Improved cellulase production by *Penicillium janthinellum* mutant

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Economic and sustainable production of bioethanol from biomass through enzymatic route depends on economics of cellulase availability, the key factor for the success of the technology. It has been realized that the bottleneck of the technology lies in obtaining highly efficient cellulase which could result in an economically feasible technology. In this study, we compared cellulase production by *Penicillium janthinellum* EMS UV-8 in shake flask in two different medium; modified Mandel and Weber (M & W) and the corn steep liquor (CSL) based medium. In CSL based medium, 3.02 FPU/mL was produced compared to 1.25 FPU/mL in modified M & W medium. Hence, CSL based medium was employed in bioreactor for cellulase production. In cellulase production, *Penicillium* sp. is comparable to the widely acclaimed *Trichoderma* sp. Changing the production medium from modified M & W medium to CSL based medium, increased cellulase production by two fold. In the bioreactor, controlled monitoring of DO and pH resulted in increase in the cellulase activity upto 5.44±0.3 FPU/ml at 168 h.

**Keywords**: Bioethanol, Bioreactor, Corn steep liquor (CSL) based medium, β-Glucosidase, Mandel and Weber (M & W) medium, Microbial degradation, Xylanase.

Cellulose is the most common organic polymer and is considered an inexhaustible source of raw material for different products\(^1\). It is the most abundant and renewable biopolymer available on earth and dominating waste material from agriculture\(^2\). Except few instances, cellulose is not present in free form in nature but is usually embedded in other structural biopolymer primarily as hemicelluloses and lignin\(^3\). Utilization of these raw materials by hydrolyzing them via microbial degradation and fermenting the resulting sugars into desired products is the most efficient and accepted technology presently, mainly in the context of environmental concerns.

Microbial degradation of lignocellulosic biomass and its downstream products is accomplished by concerted action of several enzymes, the most prominent being cellulases, produced by several microorganisms. Filamentous fungi are most exploited microorganisms for cellulose production *Penicillium*, *Trichoderma* and *Aegopterillus* are among the potential source of cellulase. Cellulases hydrolyze cellulose (β-1,4-D-glucan linkages) and produce glucose, cellobiose and cello-oligosaccharides as the primary products. There are three major cellulases as cellbiohydrolases, endoglucanases and β-glucosidases which act synergistically to convert crystalline cellulose into glucose as the final product\(^4\). Auxilliary enzymes, such as swollenin and LPMOs are also playing important roles in biomass hydrolysis.

The growing concerns about shortage of fossil fuels, emission of green house gases and air pollution caused due to incomplete combustion of fossil fuel resulted in increased focus on production of bioethanol from natural resources including algal strains\(^5-9\), particularly from lignocellulosic biomass via enzymatic route\(^8-12\). Biofuels growth by 2020 is estimated at 4% of road transport demand and USDA has predicted global ethanol production to grow up to 40% by 2022\(^6,13\). It has been stated that 10% blend of bioethanol with gasoline would reduce the carbon dioxide emission by 3-6%, which makes bioethanol a cleaner fuel in addition to being a renewable alternative to petroleum\(^14\). However, in production of bioethanol, the cost of cellulases required to hydrolyze biomass needs to be reduced and at the same time their efficiencies increased for economic feasibility of the process\(^15\). Research efforts on increasing cellulase production look up on reducing the cost by adopting such strategies as process improvement, utilizing cheaper raw materials, strain improvement by mutation or genetic manipulation\(^16,17\).

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In last few years, several pilot scale commercial bioethanol plants have come up. Economic and sustainable production of bioethanol from biomass through enzymatic route depends on economics of cellulase production, the key factor for the success of technology. Most of the commercial plants on bioethanol are buying enzymes from corporates. Still there is hardly any report on successful full fledged operation of large scale bioethanol plant earning profit.

In India, we intend to have indigenous technologies for producing enzymes and thereby ensure self sustenance. In India, there are several cellulase producing companies involving different applications from detergent to textiles which may also be employed for biomass hydrolysis however, with questionable efficiencies and specificities. It highlights the need for indigenous enzyme production technology for biofuel application.

Most of the research articles on cellulase production are focused on production studies at shake flask level but for scale-up of technology one need to study cellulase production on bioreactors. Bioreactors can be monitored and controlled. This study is based on cellulase production by a mutant strain Penicillium janthinellum EMS UV-8 on 5 L fermentor.

Material and Methods

Microorganisms and inoculum preparation

The fungal strain Penicillium janthinellum UV-EMS-8 was procured from NCIM and was cultured on potato dextrose agar (PDA) slants. Spores were harvested from a fully grown PDA slants using 1% sucrose and 0.1% tween solution. Spores were counted on haemocytometer and checked for viability and was stored at −20°C in ampoules till further use. These were used as inoculum and 10^6 viable spores per 100 mL were inoculated into the shake flask as well as fermentor for enzyme production.

All the chemicals used in the medium were reagent grade from Himedia (India) and Sigma (USA).

Medium composition and enzyme production

Enzyme production medium Mandel & Weber (M & W) as well as M6 were employed for this study. Modified M & W medium have following composition in g/L was used: KH₂PO₄, 2.0; CaCl₂·2H₂O, 0.3; urea −0.3; MgSO₄·7H₂O/ 0.3; (NH₄)₂SO₄, 1.4; peptone, 0.25; yeast extract, 0.1; tween 80, 0.1 mL/L; FeSO₄·7H₂O, 0.005; MnSO₄·H₂O, 0.0016; ZnSO₄·7H₂O, 0.0014; CoCl₂·6H₂O, 0.002; avicel, 10; and wheat bran, 25. CSL based medium composition in g/L was: NH₄(SO₄)₂, 5; MgSO₄·7H₂O, 1; tween-80, 2 mL/L; CaCO₃, 2.5; Glycerol 2.5; pH, 4.5; CSL, 27 g/L; Avicel/Cellulose, 33. Initial pH was set to 5.5 for both the medium.

Enzyme production was carried out in Erlenmeyer flask (500 mL) having 50 mL medium for comparing two media. Enzyme production with the best out of two media was carried out at level 5 L stirred tank reactor Bioflow-115 (New Brunswick Scientific, USA). Sterilization of the vessel was done in autoclave at 121°C for 30 min along with enzyme production medium. Temperature was maintained at 28°C and the pH was controlled at 4.5 by adding 1 M HCl or 1 M NaOH. Spores were used as inoculum and airflow of 2 L/min was maintained which was increased to 3 L/min after 72 h. Marine impellers were used and rpm of 250-300 was maintained. Antifoam was diluted to 2% strength (autoclaved) and was added manually during frothing. Sampling was done at regular intervals to analyze cellulase activity. Extracellular enzyme was extracted by centrifugation of the sample at 8000 rpm for 10 min.

Enzyme, protein and ammoniacal nitrogen determinations

Filter paper cellulase (FPase) was determined as per Ghosh et al.\textsuperscript{18}. Endoglucanase, xylanase and β-glucosidase activities were determined as reported earlier\textsuperscript{19}. Endoglucanase (CMCase, Endo-1, 4-b-D-glucanase; EC 3.2.1.4) activity was carried out in the total reaction mixture of 1 mL containing 0.5 mL of suitably diluted enzyme and 0.5 mL of 1% (w/v) CMC solution in citrate buffer (50 mM, pH 4.5). This mixture was incubated at 50°C for 30 min. Xylanase (1,4-b-D-xylan xylanohydrolase, EC 3.2.1.8) activity was determined under similar conditions as described above, except that 1% xylan solution was used as substrate in place of CMC. Beta-glucosidase activity was estimated using PNPG (p-nitrophenyl β-D-glucopyranoside) as substrate. The total of assay mixture (1 mL) consisting of 0.9 mL of pNP (1 mg/mL) 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 colour with 2 mL of sodium carbonate (2%). One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of glucose, xylose or p-nitrophenol produced from the appropriate substrates/min of crude filtrate under the standard assay conditions.
Protein analysis was done by following Follin and Lowry’s method and BSA was used for preparing standard. Ammoniacal nitrogen was estimated as per M W Weatherburn,\(^{20}\).

**Results and Discussion**

**Comparison of enzyme production at shake flask level**

Modified Mandel & Weber medium was employed in earlier studies for cellulase production by EMS UV-8\(^{16}\) in 5L bioreactor. CSL based medium was used for *T. reesei* RUT C-30 as well as another strain of *Penicillium* sp. (Data not published). CSL based medium was compared to M & W medium for cellulase production by EMS UV-8 at shake flask level. At four days *P. janthenellum* EMS UV-8 produced 1.25 FPU/mL cellulase and 2.75 mg/mL protein in modified M & W medium and 3.02 FPU/mL cellulase and 11.0 mg/mL protein in CSL based medium as shown in Fig. 1. The CSL based medium proved better for cellulase production when compared to modified M & W medium. Initial pH in both the medium was 5.5 but the harvest pH was 3.42 and 5.88 in M & W and CSL based medium, respectively. The CSL based medium supported cellulase production by *P. janthinellum* and the reason could be medium components which included corn steep solids (CSS) rich in micronutrients and sugars as well as protein that supports growth and production of enzymes. Also in this media, nitrogen to carbon ratio (N:C) was 5 which is regarded generally suitable for enzyme production\(^{17}\). Soluble carbon source can reduce the period of cellulase production by stimulating the initial growth of mycelium. In particular, glycerol can produce the best possible results for its inertness in cellulase synthesis\(^{21}\). A probable reason for lowered cellulase activity in M & W medium could be decrease of pH to 3.42 which is exceptionally low and would be difficult to support growth as well as cellulase production. Presence of calcium carbonate in CSL based medium resists the pH change supporting the fungal culture to grow and produce cellulases. Based on the results, it was decided to study cellulase production in bioreactor which offers several advantages over shake flasks such as controlled monitoring, maintenance of DO and pH control.

**Fermentation in a 5L fermentor**

Medium which gave better cellulase production (CSL based medium) was used for cellulase production in a 5 L fermentor with a working volume of 4 L. Spores were directly inoculated and therefore, initial lag phase was observed as spore needs germination time. CSL based medium contains cellulose which is the carbon source as well as natural inducer for cellulase production along with corn steep solids and glycerol.

*Penicillium* are being accepted as alternatives and serious competitors to the *Trichoderma* based on high titres of cellulase production in recent years. Cellulases from *Penicillium* has been shown to have better hydrolytic efficiency than *Trichoderma*\(^{22,23}\) and thereby ending the monopoly of *Trichoderma* as cellulase hyper producer\(^{24}\). *P. janthinellum* UV-EMS-8 employed in the present study produced all the components of cellulolytic enzymes required for proper hydrolysis of biomass.

Almost all the filamentous fungi producing cellulases are known to secrete cellulase economically under a regulatory control. They stop secretion in the presence of easily utilizable sugars\(^{11}\). After 24 h of inoculation with fungal spores, thick mycelial growth was visible and the viscosity of the media increased causing a drop in (dissolved oxygen) DO value which was set initially at 0.4 vvm and was increased to 0.5 vvm after 24 h. Still, DO was reduced to a minimal level; the aeration rate was increased till 0.8 vvm. RPM was also increased to 300. However, it couldn’t be increased further being deleterious to fungal filaments. Fig. 2 shows cellulase and protein production corresponding to time factor in bioreactor. FPU, xylanase, CMCase, BGL and protein increased with time and were related to each other. Till 48 h, there was no significant increase in enzyme production and the phase could be marked as growth phase only as the mycelia growth was visible and the

![Fig.1 — Comparison of cellulase production in two different medium at shake flask level.](image-url)
packed cell volume (PCV) reached 50% by 48 h. After 48 h, all the enzyme activities showed increasing trend. However, CMCase showed increasing trend since 24 h itself. Maximum enzyme production was reported at 168 h. FPUs, xylanase, CMCase, BGL and protein at 168 h was reported to be 5.44±0.3, 72±1.35, 17.11±0.75, 8.67±0.37 U/mL and 15.32 mg/mL, respectively. The exponential increase in activity was observed between 48-72 h after which the activity kept on increasing but at slower pace. Ammoniacal nitrogen analysis (Table 1) showed steep decrease in value from 700 ppm at 48 h to 370 ppm at 72 h. For enzyme synthesis, the ammoniacal nitrogen should be up to 400 ppm and this could probably the reason why further enzyme activity was not increased exponentially. After 72 h, ammoniacal nitrogen decreased further up to 180 ppm at 134 h, and thereafter there was no decrease observed as this low level was not available for the microorganisms to assimilate. At 168 h, the value remained constant, and hence the batch was terminated due to nutrition depletion as towards the end of the fermentation the viscosity was reduced and the PCV was reduced to 25% at 168 h. This could be the reason for slight increase in cellulase activity even after exhaustion of ammoniacal nitrogen. The same strain was reported to produce 3.1 U/mL FPase at 196 h with modified M & W medium in bioreactor. By changing the medium, the productivity was increased from 15.8 to 32.3 U/h/L in case of FPU. Xylanase, CMCase and BGL activities was also higher than what has been reported with M & W medium in bioreactor studies. Hence, the enzyme cocktail produced in the CSL based medium could be considered superior as the other components as also secreted at higher proportions. This particular cellulase preparation probably had the entire enzyme component needed for synergistic action for cellulosic biomass hydrolysis. *Penicillium* sp. has been commercialised in China following series of mutations and genome shuffling for further increase in cellulase titers.

Economics of cellulase production can further be improved by substituting pure cellulose avicel with pretreated biomass as well as further optimization of aeration and agitation in reactor level. This study gives an indication that cellulase productivity can further be increased by process optimization in bioreactor and the economics can be further be improved using cheaper medium components.

**Conclusion**

*Penicillium janthinellum* EMSUV-8 is a potent cellulase producer and the present study has clearly demonstrated its higher cellulase production capacity by changing the media from modified Mandel & Weber to CSL based media. CSS was the major component in the CSL based media and supported the enhanced production of all the other components of cellulase in bioreactor. As the cellulase produced was higher in titers (more than 5FPU/mL), it would be feasible to use the crude enzyme broth directly for biomass hydrolysis without doing any downstream processing.

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**References**


