Isolation and screening of fungal strains for the production of cellulolytic enzymes from sugarcane bagasse using solid-state (SSF) and submerged fermentation (SmF) methods
2.0 Introduction

Production and use of enzymes are crucial in the bioprocessing industry (Vlaev et al., 1997). Enzyme costs can be reduced by finding optimum conditions for their production (Ponce-Noyola and De la Torre, 2001). If cellulases can be obtained cheaply, readily and easily it will be of a great advantage to the bioprocessing industry. Cellulase is a multicomponent enzyme system that is well studied because of its potential industrial applications (Vyas et al., 2003). Cellulolytic enzymes are produced by variety of aerobic and anaerobic bacteria and fungi. They are detected as mesophiles and thermophiles (Bhat and Bhat, 1997). Each of these microorganisms can produce different kinds of cellulases that differ in their mode of action as well as properties like activity towards crystalline cellulose, activity and stability in acidic or alkaline pH.

Cellulases are group of enzymes that catalyze the breakdown of cellulosic substances produced either by submerged (SmF) or solid state fermentation (SSF). SmF involves the growth and fermentation by microorganisms in the presence of sufficient water so as to dissolve the whole medium components in it and is well characterized with homogenous conditions maintained throughout the processing. The physical parameters such as pH, temperature and dissolved oxygen can be effectively controlled and scaling up of the process is feasible due to defined conditions (Jain et al., 2010). The submerged culture fermentation of lignocellulosics has many advantages and the contents of fermentor are homogenous and the rate of fermentative activity is maximum under the optimum conditions of the organism used, but there are many disadvantages such as requiring high volume of liquid medium per unit weight of lignocellulose treated, and dewatering of fermented product which is a costly process. Almost all the large-scale enzyme producing facilities are using the proven technology of SmF due to better monitoring and ease of handling. Though bacteria and actinomycetes are also reported for cellulase production, the titers are very low to make the technology economically feasible. Most of the commercial cellulases are produced by the filamentous fungi like T. reesei or A. niger under SmF (Kumar et al., 2004; Cherry and Fidantsef, 2003). Cellulase production in cultures is highly influenced by various parameters including the nature of the cellulosic substrate, pH of the medium, nutrient availability, inducer supplementation, fermentation temperature, etc., and a large-scale production of cellulases requires understanding and proper controlling of the growth and enzyme production capabilities of the producer.
In contrast to submerged fermentation, the solid state fermentation involves the growth and fermentation of by microorganisms, especially fungi, on moist, water insoluble and solid substrate in the absence or near absence of free water. It offers distinct advantages as it requires no fermentation controls and lesser water volumes lead to greater product titre and is considered the self control process. In SSF, the solid state substrate particles serve as source of carbon, nitrogen, salts and growth factors and also provide mechanical support and anchorage to microbial cells (Kuhad and Singh, 2010). There are several factors (physical, chemical and biochemical), which one needs to consider for any SSF (Pandey et al., 2001). The major factors that affect microbial growth and activity in SSF include selection of suitable microorganism and substrate, pretreatment of the substrate, particle size (Inter particle space and surface area) of the substrate, water content and water activity (a_w) of the substrate, relative humidity, type and size of inoculum and temperature of uniformity in the environment of SSF, and the gaseous atmosphere (oxygen consumption rate and carbon dioxide evolution rate).

The liquid byproduct in submerged fermentation creates problems of pollution if released in the atmosphere without stabilization. The solid-state fermentation (SSF) of lignocellulosic byproducts overcomes many of the limitations of submerged culture fermentation discussed above. The solid state fermentation is attractive due to the following: low cost, lower reactor volume per unit substrate converted, direct use of bioconverted product as feed, no processing for dewatering and there is no drying of product. The solid state fermentation may be defined as a system of microbial growth on the surface of solid state particles with no free flowing water. Depending on the substrate utilized the level of moisture differs in the substrate. The moisture content of the substrate in SSF has been reported in the range of 30–80% in different systems (Zadrazil, 1977 and 1985; Kewalramani et al., 1988; Kamra et al., 1993). In recent years there has been a renewed interest in solid-state fermentation (SSF) processes for the production of bioactive compounds. SSF has been reported to be more advantageous than submerged (SmF) as it allows cheaper production of enzyme having better physiochemical properties than that produced by SmF (Kashyap et al., 2003).
2.1 Review of literature

Various microorganisms under different cultural conditions produce cellulolytic enzymes. For improving the production of enzyme, the effect of different factors involved in the fermentation medium has to be evaluated. The recent research work done in this field is reviewed here.

Cellulases can be produced through both submerged fermentation (SmF) and solid state fermentation (SSF). Among the fungal strains, most of the economically important cellulase preparations are obtained from different species of Trichoderma sp. and Aspergillus Niger. Fungal cellulases are inducible enzymes that are usually excreted into the environment (Bhat and Bhat, 1997) and depend on cellulose type (amorphous or crystalline) acting on the organism (Ortega et al., 2001). The role of the fungi Acremonium spp., Chaetomium spp., Trichoderma reesei, Trichoderma viride, Penicillium pinophilum, Phanerochaete chrysosporium (Sporotrichum pulverulentum), Fusarium solani, Talaromyces emersonii, Trichoderma koningii, Fusarium oxysporium, Aspergillus niger and Rhizopus oryzae in the cellulose degradation process in various environments has been well documented (Kuzmanova et al., 1991; Teerei and Koivala, 1995; Bhat and Bhat, 1997; Schulein, 1997; Murashima et al., 2002; Mach and Zeilinger, 2003). Fungi are the main cellulase producing microorganisms, even though a few bacteria and actinomycetes have also been reported to produce cellulases (Miyamoto, 1997; Varma et al., 1994). Cellulases from fungi and bacteria have been studied extensively, but little attention has been given to cellulase from yeasts. It was found that the optimum temperature of cellulase activities varied depending on the organism. Oikwa et al., (1998) isolated the cellulase enzyme from the yeast Rhodotrula glutinis with maximum activity at 30°C while inhibited at 40°C.

A complete cellulase system (CMCase, cellobiohydrolase and β-glucosidase) was produced by Mucor circinelloides (NRRL 26519) on lactose, cellobiose and sigmacell 50. The purified CMCase exhibited the optimum temperature and pH of 55°C and 4.0–6.0, respectively. The enzyme was stable at pH 4.0–7.0 up to 60°C. It hydrolyzed CMC and insoluble cellulose substrates (Avicel-31 Solka-floc and Sigmacell-50) to soluble celldextrins. The addition of 5mM MgCl₂ and 0.5mM CoCl₂ to the reaction mixture caused an increase of 27±5 and 44±14% in enzyme
activity, respectively (Saha, 2004), working on the same lines, Yaoi and Mitsuishi (2004) isolated xyloglucan-specific endo-β-1, 4-glucanase (XEG), xyloglucanase, having molecular weight 80 kDa and a pl 4.8 from the fungus Geotrichum sp. M128. The enzyme was not active toward carboxymethyl cellulose, avicel or barley glucan.

No literature could be found reporting cellulase activity of Penicillium roquefortii but cellulase has been reported in many Penicillium sp. including Penicillium decumbens (Mo et al., 2004; Chen and Jin, 2006), Penicillium echinulatum (Sehnem et al., 2006), Penicillium janthinellum (Wang and Gao, 2000; Oliveira et al., 2006), Penicillium brasiliense (Jorgensen et al., 2005; Jorgensen and Olsson, 2006), Penicillium occitanis (Chaibouni et al., 2005), Penicillium pinophilum, P. persicinum (Jorgensen et al., 2005) Penicillium funiculosum (Van Wyk, 1999; Van Wyk and Leogale, 2001), Penicillium ulainense (Rajal et al., 2002), and Penicillium chrysogenum (Nuero and Reyes, 2002).

There have been no publications of Cladosporium oxysporum producing cellulase but much has been published on various Cladosporium spp. producing cellulase including Cladosporium herbarum (Barbosa et al., 2001), Cladosporium sp. (Abha and Gashe, 1992). Abha and Gashe (1992) used isolated Cladosporium sp. and studied effect of different carbon and nitrogen sources on the production of cellulase. They found that Cladosporium sp. could degrade all types of native and modified cellulose. The greatest production of cellulase was when the organism was grown on carboxymethylcellulose rather than Avicel, filter paper or cotton. They concluded that KNO₃ was the preferred nitrogen source and that the surfactant Tween-80 increased cellulase production by 1.5 to 4.5-fold. The cellulase complex produced by the Cladosporium sp. was active between pH 4 to 8, with optimal activity at pH 5. The highest cellulase activity was recorded at 60°C and was stable for 24hrs at this temperature. Hurst et al., (1983) reported cellulase activity by Cladosporium sphaerospermum at both 1°C and 20°C.

Berg (1978) studied the cellulase formation and location in the fungus Phialophora malorum isolated from peat in a sub arctic bog on different carbon sources, however the genus Phialophora was split into two genera in 2003 (Harrington and McNew, 2003) and this species has been reclassified as Cadophora malorum. The cellulase was found to be partly cellfree and partly cellbound. The
fungus 291 grew on cellulose and carboxymethyl cellulose, but glucose and cellulobiose repressed cellulase formation. In shaking liquid culture the unicellular form of the fungus was dominant, in stationary liquid culture, mycelium developed and dominated. The stationary culture grew slower whereas a culture on cellulobiose or reprecipitated cellulose grew out in 5-6 days and the pH increased slightly during cultivation. Their results suggested that cellulase was only produced from mycelium. Cellulobiose and glucose caused repression of cellulase production.

Filamentous fungi such as T. reesei, A. niger, Penicillium sp., etc. have been employed for cellulase production using solid-state fermentation where a basal mineral salts medium was used for moistening the substrate. Chahal (1985) had reported a higher yield of cellulases from T. reesei in SSF cultures compared to liquid cultures. Tengerdy (1996) compared cellulase production in SmF and SSF systems and had indicated that there was about a 10-fold reduction in the production cost when SSF is employed for production. Solid-state cultures are strongly recommended as systems for producing cellulases at lower price than submerged cultures (Vintila et al., 2009) as the product concentration remains quite higher thereby reducing the step in downstream processing, in turn reducing the cost of operation. Nigam and Singh (2009) have reviewed the use of agricultural wastes as substrates for cellulolytic enzyme production under SSF and strongly believe that with the appropriate technology, improved bioreactor design and operation controls, SSF may become a competitive method for the production of cellulases. The review by Pandey et al., (1999) on SSF for industrial enzyme production also describes the application of the technology for cellulase production. SSF can thus be considered as a future technology for commercial production of cellulases considering the low cost input and ability to utilize naturally available sources of cellulose as substrate. Cellulases produced in solid-state culture shows remarkable stability towards temperature, pH, metal ions, etc. Dutta et al., (2008) claimed the cellulase produced by SSF employing Penicillium citrini showed tolerance to alkali for the first time. SSF proves to be an efficient technology for cellulase production for bioconversion, since purity is not very stringent necessity for this application, and a concentrated enzyme preparation would serve the purpose. It has also been reported that the enzyme produced using the same biomass as to be used for bioconversion proves more efficient than the one produced on other cellulosic substrate (Lynd et al., 2002).
The production of hydrolytic enzymes is directly related to the available substrates (Nybroe et al., 1992), therefore an increase in the concentration of a particular substrate should stimulate the microorganisms to produce enzymes specific for the utilization of that substrate. The agro-industrial wastes can eventually be used as substrate and act as good sources of carbon and nitrogen. The synthesis of cellulase was made by *T. reesei* QM 9414 using cellulose as carbon source. The cellulase production was carried out by using *T. reesei* Rut C-30 and *T. reesei* NG-14. The maximum growth of *T. reesei* C5 and the production of cellulase enzyme were obtained with lactose as carbon source (Muthuvelayudham et al., 2004). Similar study was done by the Muthuvelayudham et al., (2005) for the fermentative production of cellulase on different substrates such as cellulose, xylose and lactose using *T. reesei*. The lignocellulosic materials such as sugar beet pulp and alkaline extracted sugar beet pulp and cellulose were used for cellulase production (Olsson et al., 2003).

It is well-established fact that culture conditions affect significantly the production of cellulase and hemicellulases. Carbohydrates or their derivatives induce most of the cellulytic enzymes. Thus the carbon source plays an important role in enzyme production (Kubicek and Penttila, 1998). Various studies have been made by different researchers for cellulase enzyme production through either by submerged or by solid state fermentation. Bagasse was used as substrate for endoglucanase (Carboxymethyl cellulase, CMCase) production using locally isolated *Asperigillus terreus* and the various medium components and culture parameters were optimized for enhancing the cellulase yield (Youssef and Berekka, 2009). Cellulase production by members of the genus *Asperigillus* using different agricultural wastes has been reported by Gokhale et al., (1991), Jecu (2000), Ojumu et al., (2003) and Immanuel et al., (2007). Initial pH, moisture content and temperature were optimized for FPase production by *T. reesei* QM9414 and *T. reesei* MCG77 in solid state fermentation using rice bran as substrate (Himid-Esfahani et al., 2007). The cellulase and hemicellulase enzymes was produced by *Trichoderma reesei* Ru/Tc30 on steam pretreated spruce, willow, corn stover and delignified lignocellulose (Solka Floc) (Juhasz et al., 2005). Cellulase enzyme was itself as inducible on cellulosic substrates. Various substances were used for cellulase induction. It is generally accepted that cellobiose (Fritscher et al., 1990), cellobiono-8-1,5-lactone (Kubicek et al., 1988), lactose (Morikawa et al., 1995) and
sophorose (Mach et al., 1995) enhance the production of endo and exoglucanase as well as β-glucosidase. However, the highest cellulase yields have been obtained on cellulose containing carbon sources (Bhat and Bhat, 1997; Kubicek and Penttila, 1998).

Recently the interest in cellulases and hemicellulases has increased because of many potential applications for these enzymes. However, in bioethanol production, it is necessary to reduce the costs of the enzymes used to hydrolyse the raw material and to increase their efficiency in order to render the process economically feasible. In addition, there is a general interest in obtaining new, more specific, stable enzymes and to use a cheap source of inducer, such as sugar cane bagasse, and recycle all or part of the enzymes. Endoglucanases are important for the degradation of β-glucan in feed which lowers the viscosity of the intestinal contents and hence the quality of the feed is improved (Weber and Agblevor, 2005). In the fermentation process of celluloses, cellulase enzymes provides the substrate for the synthesis of methane, ethanol, glycerol, citric acid, lactic acid, vitamins, antibiotics and single cell protein and in breweries (Santosh Vyas, 2004). Cellulases and related enzymes (hemicellulases and pectinases) are used as biological control of plant pathogens and infections as a result of their ability to degrade the cell wall of plant pathogens, inhibit spore germination, germ tube elongation and fungal growth (Bhat, 2000). Cellulases and related enzymes can be used in biotechnology for developing new breeds of plant or fungal protoplasts with desired characteristics by solubilising the plant or fungal cell walls which can be merged to form hybrids (Bhat, 2000).

Microorganisms of the genera Trichoderma and Aspergillus are thought to be cellulase producers, and crude enzymes produced by these microorganisms are commercially available for agricultural use. However, attempts to use these enzymes in the degradation of cellulosic wastes have not been successful for several reasons such as low enzymatic yields, low specific activities and end product inhibition of the enzymes (Li et al., 2010). Hence research was attempted in this way in order to find more efficient cellulase producing strain capable of degrading various types of lignocellulosics. As cellulases have different important industrial applications it is imperative to screen new strains for efficient cellulase production. Thus, the present study was carried out as a first step in screening different fungal strains for cellulolytic enzyme production.

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2.2 Materials and Methods

2.2.1 Chemicals: All chemicals used in the present investigation were of analytical grade. Carboxymethyl cellulose (CMC), Potato dextrose broth (PDB) and Congo red dye were procured from Himedia Laboratories Limited, Mumbai. P-nitrophenyl-β-D-glucopyranoside was procured from Sigma, Aldrich Corp, MO, USA. All the buffer salts and microbial media components were procured from the standard commercial sources and of highest quality available.

2.2.2 Microorganisms: The cellulase producing fungal strain such as Cladosporium sp. NCIM 901 was procured from NCIM, Pune, India. The stock culture was maintained on potato-dextrose agar at 4°C. It was subcultured onto potato-dextrose agar (PDA) in petri dishes at 30°C for 1 week prior to inoculation of submerged fermentation and solid-state fermentations. Further some fungal strains were also isolated from the soil and litter samples to characterize their cellulolytic activity in comparison with commercial strains.

2.2.3 Cellulosic material: Filter paper, cellulose, carboxymethyl cellulose, Avicel and Solka floc (SW 40) were used as substrates. Sugarcane bagasse was collected from S.V. Sugar Industries, Tirupati, India. Wheat bran, rice bran, corncob, rice straw were obtained locally available cheap raw material were collected used in this study. These are collected, dried, milled and sieved into 1 mm mesh and used as fine granules.

2.2.4 Collection of soil and plant litter samples: Soil samples were collected local sugar cane fields in sterile polythene bags. The soil and plant litter samples were collected from 3 to 4 places in and around Tirupati and the collected soil and plant litter samples were pooled. The mixed soil and plant litter samples were plated for isolating the cellulolytic microorganisms.

2.2.5 Isolation of fungal strains: Fungi from local sugar cane crop field soil and plant litter samples were suspended in 0.8% saline in sterile stomacher bags. Dilutions up to $10^{-7}$ were made and pour plated at higher dilutions was done using melted CMC agar medium. The above dilutions were plated in duplicates and incubated at 30°C for 12 days. The colonies isolated from the plates were then
subcultured 3-4 times on CMC agar till active growth of isolates were obtained, and thereafter maintained on the same media as in petriplates and in slants for further experimental work. The isolated fungal cultures were screened for their cellulolytic activity.

2.2.5.1 Screening for cellulolytic ability of isolated and commercial procured fungi: A medium containing Mandels’ mineral salts solution (Mandels et al., 1974) with addition of 17.5 g/L agar and 5.0 g/L phosphoric acid-swollen cellulose and 0.5% L-sorbose as a colony restrictor and inducer of cellulose production (Wang et al., 1995) was used for the isolation of cellulolytic fungi. The cellulose substrate used in the clearing test was prepared according to the procedure as recommended by Stewart et al., (1982). The plates were seeded with 100 μL of stock suspension (10 plates for each sample) and incubated at 25°C for 4-7 days, followed by 24 h at 50°C, after which clear zones could be observed only around colonies of the active fungal strains. Cellulase activities of the more active fungal isolates were determined using a carboxymethyl cellulase activity assay (CMCase). Basal medium containing (g/L): CMC – 10, NaNO₃ – 6.5, K₂HPO₄ – 6.5, yeast extract – 0.3, KCl – 6.5, MgSO₄·7H₂O – 3.0, glucose – 0.65 and agar – 17.5, was used for plate screening. Agar blocks (5 mm in diameter) from one-week old colonies grown on CMC-agar plates were cut and inoculated in the centre of the plates. The plates were incubated at 25°C for three to five days, followed by 18 h at 50°C in the dark. Cellulolytic strains were selected on the basis of the diameter of the hydrolysis zone surrounding the colonies. For observations, plates were stained with 1% Congo red dye (15 min), followed by destaining with 1 M NaCl solution for 20 min.

2.2.5.2 Maintenance media for fungal organisms: After selection of cellulase producing fungi, the strains were separated and streaked onto slants or petri plates containing potato dextrose agar (PDA) and incubated at 28-30°C for 5-6 days until mycelial growth and then preserved and maintained at 4°C. Slant cultures were subcultured periodically.

2.2.5.3 Secondary screening for cellulolytic enzyme production: It was carried out by both solid state and submerged fermentations by fungi giving high clear zone diameter.
2.2.5.3.1 Fungal strain and inoculum preparation for SmF: Cladosporium sp. NCM 901 was used throughout the study and was maintained on potato dextrose agar slants. Spores were collected from 5-day old agar-slant cultures by washing with 10 mL of sterile water with 0.1% Tween-80 (v/v), counted in a Neubauer counting chamber (Rambault and Alazard, 1980) and diluted to give $1 \times 10^8$ spores/mL. This suspension was used as the inoculum.

2.2.5.3.2 Fungus cultivation for spore production and inoculum preparation for SSF: Fungus spore was obtained by growing the culture on potato dextrose agar under room temperature in slant cultures. The spores were harvested after 5 days of cultivation by washing with sterile distilled water containing 0.1% (v/v) Tween 80 (Smith et al., 1996). The spore suspension was passed through a 0.5mm sieve to eliminate mycelia and the spore concentration was estimated by direct microscopic counting using Neubauer counting chamber (Rambault and Alazard, 1980). 10% (v/w) of the spore suspension containing $1 \times 10^8$ spore/mL was mixed with sterile growth medium. This medium was used as inoculum for SSF process.

2.2.5.3.3 Culture conditions and enzyme production by SmF: The most efficient cellulolytic fungal strain was screened and selected on the basis of plate clearing assay. It is maintained by periodic transfer on to potato dextrose agar (0.5% dextrose), slants at 30°C. The fungus was grown in Mary Mandels mineral medium (MM medium) (Reese and Mandels, 1963). The MM medium contained (in g/L of distilled water); KH$_2$PO$_4$, 2.0; (NH$_4$)$_2$SO$_4$, 1.4; CaCl$_2$2H$_2$O, 0.3; MgSO$_4$7H$_2$O, 0.3; urea, 0.3; Proteose peptone, 0.25; Yeast extract, 0.2; Substrate, 10.0 and trace metal solution, 1mL [FeSO$_4$.7H$_2$O, 5; MnSO$_4$.7H$_2$O, 5.6 ; ZnSO$_4$.7H$_2$O, 3.34 ; CoCl$_2$.2H$_2$O, 2 mg/ L ], Tween-80, 1mL. The pH of the medium was adjusted to 5.0 after autoclaving by separately sterilized 1M Na$_2$CO$_3$. The substrate was replaced by other carbon sources in the trails.

The MM medium supplemented with 1% substrate was inoculated with spore suspension of 5-day-old sporulating slant on PDA. Enzyme production was carried out in 250 mL Erlenmeyer flask containing 100 mL MM medium with substrate (1%) as the sole carbon source. The culture was incubated at 30°C on a rotary shaker at 200 rpm. The samples were withdrawn at regular intervals. The mycelium was removed by centrifugation at 7000 rpm at 4°C for 15 min to obtain a clear supernatant. This preparation was used for measurement of enzyme activities. Results given here are the mean of at least triplicate experiments.
2.2.5.3.4 Culture conditions and enzyme production SSF: The experiments were carried out using a 250 mL Erlenmeyer flasks containing dried substrate 1 g and moistened with the 5mL of salt solution (Reese and Mandels, 1963) thus contained the following (in g/L of distilled water): KH$_2$PO$_4$, 2.0; (NH$_4$)$_2$SO$_4$, 1.4; CaCl$_2$, 2H$_2$O, 0.3; MgSO$_4$.7H$_2$O, 0.3; urea, 0.3; Proteose peptone, 0.25; Yeast extract, 0.2; and trace metal solution, 1mL [FeSO$_4$.7H$_2$O, 5; MnSO$_4$.7H$_2$O, 5.6; ZnSO$_4$.7H$_2$O, 3.34; CoCl$_2$.6H$_2$O, 2 mg/ L ]; Tween 80, 1mL. Above mentioned dried substrate was replaced by other carbon sources. The fermentation media were used along with different carbon and nitrogen sources. The pH of the solution was adjusted to 5.0.

The medium was sterilized at 121°C for 15 min. After cooling the medium was inoculated with a concentrated spore suspension of $1 \times 10^8$ spore/g dry matter from the 7 day old slant culture. The final moisture content of the medium was approximately 70-80%. The fermentation was carried out at 30°C for 6-8 days. The contents of the each flask was thoroughly mixed with 10 mL of sterile water and filtered under vacuum using 0.45 µm membrane filter (Sartorius, Germany). The filtrate as crude enzyme solution was stored at 4°C for further enzymatic assays.

2.3.6 Comparison of SSF and SmF: SSF was carried out as in section 2.2.5.3.4. SmF was carried out by using the medium composition as described in section 2.2.5.3.3. All the components were dissolved in 100 mL distilled water and the pH was adjusted to 5.0 by 0.1 N HCl/0.1 N NaOH. The fungal strain was selected by optimizing cultural conditions. The optimum conditions obtained from each experiment were used unless otherwise stated.

2.3.6.1 Effect of temperature on enzyme production: Influence of temperature on enzyme production by Cladosporium sp.NCIM 901 in solid state fermentation and submerged fermentation was studied by incubating the inoculated flasks at various temperature conditions like 20, 25, 30, 35, 40 and 45°C.

2.3.6.2 Effect of incubation time on enzyme production: Effect of incubation time on enzyme production of Cladosporium sp.NCIM 901 during solid state and submerged fermentation was determined by incubating the inoculated flasks for 8 days. The enzyme activities were estimated at every 24 h incubation period at an optimum temperature 35-37°C.
2.3.6.3 Effect of pH on enzyme production: The impact of initial pH of the medium on enzyme production by *Cladosporium* sp.NCIM 901 during solid state and submerged fermentation was studied by adjusting the pH of the salt solution with 0.1 N HCl/ 0.1 N NaOH in a range of 3.5-8.0 with half unit increment at optimum temperature and incubation time.

2.3.7 Optimization of fermentation conditions for cellulolytic enzymes during SSF production: Various parameters influencing the maximal enzyme production during SSF production were studied as follows: The effect of substrate particle size (0.5mm, 1mm and 2 mm meshed bagasse) on enzyme production was studied. The effect of substrate concentration on the enzyme production was studied by using different weights of bagasse (1-8 g/flask). The effect of initial total moisture content was carried out using different moisture content (45, 50, 55, 60, 65, 70, 75, 80 and 85%), adjusted using various volumes of buffered MM medium. The effect of supplement of different nitrogen sources were examined on equivalent nitrogen basis. The optimization of inoculum level was carried out by using different inoculum size. The effect of different cellulosic substrates as well as different surfactants on enzyme production was studied.

2.3.8 Physico-Chemical properties of the cellulolytic enzymes: The optimum incubation period of reaction mixture at which the maximum activity of the enzymes was obtained determined at different time intervals. The effect of temperature on the enzyme activity of different enzymes (FPase, CMCase and β-glucosidase) was determined after incubation of the crude extract of enzyme with its specific substrate at different temperatures (30-100°C). The optimum pH level for enzyme activity was determined by incubating each enzyme with its specific substrate at different pH levels (3-8.5) under optimal conditions. Effect of different substrate concentration on the enzyme activity was determined by using 0.1 mL enzyme incubated with different concentrations of the substrate (0.5-4.0%) under optimal conditions. The thermal and pH stabilities of different cellulolytic enzymes were also examined.

2.3.9 Enzyme assays: Filter paperase assay (FPase) was used to estimate total cellulase activity and endoglucanase activity (CMCase activity) (Ghose, 1987) in the crude enzyme preparation as given below. For filter paperase assay whatman No.1 filter paper strip of dimension 1.0 x 6 cm (50 mg) was placed into each assay tube.
The filter paper strip was saturated with 1.0 mL of Na-citrate buffer (0.05 M, pH 4.8) and was soaked for 10 min at 50°C. Half milliliter of an appropriately diluted (in Na-citrate buffer, 0.05M; pH 4.8) enzyme was added to the tube and incubated at 50°C for 60 min. And in case of carboxy methyl cellulase (endoglucanase) activity half milliliter of 1% carboxymethyl cellulose in 0.05M Na–citrate buffer, pH 4.8 was soaked for 10 min at 50°C. After that half milliliter of an appropriately diluted enzyme was added to the tube and incubated at 50°C for 30 min. Appropriate controls were also run along with the test. At the end of the incubation period, tubes were removed from the water bath, and the reaction was stopped by addition of 3 mL of 3, 5-dinitrosalicylic acid reagent per tube. The tubes were incubated for 5 min in a boiling water bath for color development and were cooled rapidly. The reaction mixture was diluted appropriately and was measured against a reagent blank at 540 nm in a UV-VIS spectrophotometer. β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21) activity was estimated using p-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate. The total of assay mixture (1 mL) consisting of 0.9 mL of pNPG (1 mM) and 0.1 mL of suitably diluted enzyme was incubated at 50°C for 30 min. The p-nitrophenol liberated was measured at 410 nm after developing the color with 2 mL of 2 M sodium carbonate (Gokhale et al., 1988). In these tests, reducing sugars were estimated colorimetrically extrapolating with 3, 5-dinitrosalicylic acid after Miller (1959) using glucose as standards. The concentration of glucose released by enzyme was determined by extrapolating with a standard curve constructed similarly with known concentrations of glucose. One unit of enzyme activity was defined as the amount of enzyme required for liberating 1 μM of glucose per milliliter per minute and was expressed as U/mL. The enzyme activity in SSF expressed as U/gds (gram dry substrate). In case of β-glucosidase enzyme assay, one unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose or p-nitrophenol from the appropriate substrates per minute under the assay conditions.

2.4 Results and Discussion:

Fungi constitute a group of microorganisms that are widely distributed in environment especially in soil (Boer et al., 2005). Since they produce wide variety of hydrolytic enzymes and hence exist in nature in saprophytic mode (Ng, 2004). The action of enzymes is so important, for instance, wood-rotting fungi are
classified on the basis of enzymatic deterioration of wood; white-rot fungi can
degrade lignin and brown-rot fungi can degrade cellulose (Tortella et al., 2005).
Fungal hydrolytic enzymes share 40% of global enzyme market and have many
industrial applications (Archer and Peberdy, 1997). Therefore, there is a need for
screening these enzymes for improved characteristics. The present investigation was
undertaken in order to harness a cheaper raw material like sugarcane bagasse into
value-added product like cellulase by fungal fermentation. It is known that certain
fungal species like *A. niger*, *T. reesei*, *Penicillium purpurigenum*, *Rhizopus oryzae*,
*Fusarium moniliforme* etc. possess cellulolytic activity, hence the purpose was to
screen similar isolates from nature and employ them for the production of
cellulolytic enzymes. In most investigations, members of the genus *Trichoderma*
Pers have been extensively studied due to their ability to secrete cellulose-degrading
enzymes. Most of the investigations have been carried out on *T. aureoviride* Rifai, *T.
viride* Pers., *T. reesei* E.G. Simmons, *T. harzianum* Rifai strains and their mutants
evaluating their ability to produce extracellular cellulolytic enzymes
(endoglucanases, exoglucanases and cellobiase) which act synergistically in the
conversion of cellulose to glucose. The cellulases secreted by *Trichoderma* have
received widespread industrial interest leading to commercial applications (Oksanan
et al., 2000; Cavaco-Paulo and Gubitz G, 2003; Nierstrasz and Warmoeskerken,
2003; Penttila et al., 2004). However, despite the efforts of many laboratories, no
commercially efficient enzyme complex has been produced. In addition, the
*Trichoderma* cellulase system is deficient in cellobiase, causing the accumulation of
disaccharide cellobiose which produces repression and end product inhibition of the
enzymes (Gruno et al., 2004). The demand for more thermostable, highly active and
specific cellulases is on the increase; therefore, cellulase systems of the other fungi
have also been investigated. The studies were concentrated mainly on soil fungi
(Lynd et al., 2002). Most fungi can be adapted to anthropogenic substrates such as
natural or waste cellulose or to substrates containing high amounts of cellulose. The
cellulase enzyme produced extracellularly and harvested easily for determination.
The results of experiments performed are systematically presented below.

2.4.1 Screening, comparison of isolated and commercial cellulolytic fungi: In
preliminary screening, both commercial and isolated fungal strains from soil and
plant litter samples were screened for their cellulolytic activity by plating them on
carboxy methyl cellulose agar (CMC-agar) medium and looking for CMC clearing zones. Identification of fungi was done based on colony characters and microscopic examination and it was concluded that *A. niger* was the dominant fungal strain in soil and plant litter samples. Besides *A. niger* the other organisms like, *Trichoderma* sp., *Rhizopus* sp., and *Pencilliurn* sp. were also identified but incidence being lesser than the dominant *A. niger*. Keeping in view of this objective, only 2 local fungal isolates were selected from natural sources (soil and plant litter) and fungal strain procured from culture collection centre were subjected to screening for their ability to elaborate extracellular hydrolases. The fungal strains were identified for their cellulolytic ability on plate clearing assay by using carboxy methyl cellulose and Congo red dye and by the formation of clear zone diameter. Plates having agar medium without any carbon source were treated as control, which showed no signs of growth and there was no clear zone formation observed, indicating no fungal growth and no enzyme activity. Based on visual observance, three isolates gave clear zones of cellulase activity having diameter 1.5mm to 3.5mm as shown in Figure 2.1.

In the first isolate, clear zone of diameter 1.5mm was observed on 4th day of its incubation at 30°C, for the second isolate the clear zone of diameter 2.2mm was observed on the 3rd day of its incubation, and for isolate 3 zone of 3.5mm was observed on 6th day; thereafter excessive growth of mycelia was seen in each plate thus covering entire plate. Thus the fungi with hyper cellulolytic activity were subjected to further screening to test quantify potency of strains for cellulase production. Isolated strains along with commercially procured standard strain which showed cellulolytic activities are listed in Table 2.1.
A- Control plate with selective media with CMC as sole carbon source (no zone formation).

B- Treated plate with *Aspergillus niger* isolate 1 (AN11) with zone formation.

C- Treated plate with *Aspergillus niger* isolate 2 (AN12) with zone formation.

D- Treated plate with *Cladosporium* sp. NCIM 901 (CS) with zone formation.

**Fig. 2.1.** Primary screening of isolated and commercial fungi for cellulolytic activity on selective media with carboxy methyl cellulose (CMC) as carbon source.
### Table 2.1. Primary screening for cellulolytic fungi

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Source</th>
<th>Cellulolytic fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Soil</td>
<td><em>Aspergillus niger</em> Isolate (ANI1)</td>
</tr>
<tr>
<td>2</td>
<td>Plant litter</td>
<td><em>Aspergillus niger</em> Isolate (ANI2)</td>
</tr>
<tr>
<td>3</td>
<td>NCIM</td>
<td><em>Cladosporium sp.</em> NCIM 901</td>
</tr>
</tbody>
</table>

#### 2.4.2 Secondary screening of cellulolytic strain:

Based on the clear zone formation and on diameter of clear zone the fungal strain *Cladosporium sp.* NCIM 901 was selected for further studies. After selecting the strain based on clear zone formation quantitative estimation of cellulolytic activity was done in submerged and solid state fermentation samples. This was carried out to select the fermentation process on which the strain showed maximum cellulolytic activity. Most enzymes are proteins and during growth in the selective broth, they are secreted extracellularly. In order to break down complex cellulose to simpler products, cellulases FPase (Filter paperase), carboxy methyl cellulase (CMCase), and β-glucosidase must be involved.

There is greater variation in SmF and SSF fermentation in terms of maintenance of cultural conditions, enzyme production and product recovery. However, the fermentation parameters (physical and chemical) were similar in both fermentations, due to the culturing either in liquid state (SmF) or solid state (SSF) will influence the fungal growth and enzyme production. Hence further studies were carried out in order to find better fermentation method that will yield that high enzyme activity. Enzyme production for secondary screening was done in both solid state and submerged culture fermentation medium. The cellulolytic enzyme complex was estimated in the culture filtrates from fungal strain, which showed cellulolytic activities. The strain was cultured both in solid fermentation and submerged fermentation media on sugarcane bagasse as substrate. Cultural conditions like temperature, pH of the medium and incubation time were similar for both types of fermentations. The following are the effect of cultural conditions for cellulolytic enzyme production in SSF and SmF. The most influencing physical parameters such as incubation time, pH and temperature were choosen for secondary screening.
2.4.3. Comparison of SSF and SmF

2.4.3.1 Effect of incubation time on cellulolytic enzyme production in SSF and SmF: For testing the optimum incubation time of Cladosporium sp. for FPase, CMCase and β-glucosidase production, the solid state and submerged fermentations were carried out up to 8 days and the enzyme activities were examined at every 24 h. FPase, CMCase and β-glucosidase activity were found to be maximum at the 5th day in both SSF and in SmF (Figure 2.2).

The results showed that the enzyme activity was very low at the 1st day and 8th day. In the first day there was no marked growth of fungus. As the nutrients utilized by fungus there was a gradual increase of the growth of the fungus as well as fungal enzyme activity up to 5 days. Further incubation up to 8 days, showed decreased level of enzyme production. The loss on enzyme activity is perhaps to the depletion of nutrients in media as well as the formation of toxic unwanted products and degradation of the enzymes (Figures 2.2, 2.3 and 2.4).

![Graph showing FPase activity in SSF and SmF](image)

**Figure 2.2.** Effect of incubation period on FPase activity in SSF and SmF.
2.4.3.2 Effect of temperature on cellulolytic enzyme production in SSF and SmF: The cultivation temperature does not only affect the growth rate of an organism, but it also has marked effects on the level of cellulolytic enzyme production. The net temperature effect is influenced not only by the environmental temperature, but also by the increase in temperature generated from the metabolic activities of the fungi growing on the solid substrates. Studies on the influence of temperature on FPase, CMCase and β-glucosidase production by Cladosporium sp. NCIM 901 were carried out. Cladosporium sp. and the data are presented in Figures 2.5, 2.6 and 2.7.
The temperature conditions varied from 20-50°C. The celluloytic enzyme activity was low at 20°C attained maximum activity at 35°C and declined after 45°C. The maximum activities of FPase, CMCase and β-glucosidase were found at 35°C in both SSF and in SmF (Figures 2.5 to 2.7). Enzyme activities were comparatively less at 20°C and 45°C. The loss of enzyme activity at high temperature is due to the denaturation, conformational changes, as enzymes are proteins.

Fig. 2.5. Effect of incubation temperature on FPase activity in SSF and SmF.

Fig. 2.6. Effect of incubation temperature on CMCase activity in SSF and SmF.
Temperature is directly related to the metabolic activities of the microorganism and influences the proper growth and product formation by the organism (Lonsane et al., 1985). Every organism has its own optimal temperature at which it grows its maximum and produces desired products maximally. Hence maintenance of optimal temperature is a must. Thermal conditions for the maximal production of FPase, CMCase and β-glucosidase were studied in Cladosporium sp. The cellulolytic enzymes such as FPase, CMCase showed increased activity at 35°C in SSF and SmF. The β-glucosidase showed maximum activity at 35°C in SSF and in SmF. This is in agreement with the results outlined by other researchers. The positive significance of temperature on cellulase enzyme production by several Asperigilli was recorded, with the temperature optima between 28-35°C (Gokhale et al., 1991; Jecu, 2000). Interestingly, Immanuel et al., (2007) reported that A. niger and A. fumigatus were capable of producing cellulase enzyme optimally at 40 and 50°C during growth on coir waste and sawdust, respectively.

During fermentation, the rise in temperature is due to exothermic reactions like large amount of metabolic heat. In solid state fermentation, the solid characteristic of the substrate takes homogeneity in the reaction and, therefore, results in the existence of temperature gradients. Such facts make the heat exchange difficult on the system (Pandey et al., 2001). Since, temperature has a primary role in the growth, product formation and sporulation of the microorganism, it is important to dissipate the heat generated immediately.
2.4.3.3 Effect of pH on cellulolytic enzyme production in SSF and SmF: Though the enzymes are proteins, the pH showed profound effect on the enzyme activity. The initial pH in the medium was varied from 3.5-8.0, for finding out the effect of pH for production of the FPase, CMCase and β-glucosidase by Cladosporium sp. NCIM 901 in both solid state and submerged fermentation. Cellulolytic enzymes showed with maximal activity at pH 5.0 in solid state and at pH 5.5 in submerged fermentation. There was less FPase activity at pH 3.5, and the activity was declined with increase in pH of 6.0-8.0 (Figure 2.8). CMCase activity showed maximal activity at pH 5.5 both in solid state and submerged fermentations (Figure 2.9). β-glucosidase activity was low at pH 3.5 and 8.0 but maximal activity at pH 5.5 in SSF and SmF (Figure 2.10). Decreased enzyme activity appeared by increasing the pH of the fermentation medium due to the fact which confirms that filamentous fungi prefer acidic environments than the neutral pH environments.

Fig. 2.8. Effect of incubation pH on FPase activity in SSF and SmF.
In addition to this, the production of enzymes is also dependent on the pH of the medium which is a critical factor. Inorganic phosphates, organic acids, hydroxide salts, gaseous ammonia, sulfuric and hydrochloric acid are added to control the pH in a fermentation system. Indirectly it is also controlled, by balancing of carbon and nitrogen sources (Ward, 1989). In the present study hydroxide salts and hydrochloric acids were used in the adjustment of pH of the fermentation medium for the production of FPase, CMCase and β-glucosidase by Cladosporium sp. NCIM 901. CMCase activity showed maximal activity at pH 5.5 both in solid state and submerged fermentations. The present result was compared with that of Cheng et al., (2006) who reported that the maximal activities of CMCase and FPase
were obtained under the fermentation conditions with initial pH of 5.5-6.0, temperature 30°C, cultivation period of 3-4 days with the initial inoculum ratio of 6% (v/v).

Fermentation is dependent on many physiological and biochemical variables. For better production of the desired product by any type of fermentation, it is necessary to study the influence of these variables on the production of FPase, CMCase and β-glucosidase. Among the various parameters, the foremost one studied was the type of fermentation, namely solid state fermentation or submerged fermentation. By the present results it was concluded that the cellulolytic enzyme production by Cladosporium sp. was much higher in SSF than in SmF. The other fermentation parameters which influence the cellulolytic enzymes production, further studies were carried out by optimizing the other cultural parameters for improved enzyme production through SSF process. The enzyme production of FPase, CMCase and β-glucosidase were higher in SSF method than SmF.

2.4.4 Influence of other fermentation parameters in SSF for the production of cellulolytic enzymes by Cladosporium sp.: SSF means that the microorganism grows on moist solid substrates in the absence of free flowing water. Studies have been subsequently carried out in an attempt to increase the production and activity of the cellulolytic enzymes produced extracellularly by Cladosporium sp. by evaluating the effects of environmental, physiological and nutritional factors on the cellulolytic enzymes production including; FPase, CMCase and β-glucosidase under SSF.

2.4.4.1 Effect of different cellulosic substrates on production of cellulases by Cladosporium sp.: Solid substrates used in SSF are insoluble in water therefore water will have to be absorbed onto the substrate particles, which can be used by the microorganisms for growth and metabolic activity (Pandey, 1992). It is also expected that the rate of water absorbed by different substrates vary from one substrate to another. This is another possible explanation for the variation in the cellulolytic enzyme production using different substrates. Previous studies reported that cellulases are inducible enzymes. They can be induced with different inducers such as cellobiose, lactose and sophorose (Mandels and Reese, 1960; Mandels et al., 1962). However, most of the microorganisms have produced highest level of cellulases when grown on cellulose (Ryu and Mandels, 1980). Other researchers reported that refined cellulosic substrates such as solka floc (Hayward et al., 2000),
Avicel (Aiello et al., 1996) are better substrates for cellulase production than agricultural residues. This may be attributed to higher lignin content in agricultural residues affecting the cellulase production (Aiello et al., 1996; Bigelow and Wyman, 2002).

The cellulolytic enzyme production profiles of Cladosporium sp. on different cellulosic substrates are presented in Table 2.2. The pretreated milled bagasse showed considerable enzyme production than other cellulosic substrates. The physical pretreatment such as milling and grinding would lead to more surface area of cellulosic substances to the fungal enzymes. However, compared with wheat bran, bagasse resulted in lower enzyme production. One of the possible explanations for the lower yields of cellulolytic enzyme production on sugarcane bagasse could be the poor nutritional status of sugarcane bagasse compared to wheat bran as the former mainly consists of cellulose and lacks the nutrients required for better fungal growth therefore, results in lower production. Similarly, Ojumu et al., (2003) during a study investigated sugarcane bagasse, corn cobs and sawdust as lignocellulosic substrates for the production of cellulase by Aspergillus flavus and reported lower CMCase production on the sugarcane bagasse.

Table 2.2: Effect of different cellulosic substrates on production of cellulases by Cladosporium sp. NCIM 901.

<table>
<thead>
<tr>
<th>Cellulosic substrate (5 g/flask)</th>
<th>FPase (IU/gds)</th>
<th>CMCase (IU/gds)</th>
<th>β-glucosidase (IU/gds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose powder</td>
<td>6.22±0.34</td>
<td>8.40±0.24</td>
<td>7.01±0.18</td>
</tr>
<tr>
<td>Avicel</td>
<td>5.22±0.22</td>
<td>5.89±0.18</td>
<td>5.35±0.13</td>
</tr>
<tr>
<td>Solka floc (SW 40)</td>
<td>5.02±0.12</td>
<td>5.24±0.16</td>
<td>5.10±0.15</td>
</tr>
<tr>
<td>Filter paper</td>
<td>5.42±0.16</td>
<td>7.05±0.32</td>
<td>5.66±0.18</td>
</tr>
<tr>
<td>CMC</td>
<td>7.30±0.38</td>
<td>9.46±0.42</td>
<td>4.30±0.10</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>8.46±0.32</td>
<td>10.2±0.44</td>
<td>8.02±0.26</td>
</tr>
<tr>
<td>Rice bran</td>
<td>5.08±0.18</td>
<td>8.12±0.21</td>
<td>6.10±0.12</td>
</tr>
<tr>
<td>Corn cob</td>
<td>4.22±0.12</td>
<td>7.24±0.30</td>
<td>4.69±0.16</td>
</tr>
<tr>
<td>Rice straw</td>
<td>3.86±0.14</td>
<td>7.05±0.16</td>
<td>4.01±0.14</td>
</tr>
<tr>
<td>Milled bagasse</td>
<td>7.63±0.43</td>
<td>9.20±0.32</td>
<td>8.62±0.28</td>
</tr>
</tbody>
</table>

Note: The enzyme assay was conducted at the end of 5th day of growth at pH 5.5 at 35°C; Data presented are the mean of triplicate.
2.4.4.2 Effect of bagasse substrate particle size on enzyme activity: The bagasse substrate was milled, powdered and used as 2 mm, 1 mm and 0.5 mm particle sizes (bagasse powder). According to studies of Poonja and Prema (2007), the size of the wheat bran particles influenced enzyme and biomass production. This influence is due to the agglomeration of particles that could inhibit oxygen transfer. Microorganism adherence and penetration, as well as the action of the enzymes, depend on the physical properties of the substrate, such as its crystalline and amorphous nature, the accessibility area, surface area, porosity, particle size, etc. (Krishna, 2005). The bagasse was subjected to three pretreatments (milling, sieving and grinding) and the resulted bagasse was subjected to fermentation by Cladosporium sp. NCIM.901 and the results are represented in Figures 2.11, 2.12 and 2.13.

Fig. 2.11. Effect of particle size (2 mm) on FPase activity in SSF.

Fig. 2.12. Effect of particle size (1 mm) on CMCase activity in SSF.
The highest cellulolytic enzyme activities on powdery form (1 mm) substrate were observed at 5th day with the enzymatic activities i.e. 6.21 IU/gds, 8.62 IU/gds and 7.82 IU/gds of FPase, CMCase and β-glucosidase, respectively. This is due to greater accessibility of enzyme to this powdery substrate than the granular form. Two factors most widely believed to control enzymatic digestibility are the accessible surface area and the crystallinity of the cellulosic fraction. Effective pretreatment processes must therefore increase the degree to which the cellulosic fraction of the lignocellulosic material is susceptible to enzymatic attack and/or decrease the extent of cellulose microfibril crystallinity. Increased accessibility of cellulose enables more enzymes to bind to cellulose fiber surfaces; whereas decreased crystallinity increases the reactivity of cellulose, i.e., the rate at which bound enzyme hydrolyzes glycosidic linkages (McMillan, 1992). Milling can improve susceptibility to enzymatic hydrolysis by reducing the size of the materials, and degree of crystallinity of lignocelluloses, which improves enzymatic degradation of these materials toward ethanol or biogas. Without any pretreatment, corn stover with sizes of 53-75 µm was 1.5 times more productive than larger corn stover particles of 425-710 µm (Zeng et al., 2007). Sidiras and Koukios (1989) showed that due to crystallinity reduction by ball milling, saccharification of more than 50% of straw cellulose with minimal glucose degradation becomes possible at mild hydrolytic conditions. The crystallinity index of solka floc by ball milling changed from 74.2 to 4.9% (Fan et al., 1980). However decreasing the particle size will reduce the enzyme production. The agglomeration of particles will inhibit the passage of enzyme towards the subsurface of the substrate particles.
2.4.4.3 **Effect of different substrate concentration:** The bagasse powder of 1 mm mesh was used to study the effect of substrate concentration on enzyme activity. The data presented in Figure 2.14 showed that lower (1-4 g/flask) and higher (6-8 g/flask) substrate concentration affected the production of cellulolytic enzymes. The production of these enzymes were increased gradually and reached their maximum production at 5.0 g of bagasse where FPase, CMCase and β-glucosidase recorded 8.42 IU/gds, 10.4 IU/gds and 9.02 IU/gds, respectively. Significant reduction in enzymes production was detected with increased amount of substrate use in the fermentation process.

![Graph showing enzyme activity vs substrate concentration](image)

**Fig. 2.14.** Effect of substrate concentration on enzyme activity in SSF.

These results are in agreement with previous results which showed high substrate concentration usually that results in lower enzyme yield due to by-product inhibition effect (Ramos et al., 1993) and enzyme inactivation (Reese, 1980). As the volume of the fermentation medium increased, the production of enzyme decreased. It might be due to the reduction in the agitation of the medium, decrease in air and mineral supply and subsequent decreased growth of the organism. Similarly, at low level of fermentation medium, the production of enzyme was also decreased. It might be due to insufficient nutrient supply in the fermentation medium for good growth of the *Cladosporium sp.* and hence enzyme production.
2.4.4 Effect of moisture level on enzyme activity: Moisture content is a crucial factor in any SSF process because this variable has influence on the growth and biosynthesis of the microbe as well as secretion of different metabolites such as enzymes. The results in Figure 2.15 indicate, maximum production of CMCase and β-glucosidase enzymes was obtained at levels of 13.42 and 12.04 U/gds, respectively at 70% moisture and at 75% the FPase was 10.64 IU/gds. These results clearly indicate that the cellulolytic enzyme production increased at moderate moisture levels (70 and 75%); while it decreased under low levels (40-50%) and higher (80-90%) moisture levels. These results are in good agreement with those reported by Jecu (2000) and Panagiotou et al., (2003), who reported that an increase in the initial moisture content of substrate from 60-75% greatly enhanced the cellulase production. However, further increase to 80% had a negative effect on the production of cellulolytic enzymes. The degree of hydration of the substrate plays an important role on the growth of the fungus and subsequently the enzyme production. Water causes the swelling of the substrate and facilitates utilization of the substrates by the proper microorganism. Increase in moisture level is believed to decrease enzyme productivity due to decreasing substrate porosity, alteration in particle structure, lowering oxygen transfer, higher soluble protein and increased bacterial contamination. On the other hand, low moisture content causes reduction in the solubility of nutrients, low degree of substrate swelling as well as reduction in soluble protein (Gervais and Molin, 2003).

Fig. 2.15. Effect of moisture content (%) on enzyme production in SSF.
The moisture content of the medium has critical function on SSF and many researchers have reported similar views on moisture content related to cellulolytic enzyme production (Jecu, 2000; Panagiotou et al., 2003). This could be attributed to the faster growth of microorganism at higher moisture content and the subsequent early initiation of enzyme production (Kalogeris et al., 1998). On the other hand, Krishna (1999) and Chandra et al. (2007) obtained the highest FPase activities of 2.8 U/g and 2.9 U/g under optimum moisture content of 70% and 50% (w/w), respectively. The result obtained showed no marginal differences in FPase activity at different levels of moisture content used in the fermentation process.

2.4.4.5 Effect of different levels of inoculum size on enzyme activity: The data illustrated in Figure 2.16 show that the maximum activities of FPase and β-glucosidase are (9.42, 10.23 IU/gds, respectively) recorded at inoculum size of 2.5 mL. While the maximum activity of CMCase was detected at inoculum size of 2 mL (11.62 IU/gds). These results are in agreement with Fadel (2000), those of who proved that CMCase productivity was highly influenced by different inoculum size of A. niger grown on radicle waste. Furthermore, Kumar and Satyanarayana (2004) recorded that effect of inoculum sizes on enzyme production depend up on other culture fermentation conditions such as; incubation period, moisture content, nature of the microbe used and characteristics of the substrate. In this connection Domigues et al., (2000) showed that fungal sporulation and its metabolic activities are influenced by size of inoculum. Low inoculum may require longer time for fungal multiplication and substrate utilization to produce desired enzyme levels.

![Fig. 2.16. Effect of inoculum size on enzyme production in SSF.](image-url)
On the other hand, higher inoculum would ensure rapid proliferation of fungal biomass. So, a balance between the proliferating biomass and substrate utilization would yield maximum enzyme activity as recorded by Ramachandran et al., (2004). Furthermore, further increase in inoculum size resulted in decreasing enzyme yield due to limitation of nutrients as reported by Ghanem et al., (2000).

2.4.4.6 Effect of inorganic and organic nitrogen sources on enzyme production by Cladosporium sp.: The results presented in Table 2.3 show that the maximum FPase, CMCase and β-glucosidase production is enhanced by the medium having (NH₄)₂SO₄, urea and yeast extract. According to Desia et al., (1982), that production of fungal enzymes is very sensitive to type of nitrogen source and its level in the fermentation medium. The present results were compared with those of other works to show that addition of NaN₃ or urea as a nitrogen source stimulated cellulase production during SSF fermentation (Rao et al., 1985; Narasimha et al., 2006). Various researchers have shown that different organic and inorganic nitrogen sources such as yeast extract (Ganguly and Mukherjee, 1995), soyameal (Gomes et al., 2000) and corn steep liquor (Hayward et al., 2000) influence the cellulase production. Experiments regarding effect of various nitrogen sources on enzyme production demonstrated that there is substantial increase in the enzyme activity when the medium is supplemented with complex nitrogen sources like yeast extract and urea (Table 2.3).

Table 2.3: Effect of inorganic and organic nitrogen sources on enzyme production by Cladosporium sp.NCIM 901.

<table>
<thead>
<tr>
<th>Nitrogen source (g/L)</th>
<th>FPase (FPU/gds)</th>
<th>CMCase (IU/gds)</th>
<th>β-glucosidase (IU/gds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄ (1.4)</td>
<td>11.4±0.52</td>
<td>14.8±0.56</td>
<td>12.4±0.42</td>
</tr>
<tr>
<td>(NH₄)₂HPO₄ (1.4)</td>
<td>4.89±0.18</td>
<td>5.06±0.21</td>
<td>4.65±0.12</td>
</tr>
<tr>
<td>KNO₃ (2.3)</td>
<td>5.42±0.26</td>
<td>6.12±0.32</td>
<td>5.22±0.18</td>
</tr>
<tr>
<td>NaNO₃ (2.8)</td>
<td>5.22±0.24</td>
<td>7.02±0.40</td>
<td>5.89±0.24</td>
</tr>
<tr>
<td>(NH₄) NO₃ (1.8)</td>
<td>5.20±0.24</td>
<td>6.65±0.38</td>
<td>5.43±0.22</td>
</tr>
<tr>
<td>NH₄Cl (2.0)</td>
<td>5.82±0.18</td>
<td>6.26±0.38</td>
<td>6.02±0.30</td>
</tr>
<tr>
<td>Protease peptone (0.25)</td>
<td>8.52±0.44</td>
<td>9.04±0.41</td>
<td>8.12±0.36</td>
</tr>
<tr>
<td>Yeast extract (0.25)</td>
<td>10.8±0.51</td>
<td>13.5±0.52</td>
<td>12.0±0.36</td>
</tr>
<tr>
<td>Soya meal (0.25)</td>
<td>8.64±0.42</td>
<td>9.63±0.48</td>
<td>8.21±0.39</td>
</tr>
<tr>
<td>Urea (0.30)</td>
<td>10.6±0.46</td>
<td>13.4±0.50</td>
<td>11.8±0.48</td>
</tr>
</tbody>
</table>

Note: The enzyme assay was conducted at the end of 5th day at pH 5.5 at 35 °C; Experiments were performed in triplicate.
2.4.4.7 Effect of various surfactants on cellulase production by *Cladosporium sp.*: The effect of the addition of surfactants on cellulolytic enzyme production by *Cladosporium sp.* NCIM 901 is presented in Table 2.4. The pH of the medium was maintained at 5.5. The results were taken at the 5th day of the fermentation period as the fungi were yield good enzymatic activity at this period stated above. Higher CMCase activity was observed. The results suggested that addition of surfactant (0.1%, v/v) was essential in order to facilitate the release of cellulases in the medium. Tween 80 was found to be the best surfactant in this regard than other surfactants used in this study. According to study carried out by Domingues *et al.*, (2000) Tween 80 influences the morphology of *Trichoderma reesei Rut C-30* as well as the enzyme production. Sukan *et al.*, (1989) demonstrated that emulsification with Tween 80 led to higher cellulase activities presumably by causing increased permeability of cell membranes and/or by promoting the release of cell-bound enzymes. However increased surfactant concentrations, may lead to decreasing the enzyme production.

**Table 2.4. Effect of various surfactants on cellulase production by *Cladosporium sp.***

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>FPase (FPU/gds)</th>
<th>CMCase (IU/gds)</th>
<th>β-glucosidase (IU/gds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 20 (0.1)</td>
<td>3.80±0.16</td>
<td>5.02±0.19</td>
<td>4.20±0.14</td>
</tr>
<tr>
<td>Tween 40 (0.1)</td>
<td>6.12±0.22</td>
<td>7.48±0.26</td>
<td>6.85±0.24</td>
</tr>
<tr>
<td>Tween 80 (0.1)</td>
<td>8.02±0.24</td>
<td>10.86±0.42</td>
<td>8.96±0.36</td>
</tr>
<tr>
<td>Triton X-100 (0.1)</td>
<td>5.32±0.24</td>
<td>8.48±0.34</td>
<td>7.42±0.24</td>
</tr>
<tr>
<td>None</td>
<td>2.46±0.06</td>
<td>3.21±0.10</td>
<td>3.02±0.08</td>
</tr>
</tbody>
</table>

**Note:** The enzyme assay was conducted at the end of 5th day at pH 5.5 at 35 °C; Experiments were performed in triplicate.

The present results were also compared with the results obtained by other works. The presence of Tween 80 in fermentation medium might have triggered the production of CMCase. Shiang *et al.*, (1990) have reported the significance of addition of Tween 80 in enhancing CMCase activity. In another work Oberoi *et al.*, (2008) found that the addition of wheat bran into the fermentation medium resulted in higher CMCase activity as was the case with the FPase activity but not much difference was observed by varying the kinnnow pulp and wheat bran ratio. CMCase
is known to be induced by certain inducers such as xylose, cellulobioside and avicel (Shiang et al., 1990). A possible explanation to this could be the surfactant effect of Tween 80 in enhancing the endoglucanase (CMCase) activity since CMCase are the first enzymes of the cellulases complex which attack on the low crystallinity areas of cellulose and thus might need a relatively lightly bound surface to act upon.

Analyzing the enzymatic activities found in this study it can be concluded that the production of FPase, CMCase and β-glucosidase is favoured in SSF by optimizing cultural conditions.

2.4.5 Physico-Chemical characterization of cellulolytic enzymes: Cellulase preparations generally contain other enzymatic activities besides cellulase and these may affect the properties of the preparations. The influence of various physico-chemical factors on enzyme activity was studied.

2.4.5.1 Effect of different incubation periods on the enzyme activity: Effect of different incubation periods on the enzyme activities are presented in table 2.5. The results showed that optimum incubation period for maximum FPase and β-glucosidase activities was 20 min, while the CMCase reached its maximum activity at 30 min. The present results compared with the results obtained by EI-Tanash (2007) who found that CMCase reaches its maximum activity after 30 min. On the other hand, El-Azab (2007) reported that the optimum incubation period for maximum avicelase and cellulase activity was 75 min, while CMCase reached its maximum activity at 30 min.

### Table 2.5: Effect of different incubation periods on the enzyme activity.

<table>
<thead>
<tr>
<th>Incubation period (min)</th>
<th>FPase (IU/gds)</th>
<th>CMCase (IU/gds)</th>
<th>β-glucosidase (IU/gds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2.06±0.24</td>
<td>6.84±0.22</td>
<td>3.21±0.06</td>
</tr>
<tr>
<td>20</td>
<td>4.86±0.18</td>
<td>8.30±0.28</td>
<td>5.26±0.13</td>
</tr>
<tr>
<td>30</td>
<td>4.20±0.14</td>
<td>9.65±0.32</td>
<td>4.04±0.11</td>
</tr>
<tr>
<td>60</td>
<td>3.02±0.12</td>
<td>7.20±0.28</td>
<td>3.62±0.09</td>
</tr>
<tr>
<td>90</td>
<td>2.52±0.10</td>
<td>3.63±0.12</td>
<td>2.90±0.04</td>
</tr>
<tr>
<td>120</td>
<td>0.09±0.02</td>
<td>0.92±0.04</td>
<td>0.54±0.02</td>
</tr>
<tr>
<td>150</td>
<td>0.02±0.01</td>
<td>0.04±0.01</td>
<td>0.12±0.02</td>
</tr>
</tbody>
</table>

**Note:** The experiments were performed in triplicate.
2.4.5.2 Effect of different pH values on the enzyme activity: The Figure 2.17 represents that the CMCase and β-glucosidase enzymes of Cladosporium sp. showed maximal activity at pH 5.5. While in case of FPase the maximum enzyme activity was noted at pH 5.0. The present results is close to the data obtained by Shah and Madamwar (2005), who reported that pH 5.0 was the optimum for A. niger CMCase and cellulase activities. Coral et al., (2002) recorded that A. niger has two major activity peaks at pH 4.5 and 7.5; they attributed the results to the presence of two subunits in enzyme preparation.

![Figure 2.17](image)

**Fig. 2.17.** Effect of different pH values on the enzyme activity.

2.4.5.3 Effect of different temperatures on the enzyme activity: Effect of different temperatures on the enzyme activities is presented in Figure 2.18. The results indicate that 50°C was the best for the maximum activity of CMCase, β-glucosidase, while the FPase showed maximal activity at 40°C. The present results were also compared with the results obtained by Sharma et al., (1991) who reported that 55°C was the optimum temperature for Trichoderma viride CMCase. However El-Azab (2007) who reported that the optimum temperature for CMCase activity ranged from 45 to 55°C. This results are also in agreement with Aboul-Enein et al., (2010), who recorded that the optimum temperature for cellulase enzyme produced by actinomycete isolate was found to be around 50 to 55°C and agreed with Jang and Chen (2003) who reported that maximum activity for cellulase were obtained at 50°C for Streptomyces transformant T3-1 and Kluepfel et al., (1986) who reported that the highest values for CMCase activities were obtained at 50°C for S. flavogriseus enzymes and the highest values for CMCellulase activities were obtained at 55°C for S. lividans.
2.4.5.4 Effect of different substrate concentration on the enzyme activity: The data in Figure 2.19 represent that enzyme activity differed by changing the substrate concentration. As shown in results that CMCase and β-glucosidase activities were increased at the substrate concentration of 3% and reached their highest activity rate. However, the FPase activity was increased at the substrate concentration of 2.5%. As stated that increase in substrate concentration made more binding sites available for the enzymes to adhere to and the rate at which product formation would be achieved therefore would be faster (Dixon and Web, 1971). El-Azab (2007) reported that the cellulases reached their maximum activity at 1.5% substrate concentration.
2.4.5.5 pH stability of the cellulolytic enzymes: The pH stability of the cellulolytic enzymes and the obtained results are presented in Figure 2.20. The results show that the maximum CMCase and β-glucosidase activities are stable at pH 5.5 and FPase activity is maximum at pH 5.0. According to George et al., (2001), CMCase from culture supernatant obtained from a species of Thermomonospora presented optimum activity at pH 5.0, whereas Jang and Chen (2003) obtained a CMCase produced by Streptomyces T3-1 with optimum activity at pH 7.0. The effect of pH on enzyme stability may be due to denaturation of the enzyme (Kalra and Sandhu, 1986).

![Fig. 2.20. pH stability of the cellulolytic enzymes.](image)

2.4.5.6 Thermostability of the cellulolytic enzymes: Stability and the activity of the enzymes at higher temperature is important aspect in the industrial point of view. Thermostability is considered very important for the selection of enzymes for industrial use. Thermal stability results of Cladosporium sp. NCIM 901 (Figure 2.21) FPase indicated that 85.4, 78.3, 24.6 and 18.21% of its activity was retained at 40, 50, 60 and 70°C, respectively after 30 min incubation. The relative enzyme activity of CMCase presented in Figure 2.22 was 91.2, 88.4, 42.4 and 24.2% at 40, 50, 60 and 70°C, respectively. However the β-glucosidase in Figure 2.23 showed relative enzyme activity of 88.4, 80.4, 28.6 and 20.4% at 40, 50, 60 and 70°C, respectively. Hashino et al., (1999) reported that, eighty percent residual activity after 30 min incubation at 45°C with Streptomyces endoglucanase. Lima et al., (2005) stated that the crude enzyme from S. drzdowiczii was able to retain 40% residual CMCase activity at 50°C after 2 h, but only 20% after 8 h incubation. Many enzymes are adversely affected by high temperatures and denatured at high temperature by losing their activity.

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For the screening and production of cellulolytic enzymes, some fungal strains were isolated from soil and plant litter samples. In this study the dominant fungal strain both in soil and litter samples was *A. niger* strain, along with other fungal strains which were less in incidence in both soil and plant litter samples. The procured strain like *Cladosporium* sp. NCIM 901 also screened for their cellulolytic ability. All the strains were grown on selective CMCagar medium for identification of cellulolytic clear zones. Two strains *A. niger* (ANI1), *A. niger* (ANI2) isolated from soil and plant litter and *Cladosporium* sp. have shown cellulolytic activities in the Congo red medium with clear zones (Figure 2.1). Based on formation of clear zone diameter, the *Cladosporium* sp. was selected for further screening studies.

In secondary screening, the *Cladosporium* sp. NCIM 901 was subjected to FPase, CMCase and β-glucosidase production using solid state and submerged fermentation methods to identify the strains with high maximal FPase, CMCase and β-glucosidase production on sugarcane bagasse. The fungal strain showed high enzymatic activity in solid state fermentation than in submerged fermentation. Hence the other fermentation parameters in SSF fermentation were studied. In general, fermentation is dependent on many physiological and biochemical variables. For better production of the desired product by any type of fermentation, it is necessary to study the influence of these variables on the production of FPase, CMCase and β-glucosidase. Among the various parameters, the foremost one studied was the type of fermentation, namely solid state fermentation or submerged fermentation. Solid state fermentation enables the maximum cellulolytic enzyme production by *Cladosporium* sp. than in submerged fermentation.

Trejo-Hernandez et al., (1991) and Solis-Pereyra et al., (1993) concluded that higher production in solid state fermentation is due to the fact that catabolic repression is less affected by solid state fermentation than by submerged fermentation. Similarly, Ramesh and Lonsane (1991) found that α-amylase produced by *Bacillus licheniformis* was less affected by catabolic repression and in final product concentration when produced by solid state fermentation than submerged fermentation. The quantity of enzyme per litre of the extract from solid state fermentation was 10 times more than with the submerged fermentation technique. However, when the same strain and identical substrates were used the yield per gram of carbon substrate was about 3 times higher for submerged fermentation (Ghildyal et al., 1985).
The pelleted growth of the fungal mycelium in submerged fermentation has the limitation of nutrient assimilation and affects the biomass production (Ward, 1989) and Alana et al., (1990) found that the production of pectin lyase was higher in surface bran culture than submerged fermentation.

By correlating the results of the present as well as the previous studies, it is clear that production of FPase, CMCase and β-glucosidase by fungi and by Cladosporium sp. NCIM 901 is higher in solid state fermentation and is less affected by catabolic repression. The product formed in solid state fermentation is more concentrated than submerged fermentation and the solid substrate provides a better ground for filamentous growth of the fungus as compared to submerged fermentation, where only pelleted growth of the fungal mycelium occurs due to agitation. Thus the fungal strain Cladosporium sp. NCIM 901 was selected and used for further studies.

The procured strain Cladosporium sp. possesses the ability to produce the cellulolytic enzymes on lignocellulosic substrates. However, the fungal strain gave better enzyme production in SSF fermentation than SmF fermentation. The pH and thermal stability of the enzymes were maximum at 5.5 and 50°C, respectively. However the strain gave higher CMCase activities than the other enzymes of cellulolytic complex. CMCase was the first enzyme released in accordance with the availability of high cellulosic content of the substrate. Finding optimum conditions of the enzymes is essential in order to utilize them in various industries.
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