6.1 Introduction

β-1, 3 glucanases represent a well-known class of enzymes widespread in bacteria and fungi, and are hydrolases specific to O-glycoside bonds between 1,3-linked glucopyranose residues found in a variety of β-glucans (Zhu et al., 2008). In fungi, several of these enzymes have roles during cell separation in unicellular organisms, and the development of cell wall architecture in yeasts and filamentous fungi (Adams, 2004). In bacteria, these enzymes take part in the degradation of polysaccharides that can be present in their natural environment and be used as an energy source (Planas, 2000). Exo-β-1, 3 glucanases cleave glucose residues from non-reducing ends, while, endo-β-1, 3 glucanases cleave β-linkages at random sites along polysaccharide chain releasing short oligosaccharides (Garciduenas et al., 1998). Exo-glucanases degrade the polysaccharides completely and release monosaccharide residues, where as endo-glucanases produce oligosaccharides by partial degradation of the exopolysaccharides (Vijayendra and Kashiwagi, 2009).
Bacterial and fungal β-1, 3 glucanases are involved in the degradation of polysaccharides that can be present in their natural environment and used as an energy source (Planas, 2000).

### 6.1.1 Structure of microbial β-1, 3 glucanase

β-1, 3-1, 4 glucanases are classified as members of family 16 with a jellyroll β-sandwich structure. The three dimensional structure of family 16 1,3-1,4-β-glucanase can be explained by the structure of a hybrid H(A16-M) 1,3-1,4-β-glucanase of mature *Bacillus amyloyticus* and *Bacillus macerans*. The core of the protein is formed by two β-sheets stacking atop each other in a sandwich like manner, which consists of seven anti-parallel strands each that are bent and create a cleft crossing one side of the protein where the substrate is found. Loops between the β-strands are mostly stabilised by β-turns and there is only one turn with an α-helical geometry. A major surface loop (residues 20-36 in *B. macerans* numbering) covers partially the binding site cleft created by the bending of the β-sheets, where the disulfide bond between Cys30 and Cys59 links this loop to the β-strand 56-64 of the protein core. On the convex side of the molecule, remote from the active site, a calcium ion is bound which plays a role in stabilising the native protein structure. Cation binding has been analysed by comparing the crystal structures and stabilities of the hybrid H (A16-M) with bound Ca2 or Na⁺ ions. Calcium is bound with nearly perfect octahedral geometry coordinating to the backbone carbonyl oxygens of Pro7, Gly43 and Asp205, a carboxylate oxygen of Asp205 (*B. macerans* numbering), and two water molecules, whereas sodium is trigonal-bipyramidally coordinated and provides lower thermal stability to the folded protein. The cleft on the concave side of the molecule defines the oligosaccharide substrate binding site. It is lined with mainly aromatic residues on its walls and with acidic residues at the bottom. The catalytic residues are located in the same β-strand where there is a strict alternation of polar (acidic) and non-polar side chains, the first pointing toward the
surface of the protein where they are able to interact with the substrate, the latter toward the hydrophobic interior (Planas, 2000).

### 6.1.2 β-1, 3 glucanases as a mycolytic enzyme

Several strains of bacteria are able to lyse and grow on viable yeast and fungal cells by producing a variety of cell wall degrading enzymes such as endo-β-1, 3 glucanases, β-1, 6 glucanases, mannanases and chitinases (Ferrer, 2006). Mycolytic enzymes produced by antagonistic microorganisms are very important in biocontrol technology (Diby et al., 2005). β-1,3 glucanases hydrolyze hydrolyzes β-1,3 glucans which is an important structural component of the cell walls in many agronomically important pests and have received considerable attention as they play a role in plant growth-promoting systems against plant pathogens. The enzymatic digestion or deformation of cell wall components of these organisms by the enzyme could present an effective method for their biological control (Lim and Kim, 1995). The structure of yeast cell wall composed of complex polymers such as β-1,3 and β-1,6 glucans, mannoproteins and smaller amount of chitin (Ferrer, 2006; Salazar and Asenjo, 2007) implies that synergistic action of these enzymes is necessary to hydrolyse its components. Enzyme systems for yeast cell lysis are usually a mixture of different enzymes such as β-1,3 glucanase, β-1,6 glucanase, protease, mannanase and chitinase, which acts synergistically for the lysis of cell wall (Ferrer, 2006; Salzar and Asenjo, 2007). A β-1,3 glucanase from Chaetomium sp. was found to degrade the cell wall of plant pathogens like Rhizoctonia solani, Gibberella zeae, Fusarium sp, Colletotrichum gloeosporioides and Phoma sp (Sun et al., 2006). An antifungal β-1, 3 glucanase enzyme was reported from the biocontrol fungus Trichoderma atroviride (O’Kennedy et al., 2011). An endo-α-D-(1→3) glucanase capable of hydrolyzing various α (1→3) glucans has been isolated from the fungus Trichoderma viride (Hasegawa and Nordin, 1969). The complementary action of GluA, GluB and GluC
gene products of β-1, 3 glucanase from *Lysobacter enzymogenes* Strain N4-7 results in the hydrolysis of β-1,3 glucans from fungal cell walls (Palumbo et al., 2003). The β-glucanase produced by *Bacillus subtilis* NSRS 89-24 plays a crucial role in the degradation of the fungal cell walls (Leelasuphakul et al., 2006).

### 6.1.3 β-1, 3 glucanases of *Pseudomonas*

β-1, 3 glucanase produced by *Pseudomonas stutzeri* YPL-1 was identified as one of the key enzymes in decomposition of fungal cell walls and the enzyme inhibited 53% mycelial growth of the fungus *Fusarium solani*. The optimum pH and temperature for β-1, 3 glucanase activity was found to be 5.5 and 40°C, respectively (Lim and Kim, 1995). β-1, 3(4) glucanase A from *Pseudomonas* sp. PE2 was found to be an essential enzyme for the degradation of *Pythium porphyrae* cell walls (Kitamura and Kamei, 2006). Mycolytic enzyme such as β-1, 3 glucanases and β-1, 4 glucanases produced by *Pseudomonas fluorescens* was found to be efficient in the lysis of *Phytophthora capsici* mycelium (Diby et al., 2005). β-1, 3 glucanase enzyme produced by fluorescent pseudomonad isolates GRC3 and GRC4 was found to have involvement in the growth inhibition/suppression of the phytopathogenic fungi *Rhizoctonia solani* (Arora et al., 2007). A β-1, 3 glucanase producing *Pseudomonas cepacia* was found to have an efficient role in the biocontrol of soilborne plant pathogen such as *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium ultimum* and decreased disease incidence caused by them by 85, 48 and 71%, respectively (Fridlender et al., 1993). A glucanase producing strain of *Pseudomonas aeruginosa* was used in the biological control of cyst forming nematode *Heterodera cajani* on sesame (Kumar et al., 2009). A *Pseudomonas fluorescens* strain producing enzymes such as β-1,3 and β-1,6 glucanases was used in the biocontrol of soil borne fungal plant pathogen *Rhizoctonia solani* (Dev and Dawande, 2010). β-1, 3 and β-1,4 glucanases produced by *Pseudomonas fluorescences* strains NRC1 and
NRC3 were found to have strong lytic effect on tomato root-rot causing fungal pathogens such as *Rhizoctonia solani* and *Phytophthora capsici* (Saad, 2006). The production of lytic enzyme β-1, 3 glucanase correlated with the antifungal activity of *Pseudomonas fluorescences* against the fungal pathogen *Rhizoctonia solani* (Nandakumar et al., 2002). β-1, 3 glucanase produced by *Pseudomonas aeruginosa* PN1 is reported to cause mycelial lysis, vacuolation and granulation of cytoplasm, hyphal deformities and branching in polyphagous fungus *Macrophomina phaseolina* (Singh et al., 2010). Chan et al. (2003) also had a similar observation. A significant relationship was observed between the level of β-1, 3 glucanase production of *Pseudomonas fluorescences* strains and its antagonistic potential towards the rice sheath blight fungus *Rhizoctonia solani* (Nagarajkumar et al., 2004). The accumulation of β-1, 3 glucanase by *Pseudomonas chlororaphis* PA-23 in canola leaf tissue was found to be responsible for the reduction of *Sclerotinia sclerotiorum* infection in pathogen inoculated plants (Fernando et al., 2007). In pea, seed treatment with *Pseudomonas fluorescens* strain 63-28 has produced chitinases and β-1,3- glucanases which accumulate at the site of penetration of the fungus, *Fusarium oxysporum* resulting in the degradation of fungal cell wall (Benhamou et al., 1996). β-1, 3 glucanase has been found to increase the resistance in Berangan banana plantlets against the fungal pathogen *Fusarium oxysporum* when treated with *Pseudomonas* sp. UPMP3 (Fishal et al., 2010).

### 6.1.4 Application of microbial β-1, 3 glucanases

Yeast lysing glucanases have enormous applications (Salazar and Asenjo, 2007). The various applications include the preparation of protoplasts, cell fusion and transformation of yeast (Kitamura, 1982), production of intracellular enzymes (Zomer et al., 1987), pre-treatment to increase yeast digestibility (Kobayashi et al., 1982), preparation of soluble glucan polysaccharides (Jamas et al., 1986), alkali extraction of yeast proteins (Kobayashi et al., 1982), production of yeast extracts.
Purification and Characterization of $\beta$-1, 3 Glucanase from Pseudomonas…..

(Conway et al., 2001), food preservation (Scott et al., 1987), and release of recombinant proteins from *Saccharomyces cerevisiae* (Asenjo et al., 1993; Ferrer et al., 1996). Yeast lysing glucanases are used for modulating the cell wall permeability as a first step in a downstream process for protein recovery from yeast. Specific activity of the lytic enzymes is a key factor that has to be considered when analyzing the possibility of using lytic enzymes for cell disruption and product release. It has to be sufficiently high to obtain fast cell breakage without allowing endogenous intracellular proteases degrading the product (Salazar and Asenjo, 2007). Purified, protease free glucanase has been used for the controlled cell lysis of the yeast *Saccharomyces cerevisiae*, which results in the selective release of cloned intracellular protein particles (Asenjo et al., 1993).

The bacterial $\beta$-1,3 glucanases have potential applications in brewing. During malting, due to the heat inactivation of endogenous 1,3-1,4- $\beta$-glucanases, large amount of high molecular weight $\beta$-glucans may cause problems such as reduced yields of extracts and lower filtration rates as well as the appearance of gelatinous precipitates in the finished beer. Thus, the level of activity of glucan hydrolases achieved during germination and the amount of their substrates are important factors for good quality brewers malt, and thermostable bacterial 1,3-1,4-$\beta$- glucanases are often added to reduce viscosity during mashing. In animal feedstuff, especially for broiler chickens and piglets, addition of enzymatic preparations containing bacterial L-glucanases improves digestibility of barley-based diets, and reduces sanitary problems such as sticky droppings (Planas, 2000).

*Pseudomonas aeruginosa* MCCB 123 was found to be a potential producer of $\beta$-1,3 glucanase having lytic action on a wide range of fungus and therefore an evaluation have been made in the application of this enzyme in fungal DNA extraction.
6.2 Materials and Methods

6.2.1 Enzyme production

β-1,3 glucanase was purified from the optimized synthetic medium composed of (in g l\(^{-1}\) Distilled water): glucose, 7.5; yeast extract, 2.5; \(\text{NH}_4\text{H}_2\text{PO}_4\), 10.04; \(\text{Na}_2\text{HPO}_4\), 0.5; \(\text{KH}_2\text{PO}_4\), 3.0; \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\), 0.2; \(\text{CaCl}_2\), 0.000625; \(\text{ZnCl}_2\), 0.01; casein, 10.0; pH, 7.0 in a 5-l fermenter (Biostat-B-Lite, Sartorius, Germany). Fermentation was carried out at 25°C, pH 7.0 ± 0.05, 300 rpm and supplied with sterile air at the rate 2.5 l min\(^{-1}\). For enzyme extraction the culture was centrifuged at 8260 g for 15 min at 4°C and the supernatant stored in 300 ml aliquots at -20°C, and used for further purification and characterization.

6.2.2 Purification of β-1, 3 glucanase

6.2.2.1 Ammonium sulphate precipitation

Partial purification of enzyme was carried out by precipitation of the cell-free culture supernatant with ammonium sulphate between 30 and 80% saturation. The precipitates were collected by centrifugation at 8260 g for 15 min at 4°C and the active fractions were pooled and resuspended in 20 mM Tris-Cl buffer, at pH 8.5. The partially purified enzyme was dialyzed against 20 mM Tris-Cl buffer, at pH 8.5 using Amicon UF stirred cell (Millipore Corporation, USA, Model 8010), with a 10 kDa cut off membrane (Omega, 25MM, 10K, Pall life sciences) and used for further purification.

6.2.2.2 DEAE-cellulose chromatography

The enzyme was then loaded on an AKTA Prime protein purification system equipped with a C16/40 (16mm×40cm) (GE Healthcare Biosciences, Uppsala) DEAE cellulose (Sigma – Aldrich Co.) column equilibrated with 20 mM Tris-Cl buffer, at pH 8.5. The column was washed with the same buffer to remove the unbound proteins, and the enzyme was eluted by applying a linear gradient of NaCl from 0 – 1000 mM at a flow rate of 0.5
ml / min., and fractions of 2 mL were collected. Active fractions were pooled and concentrated by lyophilization.

6.2.3 Characterization of $\beta$-1, 3 glucanase

6.2.3.1 Determination of molecular weight

The lyophilized active fractions of the enzyme were subjected to reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) following the method of Laemmli (1970) using 4% stacking gel and 15% resolving gel at a constant current of 12mA. After electrophoresis, gels were stained with 0.025% Coomassie brilliant blue stain R-250 and then destained in a solution of 5% methanol and 7% acetic acid. Molecular weight of the unknown protein band was determined by comparing with the molecular weight standards from Bangalore Genei.

6.2.3.2 $\beta$-1, 3-glucanase assay

$\beta$-1, 3 glucanase activity was measured by using laminarin from Laminaria digita (Sigma –Aldrich Co.) as substrate according to the modified method of Zhu et al. (2008). The laminarase activity was based on the measurement of reducing sugar cleaved from laminarin. The reaction mixture consisted of 0.5 ml of 5 mg ml$^{-1}$ laminarase (dissolved in 50 mM sodium-phosphate buffer, pH 6.0) and incubated at 50°C for 30 min. After incubation 1 ml DNS reagent was added and tubes were placed in boiling water for 10 min, cooled and 4 ml of distilled water was added and the amount of reducing sugar liberated was measured at 540 nm. Assays were carried out in triplicates. One unit of enzyme activity is defined as the amount of enzyme that catalyzed the liberation of reducing sugar equivalent to 1 µg of D-glucose per minute under standard assay conditions.

6.2.3.3 Protein assay

Quantification of protein was carried out according to the method of Hatree (1972) using Bovine Serum Albumin as standard.
6.2.3.4 Specific activity

Specific activity was calculated by dividing the enzyme units with the protein content

\[
\text{Specific activity (U/mg)} = \frac{\text{Total unit activity (U ml}^{-1})}{\text{Total protein content (mg ml}^{-1})}
\]

6.2.3.5 Effect of pH on β-1, 3 glucanase activity

Effect of pH on β-1, 3 glucanase activity was determined over a pH range of 3 to 10 using the buffers of 50 mM concentrations: sodium – phosphate (6,7), Tris-Cl (8 and 9), glycine-NaOH (9 , 10, 11 and 12) for 30 min at 37°C.

6.2.3.6 Effect of temperature on β-1, 3 glucanase activity

Effect of temperature on laminarase activity was tested by carrying out the assay at temperature ranges of 30, 40, 50, 60, 70 and 80°C for 30 min in 50 mM Tris-Cl buffer (pH 9.0).

6.2.3.7 Effect of inhibitors on β-1, 3 glucanase activity

Various inhibitors (5 mM phenyl methyl sulphonyl fluoride (PMSF), EDTA, 1, 10 phenanthroline, leupeptin, pepstatin, phosphoramidon and TLCK) were studied by including them in the assay mixture, and the relative activity was measured under standard assay conditions. Untreated enzyme was taken as the control (100% activity).

6.2.3.8 Cytotoxicity analysis of MCCB 123 β-1, 3 glucanase

HeLa cells were seeded in 96 well plates (Greiner Bio-One) containing 82 mM glutamine, 1.5 g l\(^{-1}\) sodium bicarbonate and 10% fetal bovine serum. Purified enzyme in concentrations of 0, 1, 3, 5, 10, 25, 50,100, 250 \(\mu\text{g ml}^{-1}\) (v/v) was added to the wells in triplicates. A control was kept without the enzyme addition. After 14 h incubation MTT assay was performed and the percentage of inhibited cells at each
concentration of the protease was calculated using SPSS software (SPSS package for Windows).

6.2.3.8.1 MTT assay

After replacing the medium, 50µl MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) (Sigma-Aldrich Co.) having a strength of 5 mg ml\(^{-1}\) in PBS (720mOsm) was added to each well and incubated for 5 h in dark. MTT was added to the control wells with the medium alone. The medium was removed and MTT-formazan crystals were dissolved in 200µl dimethylsulfoxide. Absorbance was recorded immediately at 570nm in micro plate reader (TECAN Infinite Tm, Austria). Probit analysis for percentage cell inhibition was done with SPSS software package (version 17).

6.2.4 Application of MCCB 123 β-1, 3 glucanase in fungal DNA extraction

6.2.4.1 Standardization of pH, temperature and incubation time for cell lysis of β-1,3 glucanase on fungal cells using *Saccharomyces cervisiae* MTCC 1766 as the reference strain

Lytic activity was carried out according to the modified method of Niwa et al. (2005). *Saccharomyces cervisiae* MTCC 1722 was grown for 48 h at 28°C. The absorbance of cell suspension was adjusted to 1.0 Abs\(600\), centrifuged the cells at 15,000 g at 4°C for 15 min and the pellets were recovered. For pH optimization, cells were suspended in 1 ml of β-1,3 glucanase enzyme (10 mg enzyme suspended in 50mM sodium acetate for pH 5 to 6, 50 mM Tris-Cl from pH 7 to 10) and incubated for 30 min at 25°C. For temperature optimization, cells were suspended in 1 ml of purified β-1,3 glucanase enzyme (10 mg enzyme suspended in 1 ml of 50 mM Tris-Cl, pH 7.0) and incubated for 30 min at various temperatures ranging from 25 to 75°C. To determine optimum incubation time for cell lysis, cells were suspended in 1 ml of β-1,3 glucanase enzyme (10 mg enzyme suspended 1ml of 50 mM Tris-Cl, pH 7.0) at
35°C and incubated up to 60 min drawing samples for DNA extraction at every 10 min interval.

After each experiment, un-lysed cells were removed by centrifugation at 15,000g for 15 min at 4°C. Into the supernatant equal volume of absolute ethanol was added, kept for 30 min and the pellet was recovered by centrifugation at 15,000g for 15 min at 4°C and dissolved in 100 µl sterile Milli Q and the presence of DNA was confirmed on 1% agarose gel and the DNA yield was determined by triplicate measurements at 260nm. Reactions without enzyme were included as controls. Optimum was determined based on band intensity and DNA yield. The band intensity was calculated using Quantity one software, BioRad, USA.

6.2.4.2 DNA extraction from fungal cultures

Fungal cultures used for DNA extraction and their culture conditions are listed in Table 3. Fungal cultures were grown until enough fungal mycelia have grown, 1ml of the culture centrifuged at 15,000g at 4°C for 15 min and mycelia were treated with 1 ml of 10 mg ml\(^{-1}\) of the purified glucanase enzyme resuspended in 50 mM Tris-Cl, pH 7.0 and incubated at 65°C for 60 min. DNA extraction and yield determination were carried out by the method as described earlier.

6.2.4.3 Nucleic acid yield and purity

Nucleic acid extracted from fungal isolates was quantified using UV-visible spectrophotometer (UV-1601, Shaimadzu). The absorbance at 260 nm (Abs\(_{260}\)) was measured for each sample and used to calculate the average total nucleic acid yield for each set of triplicate samples. To estimate the purity of extracted nucleic acid, the absorbance at 280 nm (Abs\(_{280}\)) was measured and the average ratio between the Abs\(_{260}\) nm and Abs\(_{280}\) nm (A\(_{260}/\)Abs\(_{280}\)) was calculated for each set of triplicate samples. Samples with
Purification and Characterization of β-1, 3 Glucanase from Pseudomonas…..

mean Abs$_{260}$/Abs$_{280}$ ratios between 1.8 and 2.0 were presumed to be free of contamination (Manchester, 1995; Sambrook and Russell, 2001). Samples with mean Abs$_{260}$/Abs$_{280}$ ratios below 1.8 were presumed to contain protein or other contaminants, whereas samples with ratios above 2.0 were presumed to be due to the presence of RNA (Sambrook and Russell, 2001). Nucleic acids preparations free of phenol should have Abs$_{260/280}$ ratios of 1.2 (Sambrook and Russell, 2001; Lemarchand et al., 2005).

6.2.4.4 PCR amplification of ITS region

PCR amplification of ITS region consisting of ITS 1 and ITS 2 was performed according to White et al. (1990) using primers ITS1 (5’ TCC GTA GGT GAA CCT GCGG-3’) and ITS4 (TCC TCC GCT TAT TGA TAT GC-3’). The amplification was performed using DNA Thermal cycler (Eppendorf). Reaction mixture (final volume 25 µl) contained 2.5 µl 10 X buffer, 1 µl 10 pmol each of oligonucleotide primer, 1.5 µl DNA template, 2.5 µl 2.5 mM each deoxynucleoside triphosphate, 1 µl Taq polymerase, and the remaining volume made up with sterile Milli Q water. The amplification profile consisted of initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 45 s and extension at 72°C for 1 min followed by final extension at 72°C for 10 min and the PCR products were separated on 1% agarose gel.

6.2.4.5 Microscopic examination of β-1,3 glucanase treated fungal hyphae and yeast cells under phase contrast microscope and comparison with untreated cells (control)

For the examination of cell rupture, lysed cells were observed under phase contrast microscope (Olympus) and compared with those of control (untreated cells).

6.2.5 Statistical analysis

Data generated from the experiments were analyzed using one-way Analysis of Variance (ANOVA) with post-hoc multiple comparison analysis.
performed using Tukey’s HSD. Mean of the results was compared using SPSS 17.0 package for Windows at a significance level of p<0.05. Data are presented as mean± standard deviation.

6.3 Results

6.3.1 Purification of β-1, 3 glucanase

In the present study, β-1, 3 glucanase having lytic action on a broad range of fungal cell walls was purified from an environmental isolate of *Pseudomonas aeruginosa* MCCB 123. The enzyme was purified by a two step procedure, by precipitation with ammonium sulphate followed by DEAE-cellulose chromatography. Ammonium sulphate fractions from 30-80 % showed β-1, 3 glucanase activity. Active fractions were pooled and concentrated by ultrafiltration using a 10 kDa membrane. The pooled fractions were then loaded into DEAE-cellulose chromatography column. The results of purification procedure are summarized in Table 6.1. The enzyme was purified 9.52 fold increase in specific activity. The elution profile of the β-1, 3 glucanase on DEAE-cellulose column is shown in Fig.6.1. The protease eluted between 0.70 M to 0.81 M NaCl (fractions, 70 to 81) contained β-1, 3 glucanase.

6.3.2 Characterization of β-1, 3 glucanase

The purified MCCB 123 β-1, 3 glucanase was homogenous on SDS-PAGE and its molecular weight was estimated to be 45 kDa by reducing SDS-PAGE (Fig. 6.2).

6.3.3 Effect of pH on activity of β-1,3 glucanase

The effect of pH on β-1, 3 glucanase activity was determined using buffers in the pH range of 6 to 12 at 50°C, respectively. The relative activity of the enzyme at different pH are given in Fig.6.3. The enzyme was found to exhibit activity from pH 3 to10 with its optimum at pH 7.0. Statistical analysis by One-way ANOVA indicated that there was a
significant \( p<0.05 \) difference in the \( \beta-1, 3 \) glucanase activity between pH values from 3 to 7 (Appendix 4, Tables 4.1a to 4.1c).

### 6.3.4 Effect of temperature on activity of \( \beta-1, 3 \) glucanase

The enzyme was found to exhibit activity from 30 to 80°C with its optimum at 50°C (Fig. 6.4). There was a significant \( p<0.05 \) difference in the \( \beta-1,3 \) glucanase activity in temperature ranges between 30 to 50°C (Appenedix 4, Tables 4.2a to 4.2c).

### 6.3.5 Effect of inhibitors on activity of \( \beta-1, 3 \) glucanase

There was a partial inhibition (42.98%) of enzyme activity by metalloprotease inhibitor EDTA thus proving to be metalloprotease. The enzyme retained 83.34, 84.22, 81.51 and 95.62 % activity in presence of 5mM 1, 10 phenanthroline, 50µM leupeptin, 10 µM pepstatin and 0.1 mM phosphoramidon, respectively, confirming that the enzyme did not belong to the class of serine and cysteine protease, respectively (Table 6.2).

### 6.3.6 Cytotoxicity analysis of purified \( \beta-1,3 \) glucanase

Cytotoxic effects on HeLa cells were studied at different concentrations of enzyme in the range of 0, 1, 3, 5, 10, 25, 50,100 and 250 µg ml\(^{-1}\) enzyme. There were no significant visible cytopathic effects at any of the concentrations tested (Fig.6.6) and 236.87±1.89µg ml\(^{-1}\) was the LD\(_{50}\) dose (50 % inhibition) (Fig.6.5).The addition of 1 to 3 µg ml\(^{-1}\) enzyme resulted in the increase in cell number (Fig.6.6).

### 6.3.7 Application of \( \beta-1, 3 \) glucanase in fungal DNA extraction

Optimization of pH for DNA extraction was accomplished over a pH range of 5-10 using 50mM sodium acetate for pH 5 to 6, 50 mM Tris-Cl for pH 7 to 10 at 25°C for 30 min. The enzyme exhibited good lytic activity on cells of Saccharomyces cervisiae from pH 5 to 10 with
Purification and Characterization of β-1, 3 Glucanase from Pseudomonas….. 
National Centre for Aquatic Animal Health, CUSAT 

Chapter -6 

its optimum at 7.0 with a DNA yield of 231.66±5.20 µg µl⁻¹ (Fig. 6.7). The statistical analysis revealed that pH had a significant \( p<0.05 \) difference in the DNA yield between pH 8 and 9. However, there was no significant \( p>0.05 \) difference in the DNA yield between pH 5 and 6, 7 and 8, 9 and 10 (Appendix 4, Tables 4.3a to 4.3c). The gel image of DNA extracted from at various pH is give in Fig.6.10a.

The enzyme was found to have good cell lysis from 25 to 75°C with its optimum at 65°C with a DNA yield of 310±2.5 µg µl⁻¹ (Fig.6.8). There was a significant \( p<0.05 \) difference in the DNA yield only from temperature range from 45 to 65°C (Appendix 4, Tables 4.4a to 4.4c). The gel image of DNA extracted at various temperatures is represented in Fig. 6.10b.

The DNA yield reached maximum after 60 min incubation (321.66±5.2 µg µl⁻¹) (Fig.6.9). The DNA yield was found to be significantly \( p<0.05 \) different from 20 to 30 min incubation (Appendix 4, Tables 4.5a to 4.5c). The gel image of DNA extracted at various time intervals from 10 to 60 min is represented in Fig. 6.10c.

6.3.8 DNA extraction from different fungal species

The DNA extracted from various fungal (Fig.6.11) species along with their DNA yield and quality is described in Table 6.3.

6.3.9 PCR amplification of ITS region

ITS amplification for fungi yielded an expected product size of 590 bp (Fig.6.12).

6.3.10 Microscopic examination of β-1, 3 glucanase treated ruptured fungal hyphae and yeast cells under phase contrast microscope and comparison with untreated cells (control)

For the examination of fungal cell rupture on treatment with β-1,3 glucanase lysed cells were observed under phase contrast microscope and
compared with that of control (untreated cells). The effect of β-1,3 glucanase enzyme on fungal cells is represented in Fig.6.13. The cells lost their normal appearance on treatment with enzyme when compared to that of the control.

Table 6.1 Purification profile of β-1,3 glucanase of *Pseudomonas aeruginosa* MCCB 123

<table>
<thead>
<tr>
<th>Purification step</th>
<th>β-1,3 glucanase activity (U/ml)</th>
<th>Total Protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>359.71</td>
<td>8.5</td>
<td>42.31</td>
<td>0</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Precipitation</td>
<td>188.08</td>
<td>4.09</td>
<td>45.98</td>
<td>1.08</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>98.74</td>
<td>0.245</td>
<td>403.02</td>
<td>9.52</td>
</tr>
</tbody>
</table>

Table 6.2 Effect of inhibitors on β-1,3 glucanase activity

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mM</td>
<td>57.02</td>
</tr>
<tr>
<td>PMSF</td>
<td>2 mM</td>
<td>100</td>
</tr>
<tr>
<td>1,10 Phenanthroline</td>
<td>5 mM</td>
<td>83.34</td>
</tr>
<tr>
<td>TLCK</td>
<td>0.1 mM</td>
<td>100</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>50 µM</td>
<td>84.22</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>10 µM</td>
<td>81.51</td>
</tr>
<tr>
<td>Phosphoramidon</td>
<td>0.1 mM</td>
<td>95.62</td>
</tr>
</tbody>
</table>
Table 6.3 Fungal species used for DNA extraction along with their DNA yield and quality

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>code</th>
<th>Conditions</th>
<th>Temp</th>
<th>DNA yield (µg µl⁻¹)</th>
<th>DNA purity (OD260/280)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cervisiae</td>
<td>MTCC 1766</td>
<td>YEPD</td>
<td>30°C</td>
<td>280 ± 7.5</td>
<td>1.10 ± 0.03</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>MTCC 854</td>
<td>MYA</td>
<td>37°C</td>
<td>266.66 ± 5.20</td>
<td>1.14 ± 0.03</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>MTCC 277</td>
<td>CYA</td>
<td>30°C</td>
<td>164.16 ± 8.77</td>
<td>1.07 ± 0.04</td>
</tr>
<tr>
<td>Aspergillus foetidus</td>
<td>MTCC 151</td>
<td>CYA</td>
<td>30°C</td>
<td>188.33 ± 8.77</td>
<td>1.10 ± 0.07</td>
</tr>
<tr>
<td>Aspergillus ochraceus</td>
<td>MTCC 1810</td>
<td>CYA</td>
<td>35°C</td>
<td>154.16 ± 3.81</td>
<td>1.16 ± 0.02</td>
</tr>
<tr>
<td>Phanerochaete chrysogenum</td>
<td>MTCC 787</td>
<td>MEA</td>
<td>25°C</td>
<td>156.66 ± 7.63</td>
<td>1.11 ± 0.07</td>
</tr>
<tr>
<td>Fusarium solani</td>
<td>MTCC 350</td>
<td>PSA</td>
<td>30°C</td>
<td>170.83 ± 10.10</td>
<td>1.13 ± 0.05</td>
</tr>
<tr>
<td>Acrocomonium diazepri</td>
<td>MTCC 1316</td>
<td>PDA</td>
<td>25°C</td>
<td>311.66 ± 8.77</td>
<td>1.19 ± 0.003</td>
</tr>
<tr>
<td>Heterobasidion annosum</td>
<td>MTCC 146</td>
<td>YGA</td>
<td>25°C</td>
<td>183.33 ± 5.20</td>
<td>1.12 ± 0.01</td>
</tr>
<tr>
<td>Trichoderma reesei</td>
<td>MTCC 164</td>
<td>MEA</td>
<td>25°C</td>
<td>134.16 ± 3.81</td>
<td>1.10 ± 0.02</td>
</tr>
<tr>
<td>Pencillium citrinum</td>
<td>MTCC 2553</td>
<td>CYA</td>
<td>30°C</td>
<td>146.66 ± 3.81</td>
<td>1.17 ± 0.05</td>
</tr>
<tr>
<td>Pleurotus sajor-caju</td>
<td>MTCC 141</td>
<td>PDA</td>
<td>25°C</td>
<td>218.33 ± 5.20</td>
<td>1.17 ± 0.04</td>
</tr>
<tr>
<td>Pleurotus sajor-caju</td>
<td>MTCC 1806</td>
<td>PDA</td>
<td>25°C</td>
<td>227.5 ± 5</td>
<td>1.08 ± 0.02</td>
</tr>
<tr>
<td>Daedalea flavida</td>
<td>MTCC 145</td>
<td>YGA</td>
<td>25°C</td>
<td>247.5 ± 5</td>
<td>1.07 ± 0.01</td>
</tr>
<tr>
<td>Pleurotus ostreatus</td>
<td>MTCC 142</td>
<td>YGA</td>
<td>30°C</td>
<td>226.66 ± 3.81</td>
<td>1.11 ± 0.03</td>
</tr>
<tr>
<td>Pleurotus sapidus</td>
<td>MTCC 1807</td>
<td>PDA</td>
<td>25°C</td>
<td>224.16 ± 3.81</td>
<td>1.11 ± 0.02</td>
</tr>
<tr>
<td>Pleurotus ostreatus</td>
<td>MTCC 1803</td>
<td>PDA</td>
<td>25°C</td>
<td>255.83 ± 5.77</td>
<td>1.23 ± 0.02</td>
</tr>
<tr>
<td>Pleurotus fossulatus</td>
<td>MTCC 1800</td>
<td>PDA</td>
<td>25°C</td>
<td>226.66 ± 5.20</td>
<td>1.10 ± 0.006</td>
</tr>
<tr>
<td>Coriolus versicolor</td>
<td>MTCC 138</td>
<td>YGA</td>
<td>25°C</td>
<td>219.16 ± 5.20</td>
<td>1.10 ± 0.02</td>
</tr>
<tr>
<td>Trametes hirsuta</td>
<td>MTCC 136</td>
<td>YGA</td>
<td>25°C</td>
<td>240.83 ± 6.29</td>
<td>1.06 ± 0.02</td>
</tr>
<tr>
<td>Pycnoporus sanguineus</td>
<td>MTCC 137</td>
<td>YGA</td>
<td>25°C</td>
<td>219.16 ± 6.29</td>
<td>1.11 ± 0.05</td>
</tr>
</tbody>
</table>

CYA: Czapek Yeast Extract Agar  PSA: Potato Sucrose agar  MYA: Malt extract agar
PDA: Potato Dextrose agar  YGA: Yeast glucose agar
Fig. 6.1 Elution profile of β-1,3 glucanase on DEAE-cellulose C16/40 column

Fig. 6.2 SDS-PAGE profile of purified β-1,3 glucanase. Lane 1, Molecular weight marker; lane 2, 45 kDa β-1,3 glucanase enzyme
Purification and Characterization of β-1, 3 Glucanase from Pseudomonas

Fig. 6.3 Optimization of pH for β-1,3 glucanase activity

Fig. 6.4 Optimization of temperature for β-1,3 glucanase activity

Fig. 6.5 Sigmoid curve for cytotoxicity of β-1,3 glucanase
Fig. 6.6 Cytopathic effects of β-1,3 glucanase on HeLa cell line. a) control cells without β-1,3 glucanase enzyme, b) 1µg ml⁻¹ enzyme, c) 3 µg ml⁻¹ enzyme, d) 250 µg ml⁻¹ enzyme. The addition of 1 and 3 µg ml⁻¹ enzyme resulted in the increase in the number of cells when compared to control.
Fig. 6.7 Determination of optimum pH of β-1,3 glucanase for fungal DNA extraction using *Saccharomyces cerevisiae* MTCC 1766 as reference strain.

Fig. 6.8 Determination of optimum temperature of β-1,3 glucanase for fungal DNA extraction using *Saccharomyces cerevisiae* MTCC 1766 as reference strain.
Fig. 6.9 Determination of optimum incubation time of β-1,3 glucanase for fungal DNA extraction using *Sacchromyces cervisiae* MTCC 176 as reference strain.

![Graph showing concentration of DNA vs incubation time](image)

(a)

Fig. 6.10 Optimization of pH (a), temperature (b) and incubation time (c) for cell lysis for fungal DNA extraction using *Sacchromyces cervisiae* MTCC 1766 as reference strain. Concentration of DNA in µg µl⁻¹ (average ± standard deviation is represented)

(a) pH 5: 116±6.29 µg, pH 6: 123.5±3.81 µg, pH 7: 231.6±3.20 µg, pH 8: 225.5±4.33 µg, pH 9: 185±2.3 µg, pH 10: 176±2.5 µg

(b) 25°C: 234±6.57 µg, 35°C: 241±6.20 µg, 45°C: 254.1±6.10 µg, 55°C: 292.5±8.77 µg, 65°C: 316±5.2 µg, 75°C: 294.1±6.31 µg

(c) 10 min: 241.66±8.03 µg, 20 min: 262.5±5.2 µg, 30 min: 312.5±10 µg, 40 min: 315±711.45 µg, 50 min: 320.83±7.63 µg, 60 min: 321.6±5.2 µg
Lane 1, 

Fig. 6.11 DNA extracted from fungal strains by the lytic action of $\beta$-1,3 glucanase enzyme

Fig. 6.12 PCR amplification of ITS region. Lane 1, 100 bp DNA ladder; lane 2, 540 bp amplicon of ITS region of Saccharomyces cerevisiae MTCC 1766
Fig. 6.13 (a-k). Rupture of fungal hyphae on treatment with β-1,3 glucanase of *Pseudomonas aeruginosa* MCCB 123. Control represents untreated fungal hyphae and test represents the changes in fungal hyphae on treatment with 10 mg ml$^{-1}$ of purified β-1,3 glucanase.
Control                     Test

Acremonium diospyri MTCC 1316 (Magnification 60X)
(d)

Control                     Test

Fusarium solani MTCC 350 (Magnification 60X)
(e)

Control                     Test

Phanerocheate chrysogenum MTCC 787 (Magnification 60X)
(f)
Purification and Characterization of β-1, 3 Glucanase from Pseudomonas...

Control                     Test

Heterobasidion annosum MTCC 146 (Magnification 60X)

Pencillium citrinum MTCC 2553 (Magnification 60X)

Pleurotus fossulatus MTCC 1800 (Magnification 60X)
6.4 Discussion

A β-1, 3 glucanase enzyme with a broad range of lytic activity on a broad range of fungal cell walls was purified from *Pseudomonas aeruginosa* MCCB 123. The enzyme was purified with 9.52-fold increase in specific activity. The molecular mass of the enzyme was found to be 45 kDa by SDS-PAGE.

Enzyme inhibition studies primarily give an insight into the nature of the enzyme, its cofactor requirements and the nature of the active
centre (Sigma and Mooser, 1975). In the present study, β-1, 3 glucanase activity was only partially inhibited by the metal chelator EDTA. However, the activity was less sensitive to PMSF (serine protease inhibitor), TLCK (inhibitor of lysine specific serine protease), leupeptin, trypsin soybean inhibitor and phosphoramidon (specific inhibitor of Pseudomonas aeruginosa elastase). Since the β-1, 3 glucanase activity was inhibited by the zinc chelator, EDTA, and not by any other specific class of inhibitor, it can be concluded that MCCB 123 β-1,3 glucanase belongs to the class of metalloenzyme. An exo-β-1, 3 glucanase of Rhizoctonia solani also showed 29% loss in activity in the presence of EDTA (Vijayendra and Kashiwagi, 2009), while the activity of an endo-β-1, 3-glucanase from Agaricus brasiliensis is not significantly affected by EDTA (Shu et al., 2006).

The optimum pH for β-1, 3 glucanase activity was found to be at pH 7.0. This pH optima is different from the acidic pH optimum reported for Pseudomonas stutzeri YPL-1 (Lim and Kim, 1995). Other reported microbial endo and exo β-1, 3 glucanases also have an acidic pH optimum. Ferrer et al. (1996) observed an acidic pH optimum of 4.0 for β-1, 3 glucanase activity of Oerskovia xanthineolytica LL G109 (Cellulomonas cellulans), 5.2 for β-1, 3 glucanase from Ascochyta rabiei (Hanselle and Barz, 2001); 5.0 for exo-β-1,3-glucanase of Rhizoctonia solani (Vijayendra and Kashiwagi, 2009); 4.5 for GluC β-1,3 glucanase gene product and 4.5 and 5.0 for GluB β-1,3 glucanase gene product of Lysobacter enzymogenes Strain N4-7 (Palumbo et al., 2003). Thus, MCCB 123 β-1,3 glucanase differs from other reported glucanases with respect to its pH optimum. However, the pH optimum observed for β-1, 3 glucanase described in the present study was within the range of optimal pH of 6.5-9.5 for β-1, 3 glucanase activity from Bacillus subtilis NSRS 89-24 (Leelasuphakul et al., 2006) and close to that from Chaetomium sp which was reported to have an optimum pH of 6.0 (Sun et al., 2006) and that reported from Pichia pastoris (Xu et al., 2006).
The optimum temperature for the activity of MCCB 123 β-1, 3 glucanase was found to be 50°C. Similar temperature optima were reported for the activity for β-1, 3 glucanase from Bacillus subtilis NSRS 89-24 (Leelasuphakul et al., 2006). The temperature optima for GluC and GluB β-1, 3 glucanases produced by Lysobacter enzymogenes strain N4-7 was found to be 45°C and 41°C respectively (Palumbo et al., 2003). A temperature optimum of 40°C is reported for β-1,3 glucanase from Pseudomonas stutzeri YPL-1 (Lim and Kim, 1995), Rhizoctonia solani (Vijayendra and Kashiwagi, 2009), a temperature optimum of 45°C is reported for Agaricus brasiliensis ATCC 76739 (Shu et al., 2006) and a lower temperature optimum of 30°C was observed for β-1,3-glucanase from Chaetomium sp (Sun et al., 2006).

The cytotoxicity of MCCB 123 β-1, 3 glucanase on HeLa cells was found to increase with concentration indicating that the cytotoxicity is dose dependent. LD$_{50}$ value on HeLa cell line was found to be 236.87±1.89 µg ml$^{-1}$. It is also noted that supplementation of β-1, 3 glucanase from 1-3µg ml$^{-1}$ was found to have an inducing effect on the growth of HeLa cells suggesting its possible application in cell culture.

Only a few reports are available on the production and lytic action of β-1, 3 glucanase from Pseudomonas aeruginosa (Kumar et al., 2009; Singh et al., 2010) and the reported studies have not given emphasis to the characterisation of the enzyme. In this context, characterization studies of MCCB 123 β-1, 3 glucanase and understanding its novel properties are very relevant. β-1, 3 glucanase of Pseudomonas aeruginosa MCCB 123 was found to have lytic action on a broad range of fungal and yeast strains. Since MCCB 123 glucanase is able to hydrolyse the Saccharomyces cerevisiae, it may be through the cleaving action of β (1→3)-D-glucan of the cell wall. In the yeast Saccharomyces cerevisiae, the cell wall contains β (1→3)-D-glucan, β (1→6)-D-glucan, chitin and mannoprotein (Kollar et al., 1997). Salazar and Asenjo (2007) reported
that the lysis of yeast cell wall begins with binding of lytic protease to the outer mannoprotein layer of the wall. The protease opens up the protein structure releasing the wall proteins and mannans and exposing the glucan surface below. The glucanase then attacks the inner wall and solubilise the glucan. In vitro, this enzyme cannot lyze yeast in the absence of reducing agents, such as dithiothreitol or β-mercaptoethanol, because the breakage of disulphide bridges between mannose residues and wall proteins is necessary for appropriate exposition of the inner glucan layer. When the combined action of the protease and glucanase has opened a sufficiently large hole in the cell wall, the plasma membrane and its content are extruded as a protoplast.

However, β-1,3 glucanase of *P. aeruginosa* MCCB 123 alone is sufficient for the lysis of yeast cell lysis as evident from its lytic action on different yeast strains such as *Saccharomyces cerevisiae* and *Candida albicans*. Type 1 β-1,3 glucanases are capable of readily solubilising yeast glucan and inducing complete lysis of viable yeast cells, while members of type II β-1,3 glucanases has limited capacity to solubilise glucan (Doi and Doi, 1986). Thus, MCCB 123 β-1,3 glucanase belongs to type I β-1,3 glucanase since it exhibited good hydrolytic activity on yeasts such as *Saccharomyces cerevisiae* and *Candida albicans*.

β-1,3 glucanase is reported to have lytic action on fungal cell walls (Jones et al., 1974; Reiss, 1977; Lim et al., 1991; Lim and Kim, 1995; Lahsen et al., 2001; Sun et al., 2006). The structure of fungal cell wall is highly complex which consists of thick layers of chitin, (1-3)-β-α-D-glucans, 1-6 β-glucans, lipids and peptides (Karakousis et al., 2006) and the yeast cell wall is composed of complex polymers such as β-1,3 and β-1,6 glucans, mannoproteins and smaller amounts of chitin (Ferrer, 2006) which implies that synergetic action of several enzymes is necessary to hydrolyse these components. The lytic action of *P. aeruginosa* MCCB
123 enzyme on fungal cell walls is supported by its β-1, 3 glucanase activity thereby it is shown to hydrolyze β-1, 3 glucan component of fungal cell wall causing subsequent cell rupture. β-1, 3 glucanase is reported to have action on fungal cell wall resulting in the degradation and loss of inner contents of cells (Benhamou et al., 1996). Exo and endo glucanases were found to have hydrolytic action on cell walls of plant fungal pathogens. Yang et al. (1993) reported the degradation of cell walls of *Rhizoctonia solani*, *Gibberella zeae*, *Fusarium sp.*, *Colletotrichum gloeosporioides* and *Phoma sp.* by the action of β-1, 3 glucanase. The cooperative action of endo and exo β-1,3 glucanases from parasitic fungi *Coniothyrium minitans* and *Trichoderma viride* was reported to degrade cell wall glucans of *Sclerotinia sclerotiorum* (Jones et al., 1974). Reiss (1977) reported the serial enzymatic hydrolysis of cell walls of the yeast *Histoplasma capsulatum* with α (1→3)-Glucanase and β-(1→3)-Glucanase. Lahsen et al. (2001) reported the lytic activity and antifungal activity of exo-type α -1, 3-glucanase against fungal plant pathogens.

As the structure of fungal cell wall is highly complex consisting thick layers of chitin, (1-3)-β-D-glucans, 1-6 β-glucans, lipids and peptides and tough surface layer of melanin on which most enzymes fail to lyse, development of a single universal fungal DNA extraction method has significance and has not been accomplished so far. This has paved the way for developing DNA extraction methods using a combination of different disruption methods. Karakousis et al. (2006) employed the use of digestive enzymes, mechanical disruption methods like freezing in liquid nitrogen, grinding with mortar and pestle, sonication, glass bead milling and microwaving and non mechanical disruption methods such as treatment with alkaline chemicals, detergents and other chemicals for DNA extraction from medically important fungi. DNA extraction from ectomycorrhizal basidiomycete *Tylospora fibrillosa* Donk was carried out by freezing in liquid nitrogen and three cycles of thawing at 65°C to break
the cell walls, grinding in sterile sand followed by phenol-chloroform extraction (Erland et al., 1994). DNA extraction from white rot fungi involves the use of CTAB, mercaptoethanol in the lysis buffer followed by chloroform-3-methyl-1-butanol extraction and ethanol precipitation (Kuhad et al., 2004). DNA extraction from mycorrhizal fungi was carried out by crushing in pestle followed by three alternate cycles of freezing in liquid nitrogen and incubation at 100°C for 1 min followed by freezing and final incubation at 100°C for 10 min and purification using DNA columns (Manian et al., 2001). A bead beating methods involving the use of glass beads for crushing the cells followed by phenol-chloroform extraction was used for DNA extraction from filamentous fungi (Plaza et al., 2004). Enzymatic cell lysis using lyticase followed by bead beating was employed for DNA extraction from filamentous fungi in biofilms (Saad et al., 2004). Accordingly, there exists no single method for fungal DNA extraction. Most of the lysis buffers for DNA extraction include SDS. (Dean et al., 1994; Erland et al., 1994; Haugland et al., 1999; Plaza et al., 2004).

From the above literature, it is understood that no single protocol appropriate for cell lysis for all fungi does exist and each species requires a specific method for efficient DNA extraction (Manian et al., 2001; Karakousis et al., 2006) and many of the fungal DNA extraction methods are often laborious, expensive and time consuming (Plaza et al., 2004). Existing methods for genomic DNA preparation from fungi take several hours to complete (Muller et al., 1998; Sambrook and Russell et al., 2001).

Therefore, in this context developing a single extraction method for fungal DNA is a desirable preposition. DNA could be extracted from 21 fungal species by the lytic action of the purified β-1, 3 glucanase from P. aeruginosa MCCB 123 without the addition of other chemicals and
mechanical treatments which makes this method unique among the methods reported. The extracted DNA could be directly used for PCR amplification without further purification. The above properties make this enzyme unique among the lytic enzymes used in fungal DNA extraction. Moreover, the method is inexpensive since it employs only β-1,3 glucanase as the sole reagent, the quality and quantity of DNA obtained is suitable for molecular assays and it doesn’t require the use of expensive and specialised equipment or hazardous reagents.

The quality of the extracted nucleic acid is important for further processing. Samples with mean $A_{260}/A_{280}$ ratios below 1.8 were presumed to contain protein or other contaminants, whereas samples with ratios above 2.0 were presumed to be due to the presence of RNA (Sambrook and Russell, 2001). However, nucleic acids preparations free of phenol should have $A_{260}/A_{280}$ ratios near 1.2 (Sambrook and Russell, 2001; Lemarchand et al., 2005). In the case of DNA extracted with β-1,3 glucanase from various fungal species, $A_{260}/A_{280}$ ratio was found to be in the range of 1.0 to 1.1, and phenol had not been used in the process and thus it could be concluded that the DNA extracted using this method was free of any contamination and was suitable for PCR amplification. The amplicon obtained from the PCR amplification of the ITS region was in agreement with previous workers (Dean et al., 2004).

Fungal DNA extraction using MCCB 123 β-1,3 glucanase has several advantages. First of all, the number of steps in DNA extraction procedure was minimized by replacing phenol chloroform extraction method and it also doesn’t involves the addition of any detergents or other lytic agents and other mechanical lytic methods such as grinding with sand, repeated freeze thaw cycles in liquid nitrogen. Secondly, several samples can be processed within a short time period of 30 min. Thirdly, the method yielded high quality DNA compared with standard phenol-chloroform protocol. Fourthly, this method
seems to be very cost-effective since β-1, 3 glucanase alone is used as the sole reagent. Finally, this extraction method is applicable to a broad range of fungal species. This makes this enzyme unique over all other lytic enzymes. This is the first report of a lytic enzyme alone being employed in fungal DNA extraction without the addition of detergents such as sodium dodecyl sulphate (SDS) and incorporating other mechanical lytic steps. In this context, broad range of lytic activity of glucanase on a wide range of fungal cells has immense benefits in DNA extraction.

……OCR……