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IMPACT OF BIOPROCESS PARAMETERS ON CELLULASE PRODUCTION BY *PURPUREOCILLIUM LILACINUM* ISOLATED FROM FOREST SOIL

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ABSTRACT

Searching for novel microorganisms and accurate assessment of the steps are essential approaches to trim the costs of disparate processes using industrially important enzymes. This study was aimed at the exploration of various culture parameters involving production of cellulase (exo and endo-1,4,β-D-glucanases) using fungal culture *purpureocillium lilacinum* by shake flasks culture method. Optimum pH, temperature, incubation time, inoculum volume and agitation rate was revealed to be 5.5, 30° C, 7 days, 2 ×10 ^{6} spores/ml and 150 rpm respectively. Various carbon sources used in this study, 3 % xylose (w/v) was proved to be the best source for maximum FPase (Filter paperase-1.15 IU/ml) and CMCase (carboxy-methyl cellulase-2.09 IU/ml) production. Ammonium sulphate with 0.2% (w/v) showed higher titres of FPase (1.11 IU/ml) and CMCase (1.90) IU/ml) production compared to the remaining nitrogen sources. Addition of Tween-80 at 0.02% (v/v) to the fermentation medium favoured the production of FPase (1.14 IU/ml) and CMCase (1.77 IU/ml) compared to rest of the non-ionic surfactants.

KEY WORDS: **Cellulases, fungal culture, forest soil, Czapek-Dox medium, optimal conditions.**

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INTRODUCTION

During the late 20th century, progressing market trends in enzyme fermentation technology has made tremendous development. The microbial originated enzymes are fruitful in the food processing and it is not only worthwhile quantitatively but also qualitatively[1]. Cellulases are among such enzymes that are achieving popularity in this regard. Biotechnological potential of cellulases in various manufactories such as food, feed, biofuel, brewery, textile, pulp and paper, waste management, pharmaceutical and agriculture has been the hidden agenda for energetic research focus on cellulases for past several decades[2]. The most copius organic compound in the world is cellulose that assures a type of sustainable energy that human beings can easily exploit[3]. It is a major polysaccharide constituent of the plant cell walls. It is a linear polymer of 8000-12000 glucose units linked via β-1,4 glycosidic bonds, whose natural degradation represents an important part of the carbon cycle within the biosphere. In the present techno- economic era, procurance of energy is one of the major problems which humanity is facing. All the waste cellulose is a potential source of energy and food[4]. The breakdown of cellulose into sugar can be achieved by acid hydrolysis as well as by enzymatic hydrolysis. But enzymatic hydrolysis which produces fewer by-products and proceeds under milder condition is mostly preferred[5]. Cellulase is considered a potential tool which catalyzes the hydrolysis of cellulose and related oligosaccharide derivatives, for industrial saccharification of cellulosic biomass[6]. Fungi are one of the most important groups of microorganisms in the activity of decomposition of organic matter due to their proficiency in degradation. This activity occurs mainly through their mycelium or vegetative phase. During vegetative and reproductive phases, the generation of biomass depends on the production of extracellular enzymes, which are crucial components of the deterioration of substrates, chiefly lignocelluloses[7]. Most commonly investigated cellulolytic organisms include: Fungal species- *Humicols, Trichoderma, Penicillium, Aspergillus; Bacteria- Cellulomonas, Pseudomonads, Bacilli and Actinomycetes- Streptomyces, Actinomucor*[8]. Growth period of bacteria on lignocellulose is less than that of fungi and the half-baked cellulase system makes bacteria less efficient for large scale cellulase production compared to fungi[9]. Most commercial enzymes are obtained by submerged fermentation, as the yields are higher and the costs and risks of contamination are lower. Thus, the main advantages of the submerged processes are ease in controlling the physicochemical process, greater efficiency of nutrient absorption, and excretion of metabolites through the cells, leading to lower process times and consequently, productivity gains[10]. The physico-chemical characteristics of the culture media are of fundamental importance, not only for cell growth but also for the yield of a product. This is because the cells are able to respond to physical and chemical stimuli from the external environment through biochemical mechanisms that regulate gene expression and physiology of the organism and, by extension, its performance in the formation of the target product[11]. The physical parameters like temperature, pH and incubation time, major components of production medium like carbon and nitrogen sources were found to be critically affecting the cellulase production hence need to be optimized for every isolate[12, 13]. Hence the present study was carried out to optimize the culture parameters for enhanced cellulase production by *Purpureocillium lilacinum* isolated from forest soil.

MATERIALS AND METHODS

Microorganism

The fungal culture *Purpureocillium lilacinum* (NCBI accession number: KT387301) used in the present study was isolated from forest soil, Talakona, Chittoor District, Andhra Pradesh, India.

Fungal spore inoculum preparation

The fungal spore inoculum was prepared for cellulase production from 7 days old culture slants. Sterile distilled water (2ml) was added to each fungal agar slant and shaken vigorously for preparing uniform suspension. Inoculum density was adjusted to 2×10^6 spores/ml using haemocytometer. The same inoculum size was used throughout the study.

Effect of pH

The influence of medium pH on production of cellulase by *Purpureocillium lilacinum* was studied on Czapek-Dox medium amended with 1% cellulose (substrate) with various pH ranges from 4.0 -6.5. The fermentation was carried out at 25°C for 7 days on rotary shaker at 120 rpm.

Effect of temperature

To check the effect of temperature on production of cellulase by *Purpureocillium lilacinum,* Czapek-Dox broth amended with 1% Cellulose was taken in separate Erlen -Meyer flasks and incubated at different temperatures of 25, 30, 35, 40°C. The fermentation was carried out for 7 days on rotary shaker at 120 rpm.

Effect of incubation time

The Czapek-Dox broth was inoculated with spores of *Purpureocillium lilacinum* and production of exo and endo - 1,4-β-D-glucanases was checked at intervals of 3, 5, 7 and 9 days on rotary shaker at 120 rpm at 25 °C.

Effect of inoculum size

To know the impact of fungal inoculum size on exo and endo - 1,4-β-D-glucanases production, Czapek-Dox broth was inoculated with four different inoculum sizes 1.0×10^6, 2.0×10^6, 3.0×10^6, 4.0×10^6. The inoculated flasks were maintained at 25°C for 7 days on rotary shaker (120 rpm).

Effect of agitation rate

To identify the suitable rotational speed that favours cellulase, flasks inoculated with *Purpureocillium lilacinum* were maintained on rotary shaker at 90, 120, 150 and 180 rpm. Incubation was carried out for seven days at 25°C.

Effect of carbon sources

To examine the effect of various carbon sources on exo and endo - 1,4-β-D-glucanases production, Czapek-Dox broth was taken in separate Erlen -Meyer flasks and different carbon sources which includes xylose , fructose, glucose, maltose, galactose, lactose and

cellulose were added at 3% level replacing the original carbon source in the fermentation medium. The flasks were incubated for 7 days on rotary shaker

Effect of nitrogen sources

To test the influence of different nitrogen sources on exo, endo - 1,4-β-D-glucanases production by *Purpureocillium lilacinum,* Czapek-Dox broth was supplemented with different nitrogen sources which includes Ammonium sulphate, Potassium nitrate, Ammonium nitrate, Yeast extract, Peptone, Malt extract, Tryptone and Beef extract at 0.2% level replacing the prescribed nitrogen sources in the fermentation medium. The fermentation was carried out at 25°C for 7 days.

Effect of surfactants

Cellulase production was attempted from *Purpureocillium lilacinum* grown on Czapek-Dox broth supplemented with 0.01 -0.04 % of Triton-X 100, Tween-80, Tween-20 and EDTA. The fermentation was carried out at 25°C for 7 days on rotary shaker at 120 rpm.

Cellulase assays

Filter paper activity (FPA)

Filter paper activity of the culture filtrates was analyzed according to Ghose method[14] .Whattman filter paper strips of 1×6 cm (50 mg) were suspended in 1.0 ml of 0.05M sodium citrate buffer (pH 4.8) at 50 °C in a water bath. An aliquot of 0.5 ml of culture filtrate with appropriate dilution was added to the reaction mixture and incubated for 60 minutes at 50°C. After incubation, the liberated reducing sugar was estimated by the addition of 3, 5-dinitrosalicylic acid[15.] After cooling, the color developed in tubes was read at 540 nm by using the spectrophotometer (Thermo scientific). Appropriate control without enzyme was simultaneously run. Activity of cellulases was expressed in filter paper units. One filter paper unit (FPU) was defined as the amount of enzyme required to release one micro mole of reducing sugar from substrate/ml /min.

Endoglucanase assay

Activity of endoglucanase in the culture filtrates was quantified by Ghose method[14]. The reaction mixture with 1.0 ml of 1% carboxy methyl cellulose in 0.2 M acetate buffer (pH 5.0) was pre-incubated at 50°C in a water bath for 20 minutes. An aliquot of 0.5 ml of culture filtrate with appropriate dilution was added to the reaction mixture and incubated at 50°C in water bath for 30 minutes. Appropriate control without enzyme was simultaneously run. The reducing sugar produced in the reaction mixture was determined by Miller method[15]. 3, 5-dinitro-salicylic acid reagent was added to aliquots of the reaction mixture and the color developed was read at wavelength 540 nm by using the spectrophotometer (Thermo scientific). One unit (IU) of endoglucanase activity was defined as the amount of enzyme required to release one micromole of reducing sugar from substrate/ml /min.

RESULTS AND DISCUSSION

Cellulases are inducible enzymes and their induction and activity depends on the nature of substrate. Enzymes of microbial origin is greatly influenced by physical factors such as temperature, pH, incubation time and inoculum density as well as media components, especially carbon and nitrogen sources. The production of enzymes in inexpensive and optimized media on large scale is critical for the process to be commercially viable. So the influence of various physico-chemical parameters such as pH, temperature, incubation periods, inoculum size, agitation rate, carbon and nitrogen sources and surfactants were studied.

Effect of pH

Initial pH of the medium exerts a strong influence on the mycelial growth and enzyme production. It also has a role in transport of various components across the cell membrane. In the present study the effect of different pH ranges on cellulase production by *Purpureocillium lilacinum* was studied and represented (fig.1). In this Cellulase activity increased with increase in pH up to 5.5 and later declined at higher pH . Higher Exo and Endo-1,4-β-D-Glucanases of 1.22 IU/ml, 1.82 IU/ml was produced at pH 5.5 respectively. Similarly, *Trichoderma viride* produced exoglucanase with 2.16 U/ml and endoglucanase 1.94 U/ml at pH 5.5[16]. Optimum pH for cellulase production by *A. terreus and Trichoderma reesi* was reported at pH 5.5[17,18] . Similarly, *Aspergillus* and *Fusarium* sps. yielded maximum endoglucanase when the medium was maintained at pH 5-5.5[19] .The above results were in agreement with the present study.

Effect of temperature

Temperature is an important physiological parameter that affects microbial growth and has profound effect on metabolic activities of microorganisms. The effect of temperature on cellulase production was studied and the results were depicted (fig.2). Improvement in the cellulase activity was recorded up to 30°C and decreased with further increase in temperature. In the current investigation, the optimum temperature for FPase (1.14 IU/ml) and CMCase (2.03 IU/ml) activities was found to be 30°C. Maximum enzyme activity by *Aspergillus heteromorphus* was observed at 30°C, yielding filter paper activity of 2 IU/ml and CMCase activity of 83 IU/ml[20]. Cellulase production by *Fomitopsis* sp. under SSF was maximum at 30°C[21]. Cellulase production was increased by *Aspergillus niger* up to 30°C and thereafter the production of enzyme declined[22]. These reports are in corroboration with the present findings.

Effect of incubation time

The results obtained with varying incubation time intervals revealed that the production of FPase and CMCase by *Purpureocillium lilacinum* increased with increase in incubation time and optimum enzyme recovery period was identified to be 7 days (fig.3). Higher titres of FPase of 0.89 IU/ml and CMCase of 1.49 IU/ml recorded at 7th day of interval. Further increase in incubation caused decrease in the enzyme production. Similarly, maximum cellulase production by *Trichoderma viride* and *Aspergillus niger* was achieved after 7 days of incubation under ssf and smf respectively[23, 24]. The suitable incubation period for higher cellulase activity by the isolate *Trichoderma* sp. was 6 days[16]. Prolonged incubation would led to depletion of nutrients, cell death, proteolytic digestion or thus may be because of the denaturation of the

enzymes at various pH which is a common phenomenon during fermentation due to the release of acidic by-products in media[25, 26] .

Effect of inoculum size

The fungal Inoculum volume is an important biological criterion as it ascertains both biomass as well as cellulase production. In the present study, effect of different sizes of inoculum was explored (Figure 4). Higher FPase and CMCase activities of 0.77 IU/ml and 1.39 IU/ml were noticed when 2.0 $\times 10^6$ spores/ml was used. Further increase in inoculum volume caused reduction in fungal enzyme production. Similarly, *Aspergillus niger* gave maximum cellulase production on Czapek-Dox medium when inoculum size of 2.0 $\times 10^6$ spores was used[24]. Mutant *Aspergillus niger at* 2.0 $\times 10^6$ spore density gave higher titers of cellulase production on pea seed husk[27].These reports were in similarity with the present results. In contrast, cellulase production was found to be optimal when flasks were inoculated with 4% of inoculum size by A. *Flavus* and *A.niger*[28, 29]. 5% (v/w) of inoculum size was found to be optimal for cellulase activity of 0.0413 U when fermented oil palm biomass by *Trichoderma harzianum*[30]. The decrease seen with larger inoculum volume could be due to the clumping of cells which could have reduced sugar and oxygen uptake and enzyme release[31].

Effect of agitation rate

The shaking speed increases the dissolved oxygen in the culture, which is necessary for cell membrane components and uniform distribution of the medium contents such as nutrients and catabolites[32]. As regards agitation, variation of rotational speed from 90 to 180 rpm affected enzyme production (fig.5). Accelerated FPase (1.17 IU/ml) and CMCase (2.25 IU/ml) production was recorded at 150 rpm. Similarly, optimum rotational speed for CMCase production by *Aspergillus hortai* was found to be 150 rpm[33]. In contrast, cellulase production in agitated shake flask fermentation was higher at agitation rate of 200 rpm [17]. Agitation rates below or above 150 rpm resulted in low cellulase yields. The reason may be the difficulty in maintaining sufficient dissolved oxygen (DO) level for cell growth. Higher agitation rates resulted in a slight decline in enzyme levels, which could be due to mycelial damage[34] .

Effect of carbon sources

The good utilizable form of carbon source by *Purpureocillium lilacinum* was assessed and represented (fig.6). Out of the eight carbon sources used in the present study, 3 % xylose was proved to be the best for maximum FPase (1.15 IU/ml) and CMCase (2.09 IU/ml) production. fructose stands next to xylose as a good carbon source for FPase (1.00 IU/ml) and CMCase (1.89 IU/ml) activities. Lactose, galactose and glucose follow fructose in this regard. Maltose and cellulose were noticed as poor carbon sources for FPase and CMCase production. The combination of cellulose with xylose led to the best production of FPase with activities of 7.02, 24.86 and 23.48 FPU L-1, respectively to the *Trichoderma* strains 94144, 97177 and Tm3 in 10 days of fermentation[35]. The same trend was also found in the production of CMCase. These findings are in matching with the present results.

Effect of nitrogen sources

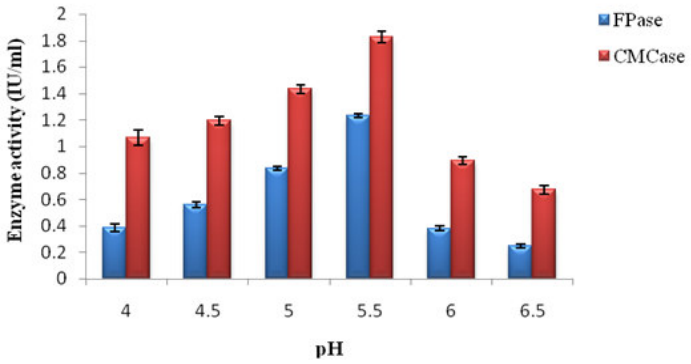
Necessity of specific nitrogen source varies from organism to organism or even among the same species for maximum enzyme production[36]. Of the entire tested nitrogen sources, supplementation of Ammonium sulphate had the highest impact on FPase (1.11 IU/ml) and CMCase (1.95 IU/ml) production (fig.7). Potassium nitrate exhibited slightly lower FPase (0.98 IU/ml) and CMCase (1.86 IU/ml) activities compared to NH_4SO_4. Yeast extract served as good nitrogen supplement after KNO_3. Peptone, beef extract, tryptone, malt extract supported poor exo and endo- 1, 4-β-D-glucanases production by *Purpureocillium lilacinum.* Inorganic nitrogen sources like NH_4SO_4 supported maximum production of FPase (0.926 U/ml/min) CMCase (1.68 U/ml/min)[37]. Combination of 20% apple waste with 0.3% ammonium sulphate gave maximum cellulase activity of 2.28 IU/ml by *Aspergillus fumigates* JCF[38]. It was reported that good cellulase yield can be obtained with ammonium sulphate as the nitrogen source[39] . When compared to organic and rest of the inorganic nitrogen sources NH_4SO_4 acted as the best nitrogen source[40]. This is in absolute agreement with the present findings.

Effect of surfactants

In the present study the impact of different surfactants on cellulase production was studied and narrated (fig.8, fig.9). All the surfactants used in the present study improved cellulase production except EDTA when compared to the control. 0.02 % tween-80 supported enhanced FPase and CMCase activities of 1.05, 1.57 IU/ml respectively. Triton-X 100 stood next to tween-80 in favouring FPase and CMCase production (1.14, 1.77 IU/ml respectively). Tween-20 (0.02%) followed triton-X 100 in elevating levels of cellulase production. Cellulase production was enhanced with the addition of 0.2 - 1% and (v/v) of Tween-80 by *Trichoderma reesei* strain QM-9414 and *Streptomyces flavogriseus* respectively[41]. The production of cellulase by A.*terreus* was increased by two-fold with the addition of tween-80 (2ml L-1) as surfactant, as compared to fermentation without surfactant[17]. These results were in matching with the present findings.

Figure 1

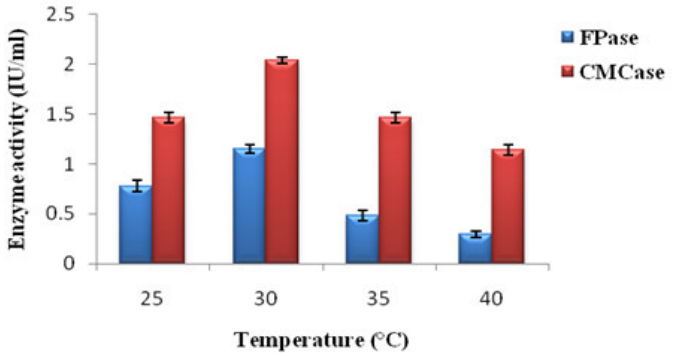
Effect of PH on production of exo and endo - 1,4-β-D-glucanases by Purpureocillium lilacinum



**Values represented in the figure are the average mean of three replicates with standard deviation.*

Figure 2

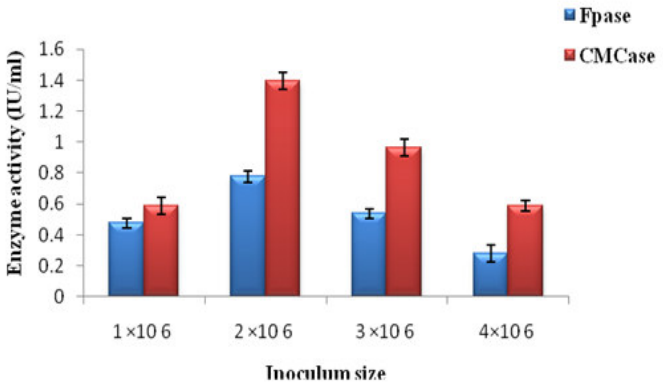
Effect of temperature on production of exo and endo - 1,4-β-D-glucanases by Purpureocillium lilacinum



**Values represented in the figure are the average mean of three replicates with standard deviation.*

Figure 3

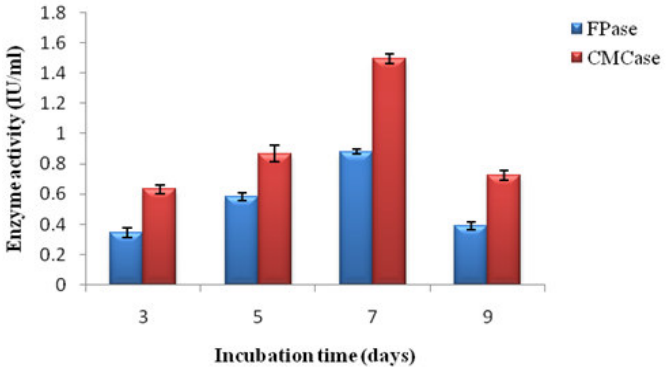
Effect of incubation time on production of exo and endo - 1,4-β-D-glucanases by Purpureocillium lilacinum



**Values represented in the figure are the average mean of three replicates with standard deviation.*

Figure 4

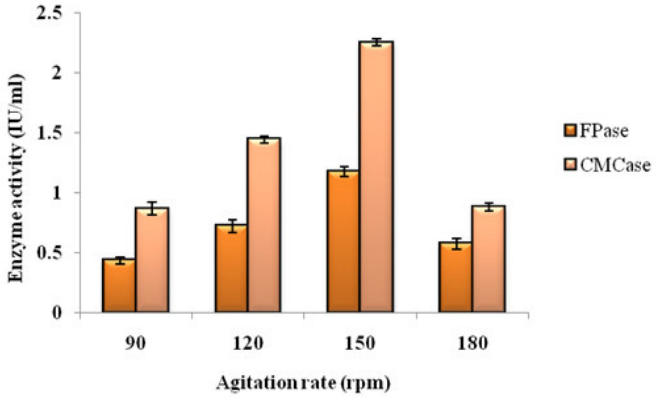
Effect of inoculum size on production of exo and endo - 1,4-β-D-glucanases by Purpureocillium lilacinum



**Values represented in the figure are the average mean of three replicates with standard deviation.*

Figure 5

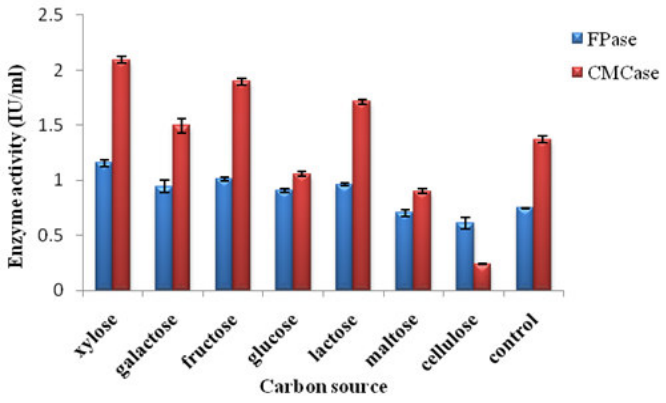
Effect of agitation rate on production of exo and endo - 1,4-β-D-glucanases by Purpureocillium lilacinum



**Values represented in the figure are the average mean of three replicates with standard deviation.*

Figure 6

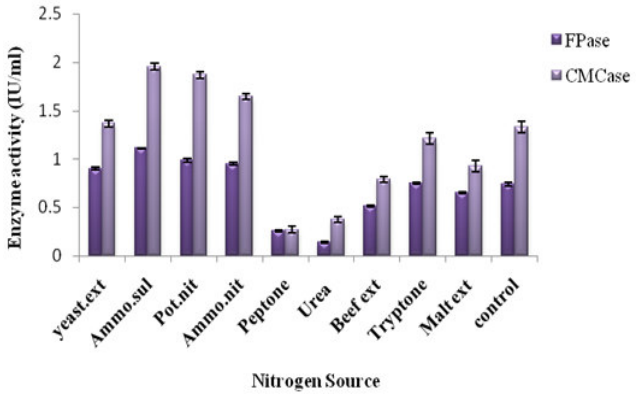
Effect of Carbon sources on production of exo and endo - 1,4-β-D-glucanases by Purpureocillium lilacinum



**Values represented in the figure are the average mean of three replicates with standard deviation.*

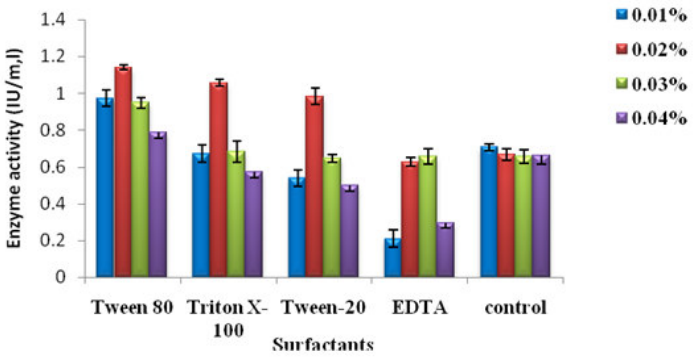
Figure 7

Effect of Nitrogen sources on production of exo and endo - 1,4-β-D-glucanases\ by Purpureocillium lilacinum



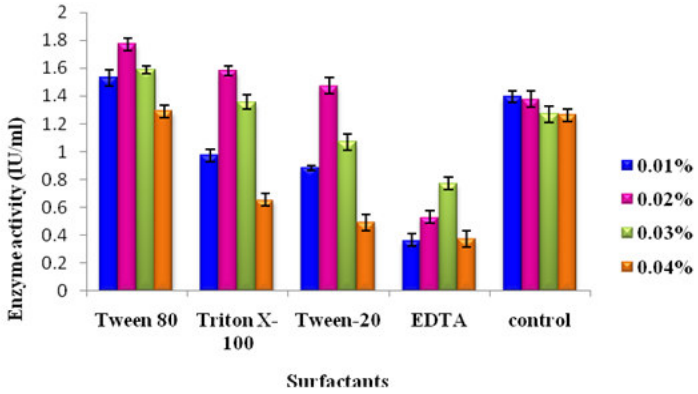
Values represented in the figure are the average mean of three replicates with standard deviation.

Figure 8
Effect of surfactants on production of exo - 1,4-β-D-glucanase (Fpase) by Purpureocillium lilacinum



Values represented in the figure are the average mean of three replicates with standard deviation.

Figure 9
Effect of surfactants on production of endo - 1,4-β-D-glucanase (CMCase) by Purpureocillium lilacinum



Values represented in the figure are the average mean of three replicates with standard deviation.

CONCLUSION

The effect of various bioprocess parameters on cellulase production by *Purpureocillium lilacinum* was studied in the present investigation. Physical factors, pH 5.5, temperature 30° C, incubation period 7 days, inoculum size 2.0 $\times 10^6$ spores/ml, 150 rpm agitation speed was found to be optimal for cellulase production. Xylose as a carbon source induced the higher titers of FPase and CMCase activities. Among the nitrogen sources used in this study ammonium sulphate gave maximum cellulase production. Addition of 0.02% tween – 80 favoured elevated level of enzyme production. This could be the first report on cellulase production by *Purpureocillium lilacinum* isolated from forest soil. Thus, the findings obtained in this study allowed us to conclude that the *Purpureocillium lilacinum* isolated from forest soil is a potential candidate for cellulase production. The efficiency of the fungal culture could be further increased by genetic manipulation studies.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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