</td>
<td>IDT, USA</td>
</tr>
<tr>
<td>Putrescine</td>
<td>Sisco research laboratory, India</td>
</tr>
<tr>
<td>Sephracryl S-300</td>
<td>Amersham biosciences, UK</td>
</tr>
<tr>
<td>2,4,6-trinitrobenzenesulfonic acid</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Difco Industries, Detroit, MI, USA</td>
</tr>
</tbody>
</table>

All other chemicals used were of analytical grade.
2.2 Organisms and culture conditions

The parent strain *Benjaminiella poitrasii*, a 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. 

*Escherichia coli* (JM 109 strain) was used as a host strain for the cloning experiment. Cells were taken from glycerol stocks stored at -70°C, spread on Luria Bertani (LB) medium (0.5% yeast extract, 1% tryptone, 1% NaCl, 1.5% agar) and incubated at 37°C until colonies were observed (16-18 h). For bacteria containing plasmid, the LB medium was supplemented with ampicillin (100 µg/ml). For the plasmid preparation, a single colony was inoculated into 5 ml LB medium containing 100 µg/ml ampicillin and grown for 16 h with shaking (180 rpm) at 37°C. For permanent storage, bacterial cells were stored at -70°C in 30 % (v/v) glycerol.

2.2.1 Spore suspension

The sporangiospores of *B. poitrasii* required for yeast to hypha transition studies were obtained from 7 d old parent strain grown on YPG 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 8 x 10⁶ sporangiospores/50 ml of culture medium.

2.2.1.1 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 purity of the morphological was confirmed microscopically.

2.2.2 Yeast - hypha transition studies

The yeast form of *B. poitrasii* was obtained by inoculating 8 x 10⁶ spores/50 ml of YPG (1 %, glucose) medium and incubated on rotary shaker (180 rpm) at 37°C for 24 h. For the yeast to hypha transition, the yeast cells obtained (8 x 10⁶ cells/50 ml) were washed in YP medium and inoculated into YPG (0.1%, glucose) or YP medium with varying temperature, pH, glucose concentration or other factors and incubated on rotary shaker (180 rpm) at 28°C unless otherwise mentioned.
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.

Hyphal cell was also grown in synthetic medium (0.3 % NaNO₃, 0.1 % K₂HPO₄, 0.05 % MgSO₄, 0.05 % KCl, 0.001 % FeSO₄ and 0.1 % glucose, pH 6.2) at 28°C for 24 h that was used for the microscopic studies on the cell surface.

2.2.1.1 Screening of inhibitors

For the screening of inhibitors, different concentrations of inhibitors (0-40 μg/ml) were added in 3 ml of YPG (0.1% glucose) from the stocks prepared in DMSO (4 mg/ml) and equal volume of DMSO was added in a control tube. Inoculum was washed with YP and inoculated as mentioned section 2.2.2 and incubated at 28°C for 12 h. Percentage germ-tube formation was calculated after 12 h. Inhibitory concentration was calculated with reference to the value obtained from the control sample. Inhibitory concentration was measured as concentration required to prevent 50 % germ-tube formation. Inhibitors used were chemically synthesized as reported by Vatmurge et al., (2008) and Bavikar et al., (2008). The compound tested were β-lactam–bile acid conjugates (B-compounds), bile acid dimers linked with triazole and bis-β-lactam (D-compounds) and cholic acid derivatives (S-compounds).

2.2.1.2 Growth conditions for the fluorescence, atomic force microscopy and flocculation.

The spores were inoculated into 50 ml of liquid YPG (1 % glucose) medium and incubated at 37°C for 24 h to obtain yeast (Y) cells, which were washed with ice-cold YP and used as an inoculum (8 x 10⁶/50 ml), unless otherwise mentioned. To obtain hyphal cells, YP (0.3 % yeast extract and 0.5 % peptone, pH 6.4) or synthetic (0.3 % NaNO₃, 0.1 % K₂HPO₄, 0.05 % MgSO₄, 0.05 % KCl, 0.001 % FeSO₄ and 0.1 % glucose, pH 6.2) medium were inoculated at 28°C for 24 h as described by Khale et al. (1990). The yeast form cells obtained in YPG medium were inoculated (8 x 10⁶/50 ml) again in YPG (1 % glucose) medium and incubated at 28°C for 24 h on rotary shaker (180 rpm). The monomorphic (Y-5) mutant cells were also inoculated (8 x 10⁶/50 ml) in YPG medium at 28°C and grown for 48 h.

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Flocculation experiments were performed in YPG with and without 1 mM glutamate (substrate for NAD-GDH), 1 mM 2-ketoglutarate (substrate for NADP-GDH), or 2.5 mM isophthalic acid (NAD-GDH inhibitor). The quantitative difference in the mannan contents of the cell wall of Y cells grown with and without addition of benomycin-A (mannan synthase inhibitor) (0.5 µg/ml) to 10 ml of YPG medium and grown at 28°C for 24 h were studied using fluorescence microscopy.

2.2.1.3 Temperature and/or glucose exposure of yeast form cells

Experiment was done with the yeast form cells grown in YPG (0.1% glucose) for 18 h. To obtain a homogenous population based on size (8-12µ) the 18 h grown culture was filtered using glass fiber filter (G1, Jensil India) as described in section (2.2.1). The filtration by G1 filter yielded a uniform sized yeast population and was totally devoid of hyphal contamination. The Y-H transition was studied at 28°C for 8 h in a transition medium YPG (0.1% glucose). The inoculum yeast cells were pre-incubated under different conditions as follows: i) At 22°C for 10 min (T) in transition medium (control at 28°C in transition medium for 10 min); ii) YPG (10% glucose) for 10 min (G) at 28°C (control at transition medium for 10 min); iii) both treatments given simultaneously (GT, YPG (10% glucose) at 22°C for 10 min); and iv) both treatments in the sequence of temperature followed by glucose (T+G) and glucose followed by temperature (G+T). The cells exposed to glucose treatment were centrifuged (1000 g, 5 min) and washed with YPG (0.1% glucose) medium before subsequent treatment. The morphological outcome was observed by counting the number of germ tube forming cells and the budding yeast cells after 8 h. As the lowering of temperature favoured hyphal form, the temperature effect was measured by counting increase in the germ tube forming cells. The glucose effect was studied using change in per cent budding cells. The reversal effect during the sequential treatment was studied by measuring the percent reduction in the morphological outcome (germ tubes/budding) of the exposure given first.

2.2.1.3 Effect of polyamines synthesis inhibitors on Y-H transition

To study the effect of inhibitors on GDH activity, trifluoperazine (TFP, 40 µM) a ca-calmodulin dependent protein kinase inhibitor and H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine, 100 µM), a cAMP dependent protein
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kinase inhibitor were prepared in DMSO (dimethyl sulfoxide) and added during the temperature and glucose exposure (control with DMSO without inhibitor) at subinhibitory level. The concentrations selected affected differentiation but not growth. To study the effect of inhibitors on germ tube formation and budding, the inoculum cells were washed twice before their transfer to the transition medium and were counted as mentioned in section 2.2.2.

2.3 Molecular methods

2.3.1 Isolation of genomic DNA from B. poitrasii

Yeast form cells of B. poitrasii were used to isolate genomic DNA. Cells were harvested by filtration through Whatmann No. 1 paper, washed with sterile water, frozen immediately in liquid N2, and stored at -80° C until use. The cells were ground using mortar and pestle under liquid N2. The ground cells (1g) were then transferred to 10 ml of extraction buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate (SDS), 50 mM DTT, 1% β-mercaptoethanol) and mixed by inversion 2-3 times. This was followed by three phenol:chloroform (1:1) and two chloroform extractions. The DNA was precipitated with 0.8 volumes of isopropanol. The pellet was then washed with absolute ethanol, air-dried, and resuspended in TE (Tris pH 8.0, 10 mM; Tris, EDTA 1 mM).

2.3.2 Plasmid DNA isolation from E. coli

Plasmid isolation from E. coli transformants was carried out by the alkaline lysis method described by Sambrook and Russel (2001). Cells were grown for 16 h in 5 ml LB-ampicillin as described in Section 2.2. A 1.5 ml portion of culture was transferred into a 1.5 ml microfuge tube and spun down at 11600 x g for 30 s in a microcentrifuge. The pellet was resuspended in 100 |al ice-cold solution consisting of 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0. After the addition of 200 |al freshly prepared 0.2 M NaOH, 1% SDS, the suspension was mixed by inversion and placed on ice. A 150 |al of stock solution containing 60 ml potassium acetate (5M), 11.5 ml glacial acetic acid and 28.5 ml distilled water was added and the tube vortexed in an inverted position for 10 s to disperse the solution through the viscous lysate. The tube was then kept on ice for 3-5 min. The lysate was spun at 11600 x g for 5 min and the supernatant was transferred to a clean microfuge tube. An equal volume of phenol/chloroform (1:1) saturated TE, was added after vortexing. The
suspension was then centrifuged at 11600xg for 2 min. The aqueous phase was removed and the DNA was precipitated with two volumes of 96% ethanol at room temperature for 2 min. The DNA was pelleted by centrifugation at 11600 x g for 5 min. The pellet was washed with 70% ethanol and dried at 37°C for 10 min. The DNA was redissolved in 50 ml TE containing DNAase – free pancreatic RNAase (20 mg/ml).

2.3.3 Isolation of total RNA from *B. poitrasii*

To isolate whole RNA, *B. poitrasii* yeast cells were inoculated in YPG (0.1% glucose) medium and incubated at 28°C for 12 hrs. Yeast and hyphal cells were separated as described in section 2.2. Cells were harvested by filtration through Whatmann No. 1 paper, washed with sterile water, frozen immediately in liquid N2, and stored at -80°C until use. The cells were ground using mortar and pestle under liquid N2. The RNA was then isolated with RNeasy spin column (Qiagen, USA) according to the manufacturer’s instructions. The RNA preparations were stored at -80°C until use. Quality of RNA was checked by spectrophotometric and agarose gel electrophoresis analysis.

2.3.4 cDNA synthesis

For synthesis of cDNA from total RNA, the isolated RNA samples were first treated with DNase (Promega, USA) to remove any contaminant DNA as per manufacturer’s instruction. For cDNA synthesis, the 1-2 μg of RNA was taken and reaction was carried out with the help of reverse transcriptase (Sensiscript, Qiagen, USA) first strand synthesis kit as per manufacturer’s instruction. Synthesis of cDNA was confirmed with ITS1 and ITS4 primers and analyzed by agarose gel electrophoresis.

2.3.5 Polymerase chain reaction (PCR) and RT-PCR

To amplify NADP-GDH gene fragment from *B. poitrasii*, degenerate PCR primers directed towards conserved region in NADP-GDH were used for the PCR amplification of the genomic DNA. The primers were: forward GDHC1 5’- CYG ACT TTG ACC CCA AGG G-3’ and reversed oligo (dT)12. The PCR reactions were performed in 20 μl volume containing reaction buffer (with 1.5 mM MgCl₂), forward and reverse primers (1μM of each, obtained from IDT, USA), 200μM of each dNTPs and 1 unit of Taq polymerase (all procured from Bangalore Genei, India) and 100 to
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150 ng of DNA or cDNA. The amplification was carried out using the thermal cycler (Myccycler, Biorad, USA), programmed for 1 cycle of 94°C for 3 min, followed by 35 cycles each of 94°C for 30 s, 50°C for 45 s and 72°C for 1 min. This was followed by the final cycle of 72°C for 7 min. The products obtained at the end of the PCR reactions were run on the gel to determine the existence of the amplified fragments of the desired length.

2.3.6 Cloning of DNA

Cloning of the PCR amplified fragments was done in two steps: (a) ligation reaction and (b) transformation. Ligation reactions were performed at 4°C using pGEM-Teasy vector from Promega (USA) according to manufacturer’s instructions. For the transformation reactions, *E. coli* JM109 competent cells were prepared by Inoue method as described by Sambrook and Russell (2001). The transformation reactions were also performed according to manufacturer’s instructions. The transformed cells were plated on LB media containing X-gal (40 μg/ml), IPTG (0.5 mM) and ampicillin (100 μg/ml) and incubated at 37°C for 24h. After incubation, white colonies on the plates were identified as those containing recombinant DNA.

Plasmid samples bearing the expected insert were used for the sequencing. Sequencing was done on an AB3730 DNA analyzer using the Big Dye terminator kit (Applied Biosystems, Inc. Foster City CA)

2.4 Biochemical methods

2.4.1 Cell extract preparation

Cell extracts of yeast and hyphal forms of *B. poitrasii* were obtained by the modified 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 100 mM, pH 7.4 containing 1.0 mM EDTA, 1 mM PMSF and 3 mM DTT. 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 20 min to obtain cell extract. Extract was used for the estimation of enzyme activities. In the case of glutamate synthase, DDT was omitted from the extraction buffer.
2.4.2 Enzyme assays

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

The intracellular NAD- and NADP-dependent GDH (E.C 1.4.1.2 and E.C 1.4.1.4, respectively) was assayed using standard procedure, described by Khale et al. (1992). The reductive amination of 2-ketoglutarate was measured by monitoring the decrease in the A340 of NAD(P)H. The reaction mixture (1ml) contained 50-100 μl of crude extract, 200 mM NH₄Cl and 30 mM 2-ketoglutarate in 100 mM Tris-HCl 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 2-ketoglutarate was omitted from the reaction mixture was run for each assay. One unit (U) of NAD- or NADP-GDH was defined as the amount of enzyme required to oxidized 1 nmol of NADH or NADPH per min per mg protein.

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

The intracellular glutamate synthase was assayed using standard procedure described by Khale et al. (1992). GOGAT was assayed by monitoring NADH and NADPH oxidation at 340 nm. The reaction mixture (1ml) contained 50 μl of crude extract, 10 mM 2-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. One unit (U) of NAD- or NADP-GOGAT was defined as the amount of enzyme required to oxidized 1 nmol of NADH or NADPH per min per mg protein.

2.4.2.3 Glutamine synthetase (GS, E.C 6.3.1.2)

The GS was assayed using standard procedure, described by Khale et al. (1992). A fresh concentrated assay mixture containing 18 mM hydroxylamine HCl, 0.27 mM MnCl₂, 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 1 ml of stop mixture (5.5 g FeCl₃·6H₂O, 2.0 g TCA and 2.1 ml concentrated HCl in a final volume of 100 ml). Samples were centrifuged to remove precipitate, and A₅₄₀ of γ-glutamyl hydroxymate in the supernatant was read.

One unit (U) of GS activity was defined as the amount of enzyme required to produce 1.0 nmol of γ-glutamyl hydroxymate per min per mg protein.

2.4.2.4 Ornithine decarboxylase (ODC, E.C.4.1.1.17)

2.4.2.4a Spectrophotometric assay

The reaction mixture (400 µl) contained 5 mM DTT, 1.5 mM EDTA, 0.1 mM pyridoxal phosphate (PLP) and 0.5 mM cyclohexylamine in 0.15 M potassium phosphate buffer (pH 7.1). The 400 µl crude cell extract was added to 400 µl reaction mixture and the reaction was initiated by the addition of 9.6 µmol L-ornithine HCl (400µl). Reaction was carried out at 37°C for 30 min. and terminated by the addition of 800 µl of 1 M HClO₄. The reaction mixture was centrifuged at 5000 g for 10 min and 0.5 ml of the supernatant was used for the estimation of putrescine. The heat killed enzyme in the reaction was used as a control.

For the estimation of putrescine, 2,4,6-trinitrobenzenesulfonic acid (TNBS) was used as described by Ngo et al. (1987). The 0.5 ml putrescine and 1 ml 4 N NaOH were mixed under vigorous shaking. 2 ml 1-pentanol was then added and the sample was mixed and vortexed for 20 s. The emulsion was centrifuged at 2000 g for 5 min, and 1 ml of upper organic phase was transferred to the test tube containing 1 ml 0.1 M sodium borate buffer, pH 8.0 and was mixed briefly. 1 ml 10 mM TNBS dissolved in 1-pentanol was added, vortexed for 20 s and 2 ml dimethyl sulfoxide (DMSO) was added and vortexed for 20 s. The sample was centrifuged at 2000 g for 5 min and the absorbance of the trinitrophenyl (TNP) putrescine adduct present in the organic phase was measured at 420 nm. The putrescine (6-250 nmol) in 0.1 M potassium phosphate buffer, pH 7.0 was used to plot a standard graph.

One unit of ODC activity was defined as the amount of enzyme required to produce one nmole of putrescine per min per mg protein.
2.4.2.4b Radiometric assay

In this method the release of $^{14}$CO$_2$ from $^{14}$C-labeled ornithine was measured as described by Voige (1997) with some modifications. A 400 µl crude cell extract was added to 100 µl reaction mixture containing varying concentration of L-ornithine and 25 mCi L-[1-$^{14}$C] ornithine (52 mCi/mmol) prepared in K-phosphate buffer (pH 7.1) containing 0.1 mM EDTA, 2 mM DTT, 0.2 mM PLP and 1 mM PMSF in a glass tube (14 x 88 mm). Reaction tubes were fitted with a rubber stopper and a glass microfibre filters (Whatman GF/C, 2.5 cm) soaked in 200 µl 2N KOH. After 30 min incubation at 37°C, the reaction was stopped by adding 800 µl of 1M HClO$_4$, vials were incubated further for another 30 min to trap CO$_2$ released. The $^{14}$CO2 released was trapped on filter paper disc. At the end of incubation, filter papers are transferred to vials containing 3 ml scintillation fluid (4g PPO (2,5 diphenyloxazole) and 0.1g POPOP (1,4-bis[5-phenyl-2-oxazolyl]- benzene) in 1:1 toluene) for radioactivity counting. The heat killed enzyme was used as a control. One unit of ODC activity was defined as one nmole of CO$_2$ released per min per mg protein. The amount of radioactivity in each vial is determined by liquid scintillation spectrometry (Beckmann LS-500). All the experiments were carried out in triplicate for three times.

2.4.3 Estimation of polyamines

The standard polyamines and polyamines in cell extracts were benzoylated by a slightly modified method of Flores and Galston (1984). One ml of 2N NaOH and 10 µl of benzoyl chloride were added to 200 µl of the polyamine aliquots and vortexed for 30 sec. After incubation at 25°C for 20 min, 2 ml saturated NaCl was added to the samples to stop the reaction. The benzoyl polyamines were extracted in 3 ml of diethyl ether. The samples were centrifuged at 1500 x g for 5 min and 1.5 ml of ether phase was collected and evaporated over a water bath (60°C) to dryness. The benzoyl polyamines were redissolved in 200 µl of 64 % (v/v) methanol (HPLC grade; Merck, Germany) and the extract was analyzed by HPLC. The benzoylated samples were stored at –20°C.

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

2.4.4 Estimation of protein

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

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(A280 nm - A340 nm) - (A260 nm-A340 nm)]

2.4.5 Enzyme purification

2.4.5.1 Isoelectric focusing

The isoelectric focusing of purified NAD-GDH was carried out using preparative isoelectric focusing unit on Horizontal Rotofor 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 of 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.

2.4.5.2 Ammonium sulphate fractionation

The precipitation of NAD-GDH from the cell extract of hyphal form of B. poitrasi was carried out as follows. The (NH₄)₂SO₄ 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 8000 x g at 4°C. This was followed by addition of (NH₄)₂SO₄ to 55 % saturation to the supernatant. This mixture was stirred for 45 min and the precipitate was separated by centrifugation at 8000 x g at 4 °C. The precipitate thus obtained was dissolved in buffer B (20 mM potassium phosphate
buffer (pH 7.4) 1mM EDTA and 3mM DTT) and dialysed against 1 liters of the same buffer to remove salt.

2.4.5.3 DEAE ion exchange chromatography

Dialyzed fractions were applied to a DEAE fast-flow column (23 by 2.8 cm) equilibrated with buffer B. After sample application, the column was washed with 3 column volumes of buffer B. NAD-GDH was subsequently eluted with a linear KCl gradient of 4 column volumes (0–0.5 M). Fractions with NADP-GDH activity were pooled and dialyzed against 1 liters of buffer B. It was then concentrated with the help of YM-30 membrane in Amicon ultrafiltration unit (Millipore, US). The sample obtained was applied on the blue sepharose column.

2.4.5.4 Blue sepharose affinity chromatography

A reactive blue sepharose column (10 by .2 cm) was equilibrated with buffer B. After application of the sample from the previous step, the column was washed with 3 volumes of buffer B. NAD-GDH was eluted with a linear KCl gradient of 4 column volumes (0–0.5 M). Fractions with NAD-GDH activity were pooled, dialyzed against buffer B, concentrated by ultra-filtration Amicon YM30 membrane and dialyzed against 1 liter of buffer B.

2.5 Analytical methods

2.5.1 Assay of flocculation

The yeast form cells (Y and Y-5) were obtained as mentioned in section 2.2. Cells were washed with deionized water until there was no absorption at OD₆₀₀ in the washings. The washed yeast cells were resuspended at 1.3 - 2 x 10⁶ cells/ml in 0.1% Tween 80 prepared in deionized water. The initial OD₆₀₀ was recorded and readings were taken at an interval of 5 min for 60 min.

2.5.2 Zeta potential

Zeta potential measurements were carried out according to Smijs et al., (2007) with Brookhaven particle size analyzer (BIC, US) on the yeast and hyphal forms grown in different media. Yeast cell samples were prepared as mentioned under section 2.5.1. Hypha were harvested, washed and resuspended in deionized water. The hyphal suspensions were sonicated for 6 cycles of 5 sec each (50% amplitude, Branson Sonifier, US). Hyphal cell samples were prepared by washing until there was no absorption detected at OD₆₀₀ in the supernatant. Washed cells were suspended
in 20mM buffer solutions of different pH values (pH 3-9) containing 30% glycerol prior to zeta potential measurement. For every sample two measurements were carried out for every indicated pH and every measurement contained 5 runs.

2.5.3 Microscopy

2.5.3.1 Fluorescence microscopy

Fluorescein isothiocyanate (FITC) labeled wheat germ agglutinin (WGA) and concanavalin A (Con-A, 0.5 mg/ml in buffer A (1 mM CaCl₂, MgCl₂, and MnCl₂ each in 20 mM Tris-HCL buffer, pH 7.0)) were used for staining as described by Mormeneo et al. (1996). After 30 min of incubation in dark, cells were washed with the buffer A to remove excess stain. Fluorescence was observed under epifluorescence microscope (Leitz LaborLux S, Germany) using I3 filter with an excitation range of 450-490 nm. Fluorescence as well as optical images were recorded by the Canon power shot S80 digital camera (Japan) attached to the microscope.

2.5.3.2 Atomic force microscopy (AFM)

B. poitrasii yeast and hyphal cells harvested from different media were washed twice with buffer A. Air-immobilization technique was used to fix the cells on a coverslip. Cells were (resuspended in 5μl of buffer A) placed on a coverslip, and air-dried under sterile condition. The AFM scans were obtained in air (23°C, RH 45%), using Multiview 1000 system (Nanonics Imaging Ltd. Israel). Scanning was done in tapping mode with SuperSensor™ NSOM/AFM probe with >1-20 N/m force constant and 50 nm apex radius at set resonant frequency of 25-70 kHz. Cells were selected after observation under light microscope (Olympus) attached to the AFM. AFM images were acquired by Quart software (Cavendish Laboratories, UK). Height images were acquired by line scans of 256 x 256 lines per unit scan area and 512 x 512 lines per unit scan area. The images were further processed and analyzed using WSxM software (Horcas et al, 2007). Roughness of the cell surfaces was compared from their Root Mean Square of the roughness values (RMS values) from the height images acquired by the AFM.

2.5.4 Agarose gel electrophoresis

Agarose gels were prepared by melting agarose in TAE buffer (Tris-acetate, 40 mM; EDTA, 1mM; pH 8.0). To cast and run the gel, Bio-Rad Mini, Midi or Maxi
electrophoresis cells were used (Biorad, UK). The concentration of agarose varied according to the size range of DNA molecules being separated. These were: uncut DNA, greater than 8kb (0.5-0.8%); 8-0.5 kb (0.8-1.2%) and 2-0.4 kb (1.2 – 1.6%) containing 5 μg/ml ethidium bromide. The solidified gel was placed into the electrophoresis cell. TAE buffer was poured onto the gel up to approximately 5 mm over the gel. DNA samples were loaded into the wells after being mixed with 6x gel loading buffer. As molecular weight markers, 200 ng of a Generuler ladder (Fermentas, USA) was used. DNA samples were electrophoresed through the gel at 5 V/cm for 1 h. The DNA was visualized under ultraviolet light on a Gel Doc XR system (Biorad, USA).

2.5.5 Polyacrylamide gel electrophoresis

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).

2.5.6 Activity staining for NAD- and NADP- dependant 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⁺ 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).