

ORIGINAL ARTICLE

Cellulolytic Enzymatic Activity of Soft Rot Filamentous Fungi *Paecilomyces variotii*

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ABSTRACT

Cellulases are a group of hydrolytic enzymes capable of hydrolyzing the most abundant organic polymer i.e. cellulose to smaller sugar components including glucose subunits. Cellulases have enormous potential in industries and are used in food, beverages, textile, laundry, paper and pulp industries etc. This study was aimed to investigate the cellulolytic enzymatic activity of soft rot wood degrading filamentous fungi *Paecilomyces variotii* 103-7 strain from naturally degraded wood. Furthermore, optimal cultural condition for enzyme activity and induction of enzyme synthesis were also determined. The mycelial plugs of these isolate was grown in submerged cultures of modified czapeck dox liquid medium containing the appropriate carbon source (cellulose, CMC, glucose and sucrose) and nitrogen source (peptone, yeast extract, urea and sodium nitrate) at different temperature, pH and incubation period. In fermentation with the addition of Tween 80 (0.1%) as surfactant, the production of cellulase was increased by two fold as compared to fermentation without surfactant. The enzyme activity assay was carried out on the culture filtrate obtained. Cellulase production in agitated shake flask fermentation at 150 rpm was two times higher as compared to fermentation in static flask. This study showed that the wood degrading soft rot fungi *Paecilomyces variotii* is capable of producing cellulases in submerged cultures.

Key Words: Cellulase enzyme, pH, Temperature, *Paecilomyces variotii* 103-7, Submerged fermentation.

INTRODUCTION

Plant biomass is made up of mostly polysaccharides. The most abundant organic polysaccharide in the biosphere is cellulose [1] and is the major polysaccharide found in the plant cell wall giving the structural rigidity and strength to plants. Cellulose is an unbranched glucose polymer composed of β -1,4-glucose units linked by a β -1,4-D-glycosidic bond. A number of plant pathogenic organism are capable of producing multiple groups of enzymes, called cellulase that act to hydrolyze the β -1,4-D-glycosidic bonds within the cellulose molecules [2, 3].

The cellulases are classified into three types:

- (i) Endoglucanase (EC 3.2.1.4)
- (ii) Exoglucanase (EC 3.2.1.74)
- (iii) β -glucoside glucohydrolases (EC 3.2.1.21)

Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends [4]. Exoglucanases act by hydrolyzing the reducing or non-reducing ends of cellulose polysaccharide chains, liberating either glucose or cellobiose as major products [5]. β -glucosidases hydrolyze soluble celloextrins and cellobiose to glucose.

The potential biotechnological applications of these enzymes in food and pharmaceutical industries, essential oils, pulp and paper industries, biomass conversion of agricultural and industrial wastes to chemical feedstock, biofuels, animal feeds and pollution control are well documented [6].

Cellulases are produced by a wide range of microorganism particularly fungi. *Paecilomyces* is a genus of nematophagous fungus which kills harmful nematodes by pathogenesis, causing disease in the nematodes. Therefore the fungus can be used as a bionematicide to control nematodes by

applying it to soil. *Paecilomyces variotii* are the member of ascomycetes (soft rot) and versatile filamentous fungi found in soil, water, air, fungal degraded wood, plant residues, municipal soil waste etc. These species also called as soft rot fungi because they possess the ability to degrade cellulose. These fungi produce many useful enzymes like- amylases, cellulose, amylo-glucoasidases, pectinase, invertase, lactases and glucoamylase.

In this investigation, a cellulose producing strain of *P. variotii*, isolated from degraded wood, were subjected to optimization of media and cultivation parameters for cellulose production.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were of analytical grade. Potato Dextrose Agar and crystalline cellulose were obtained from Merck, Germany. Carboxymethyl cellulose was obtained from Whatman Ltd, England. All other chemicals and reagents were obtained from Sigma Chemicals co. Ltd, England. The strain of *P. variotii* 103-7 was isolated from naturally degraded wood in Gwalior. It was grown at $28 \pm 2^\circ\text{C}$, maintained on potato dextrose agar slants containing potato 20 % (w/v), dextrose 2 % and agar 1.5% at $4 \pm 2^\circ\text{C}$. The slants were freshly made once a month.

Cultivation and enzyme production

The extracellular enzymes were produced through submerged fermentation. The organism was grown on modified czapek dox liquid medium containing (g/L): NaNO_3 , 3.0g; KCl, 0.5g; KH_2PO_4 , 1.0g; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01g and 10.0 g of cellulose. One liter of the media was supplemented with 1.0ml of trace solution containing (g/L) ZnSO_4 , 1.0g and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.5g. The pH of the medium was adjusted to 5.6 prior to sterilization. 250 mL Conical flasks containing 100 mL of respective media were autoclaved at 121°C for 15 minutes, cooled and inoculated with 1.0 mL of spore suspension in 0.1% Tween 80 ($2-4 \times 10^6$ spores per mL) of the pure fungal isolates. The cultures were incubated for 72 h with continuous agitation at 150 rpm. Cells were harvested by centrifugation at $10000 \times g$ rpm for 20 minutes at 4°C using ultracentrifuge. The cell free culture supernatant was used as source of crude extracellular enzyme. Three replicates were prepared for each experiment.

Measurement of cellulolytic enzymatic activity

Filter paper activity (*FPase*) for total cellulose activity in the culture supernatant was determined according to the standard method [7]. Aliquots of appropriately diluted cultured supernatant as enzyme source were added to Whatman filter paper No. 1 strip (1×6 cm; 50 mg) immersed in 1 mL of 0.05 sodium citrate buffer of pH 5.0. After incubation at $50 \pm 2^\circ\text{C}$ for 1 h, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method [8]. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 μM of reducing sugar from filter paper per ml per minute. Carboxymethyl cellulose (*CMCase*) activity was measured using a reaction mixture containing 1 ml of 1% carboxymethyl cellulose (CMC) in 0.5 M citrate acetate buffer (pH 5.0) and aliquots of suitable diluted filtrate. The reaction mixture was incubated at $50 \pm 2^\circ\text{C}$ for 1 h, and the reducing sugar produced was determined by DNS method. β -glucosidase activity was assayed by the method of Pointing [9]. One unit (IU) of endoglucanase activity was defined as the amount of enzyme releasing 1 μmole of reducing sugar per minute.

Optimization of culture conditions for cellulolytic enzymatic activity

Effect of incubation period

Fermentation period is important parameter for enzyme production by *P. variotii*. In this study, experiments fermentation was carried out up to 7 days and production rate measured at 24 h intervals.

Effect of pH and temperature

The most suitable pH of the fermentation medium was determined by adjusting the pH of the culture medium at different levels in the range of pH 3-8 using 1-5 M NaOH and 1 N HCl. In order to determine the effective temperature for cellulolytic activity by the *P. variotii*, fermentation was carried out at 5°C intervals in the range of 15 to 40°C .

Effect of carbon sources

Effect of various carbon compounds viz. cellulose, carboxymethyl cellulose, glucose and sucrose were used for studying. The broth was distributed into different flasks and 0.5 to 1.5% of each

carbon sources were then added before inoculation of the strain and after culture inoculation, the flasks were incubated for 7 days at 28 ± 2 °C.

Effect of nitrogen sources

In the present study, to detect the appropriate nitrogen sources for cellulolytic enzymatic activity by the *P. variotii*. The influence of peptone, sodium nitrate, yeast extracts and urea were studied. The fermentation medium was supplemented with different nitrogen compounds at 0.5 to 1.5% level, replacing the prescribed nitrogen source of the fermentation medium.

Statistical analysis

Data presented on the average of three replicates ($\pm$ SEM) obtained from three independent experiments.

RESULTS AND DISCUSSION

Effect of incubation period

The incubation period is directly related with the production of enzyme and other metabolic up to certain extent. *P. variotii* showed the most active cellulolytic species along different incubation period. *P. variotii* inoculated into modified czapek dox agar medium in 250 mL conical flask and incubated at 28 ± 2 °C for a period of 7 days. The cellulose activity was measured at regular intervals. However the maximum yield of exoglucanase (0.84 μ g/mL) and endoglucanase (0.88 μ g/mL) activity was obtained after 3 days. However maximum β -glucosidase (0.88 μ g/mL) activity was shown after 3-6 days incubation in Figure 1.

The incubation periods to achieve peak cellulose enzymatic activity by the *P. variotii* were 3-6 days which were suitable for commercial point of view [10]. It might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretory machinery of the enzymes [11].

Effect of pH

Cellulase yield by *P. variotii* appear to depend on pH value. Result illustrated by Figure 2 clearly show that cellulose production, expressed as enzyme activity, gradually increased as the pH value from 5-6 and reached its maximum at pH of 5 being exoglucanase (1.72 μ g/mL), endoglucanase (1.68 μ g/mL) and at pH 6 activity of β -glucosidase (1.79 μ g/mL) is maximum. The enzyme activity gradually increased when increasing the pH up to the optimum followed by a gradual fall in activity. It was also noted that the enzyme activity was decreased at pH 7-8. Effect of pH on cellulose production by these fungi supports the findings of Lee et al., [5] who reported that exoglucanase and endoglucanase activities exhibit a pH optimum of approximately 5, while the pH optimum of β -glucosidase was pH 6.

Effect of temperature

Like pH, temperature is also an important factor that influences the cellulose yield. Maximum enzyme production by *P. variotii* was found to be exoglucanase (1.54 μ g/mL), endoglucanase (1.95 μ g/mL) and β -glucosidase (1.88 μ g/mL) activities at temperature 30 °C (Figure 3). At higher temperature, 35 °C and 40 °C, cellulose enzyme activities was reduced as compared to that obtained at optimal temperature (30 °C). Many workers have reported that the optimal temperature for cellulose production also depends on the strain variation of the microorganism [12, 13].

Effect of carbon sources

Carbon sources play a vital role in the cell metabolism and synthesis of cellulose. The effect of carbon sources on the production of enzyme by *P. variotii* was investigated. Carbon sources tested for production of cellulase enzyme were cellulose, carboxy-methylcellulose, glucose and sucrose ranging from 0.5-1.5% (w/v). Sucrose was the most effective as a sole carbon source for the cellulolytic enzymatic activity, results increased in enzyme activity, being exoglucanase (2.60 μ g/mL), endoglucanase (2.11 μ g/mL) and β -glucosidase (2.01 μ g/mL) were obtained in culture medium containing 1% sucrose followed by cellulose, CMC and glucose (Figure-4). Among the different carbon sources used, the glucose was the second best carbon source (1%) for cellulose production by *P. variotii* followed by cellulose and CMC (Figure 4). Cellulase production commenced on reaching nitrogen limiting conditions and the yield of cellulose decreased when excess peptone was presented; various inorganic nitrogen sources have been optimized by different workers for cellulose production [14, 15].

Effect of nitrogen sources

To evaluate the effect of nitrogen source on cellulose formation, the nitrogen sources in the modified Czapek dox medium was replaced by different nitrogen sources. The nitrogen sources tested ranged from 0.5-1.5% (w/v) peptone, yeast extract, urea and sodium nitrate. The result in Figure-5 showed that a concentration of 1.0% peptone led to maximum production of cellulose enzyme, including exoglucanase (2.21 $\mu\text{g/mL}$), endoglucanase (1.93 $\mu\text{g/mL}$) and β -glucosidase (1.90 $\mu\text{g/mL}$), were yeast extract also produce second most cellulose producing nitrogen source by *P. variotii* (Fig.5). The activity of exoglucanase, endoglucanase and β -glucosidase was not detected when urea was used as a nitrogen source.

P. variotii

Cellulose is world's most abundant organic substance and comprises a major storage form of photosynthesized glucose. It is a major component of biomass energy. Because of large proportion of vegetation added to soil is cellulose therefore, decomposition of cellulose has a special significance in the biological cycle of carbon. In industry, these enzymes have found novel application in production and processing of chemicals, food and manufactured goods such as paper, rayon etc. and extraction of valuable components from plants and improvement of nutritional values of animal feed [16]. Fungi are well known agents of decomposition of organic matter in general and of cellulosic substrate in particular as reported by Lynd et al., [17]. The most common and most potent cellulose producers are *Aspergillus*, *Trichoderma*, *Penicillium*, *Paecilomyces* and *Fusarium* species [18].

Many research works has been focused on the optimization of enzyme production in fungi due to the continued demand for biotechnological and industrial application of enzymes. Therefore further studies will also involve the development of mutant strains of these organisms with enhanced production of cellulase enzyme for lignocellulosic materials.

The organisms used in this study (*P. variotii*) were able to grow in the various carbon sources employed. This is an indication the cellulolytic enzymes were secreted by the isolates to depolymerize the carbon sources to simple sugars for growth, however in this present study, the enzyme production capacities of the *P. variotii* strains on four different carbon sources (CMC, Cellulose, Glucose and Sucrose) under the submerged culture conditions were comparatively examined. These observations are well agreement with the results of the present study. It is therefore evident that the presence of sucrose is responsible for the highest support for enzyme production by the *P. variotii*. Low level of cellulolytic enzymes in the presences of CMC in this study could be attributed to repression of synthesis of cellulolytic enzymes. The difference in the production of cellulolytic enzymes on a variety of lignocelluloses by different organisms could be assessed to various factors such as variable cellulose content in lignocelluloses derived from different plant sources, heterogeneity of structure and cellulolytic abilities of the organism at different degree.

In case of submerged fermentation, the maximum activity of cellulose enzyme was achieved after incubation period of 72 h. This is due to the fact that during this phase the microbes were in stationary phase. The result is in accordance with those of earlier work conducted on cellulolytic fungi by Aurangzeb et al., [19]. Further increase in the incubation period did not show any enhancement [20]. Sugar produced during fermentation may also be responsible for the feedback inhibition of the enzyme production as reported by Podukhe and Soman [21].

The optimum pH obtained from this study was in the range of those reported for cellulose production by *T. Reesei* [22]. Cellulase production by *Aspergillus niger* MS82 was maximal when the initial culture pH was adjusted to 6.0 or 7.0 [23]. On the other hand, Juhasz et al., [24] claimed that the maximum cellulose production was obtained at pH ranging from 3.0 to 5.0. Generally, the pH of the culture increased during the first two days of cellulose fermentation by fungi due to utilization of carbon and nitrogen sources for growth. After an active growth was achieved, the culture pH decreased due to the formation of carboxylic groups and carbonic acids from lignin [25]. At this stage, the fungus started to utilize the crystalline portion of cellulose and starts secreting cellulose. During the fermentation, the culture pH was reduced to acidic when cellulose was consumed by the fungi. Reduction in culture pH was due to the absorption of ammonium ions by the fungal mycelium [26]. At culture pH of below 5, inhibition of growth and inactivation of cellulases occurred. Therefore, appropriate pH control strategy is necessary for the enhancement of cellulose production.

Most work concerning the effect of incubation temperature on growth of filamentous fungi supports the finding that is within limits, increased incubation temperature results in increased growth rate [27, 28]. However, in most published works the effect of incubation temperature on cellulose is not fully discussed. Reduced activity of FPase and β -glucosidase in fermentation by *Penicillium pinophilum* strain NTG 111/6 was observed with increased in incubation temperature from 30 to 35 °C [27]. The optimum temperature (28 °C) for growth and cellulose production by *A. terreus*, as reported in previous study, was similar to those observed in *T. Ressei* RUTC30. The optimum temperature for cellulose production by *T. Ressei* was 28-30 °C [29] while the optimum temperature for cellulose production by *T. harzianum* Rut C-8230 was 28 °C [30].

Result from this study have indicated that *P. variotii* is capable of producing high activities among all the three main component of cellulase enzyme (FPase, CMCase and β -glucosidase) in submerged fermentation using modified Czapek dox liquid medium. Sucrose was the preferred carbon source as compared to glucose, CMC and cellulose similar peptone was the preferred nitrogen source as compared to urea, yeast extract and NaNO_3 for cellulase production. The preferred initial culture pH 5.0 for FPase and CMCase activity and pH 6.0 for β -glucosidase activity. The incubation period and temperature for cellulase activity by *Paecilomyces variotii* was 3 days and 30 ± 2 °C, respectively.

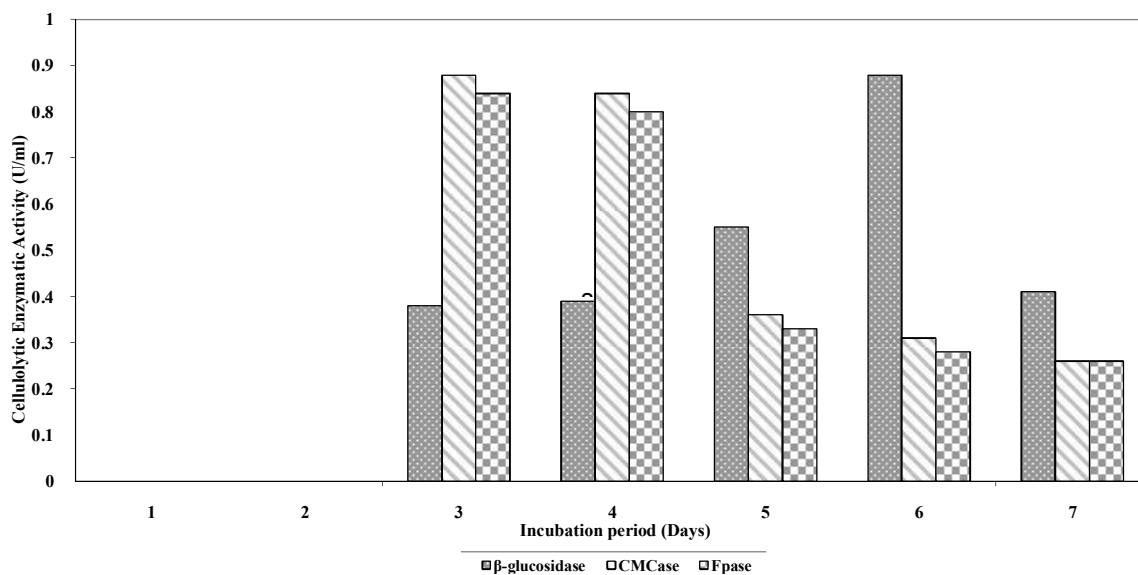


Fig. 1: Effect of incubation period in enzymatic activity ($\mu\text{g/mL}$) by *P. variotii*

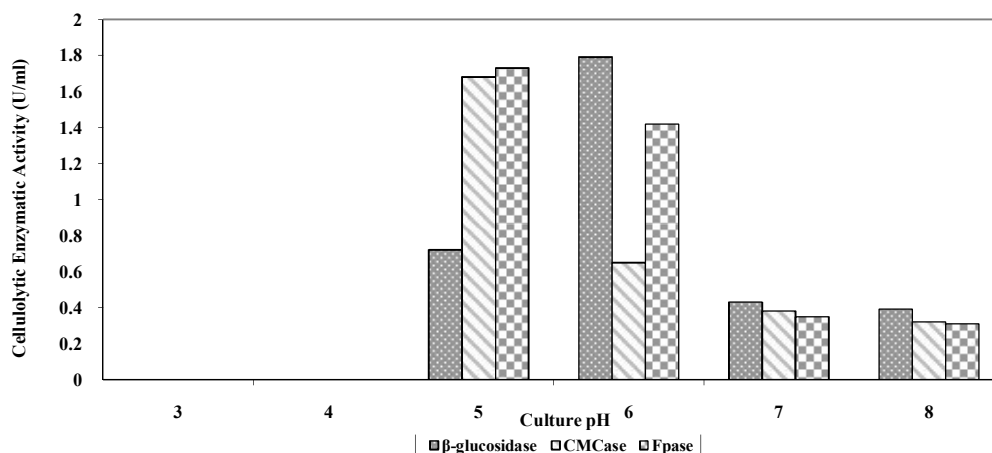


Fig. 2: Effect of pH in enzymatic activity ($\mu\text{g/mL}$) by *P. variotii*

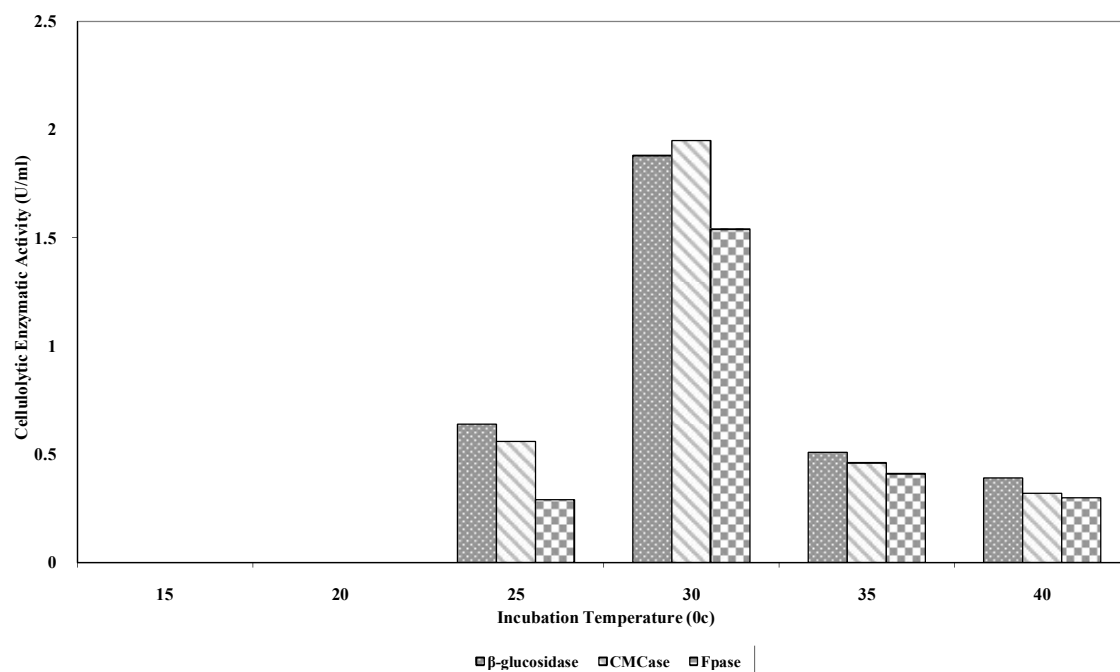


Fig. 3: Effect of incubation temperature in enzymatic activity ($\mu\text{g/mL}$) by *P. variotii*

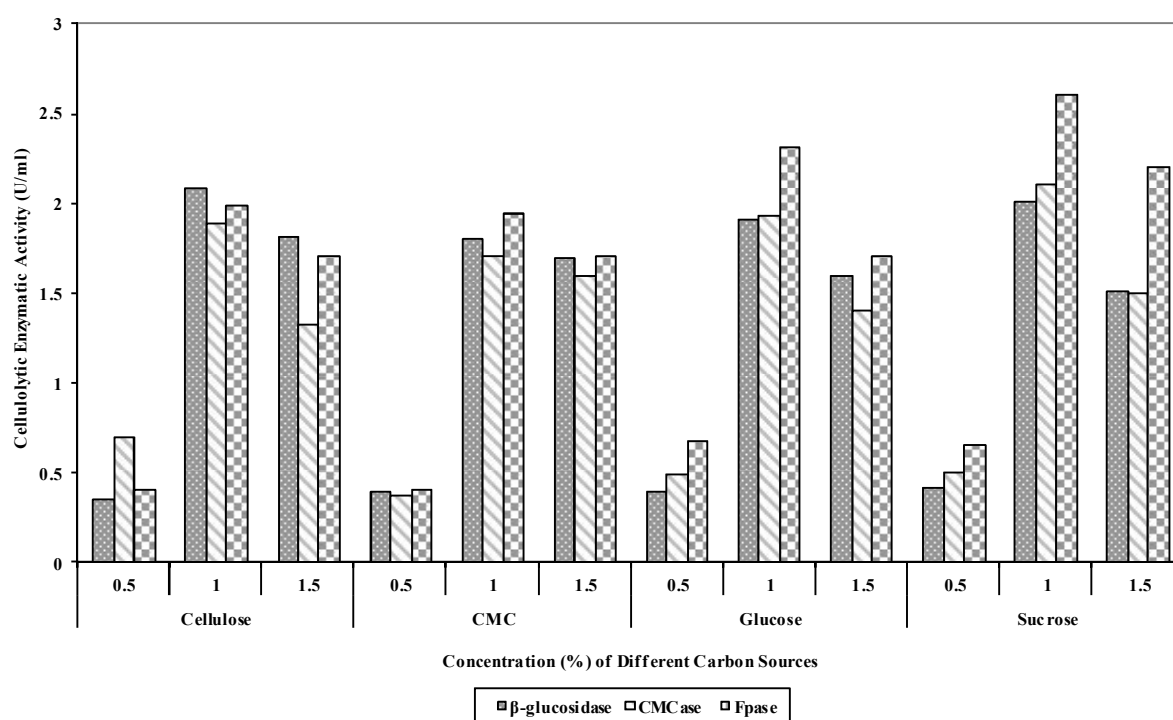


Fig. 4: Effect of carbon sources concentration (%) in enzymatic activity ($\mu\text{g/mL}$) by *P. variotii*

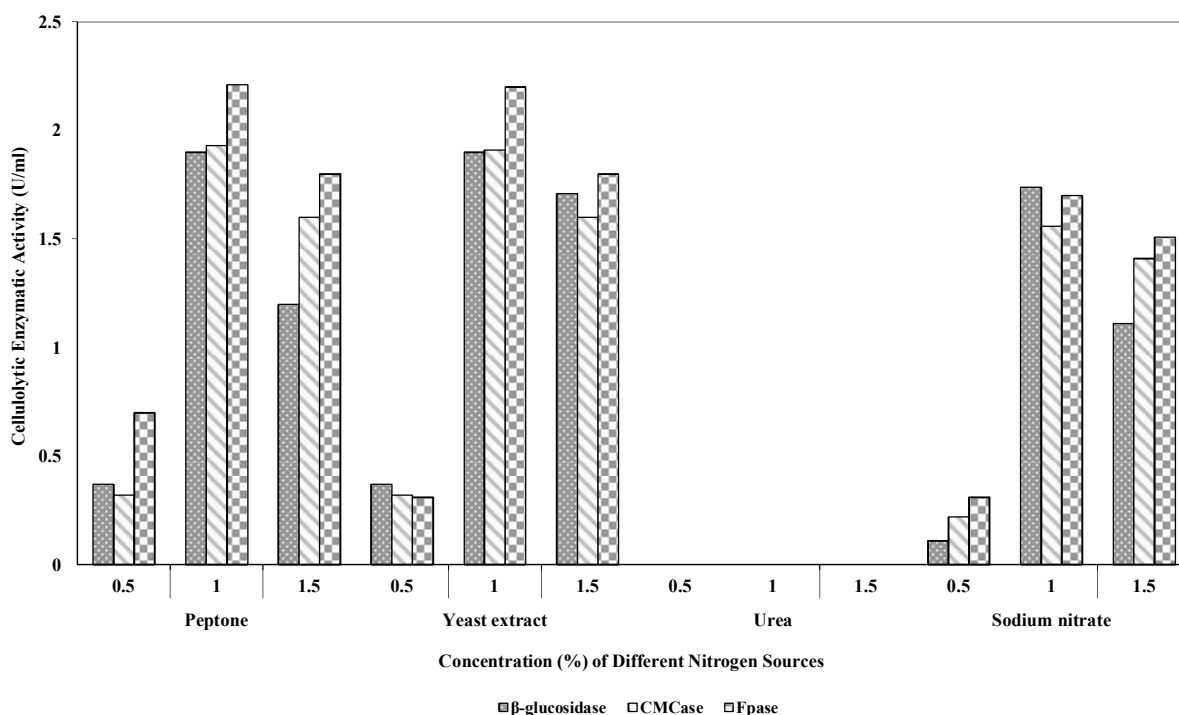


Fig. 5: Effect of nitrogen sources concentration (%) in enzymatic activity (μg/mL) by

CONCLUSION

Since this newly isolated strain (*P. variotii*) has the ability to produce high activities of all three main components of cellulase in low cost substrate, it has great potential to be used as industrial strain for cellulase production. Development of large scale fermentation process in bioreactor is the subject of our current research, emphasizing on the selection of various control strategies such as dissolved oxygen tension and pH for the improvement of the production.

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