Production of Cellulolytic Enzymes by *Aspergillus niger* on Solid and Submerged State Fermentation

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Received July 14, 2008 / Accepted August 6, 2008

Microbial production of cellulolytic enzymes by *Aspergillus niger* in solid state fermentation (SSF) and submerged state fermentation (SF) in laboratory scale was compared. Czapek Dox liquid broth amended with cellulose (0.5%) was used for cultivation in SF, whereas rice bran was used as a solid support, moistened with cellulose, amended Czapek Dox broth for growth in SSF. The production of Carboxymethyl cellulase, Filter paperase and β -Glucosidase was monitored at regular intervals. The peak production of the enzymes occurred within 3 days of incubation in SSF as against ≥ 7 days in SF. SSF gave higher yields of enzymes in comparison to SF. Maximum titres of 0.40, 0.62 and 0.013 U/ml in respect of FPase, CMCase and β -glucosidase in SSF were recovered as against 0.13, 0.06 and 0.0013 U/ml in SF respectively, at their respective peak time intervals. Hence, SSF appeared to be a better choice for production of cellulolytic enzymes by *Aspergillus niger*.

Key words : *Aspergillus niger*, submerged state fermentation, solid state fermentation, filter paperase, carboxymethyl cellulase, β -glucosidase

Introduction

Cellulose is the most abundant polymer in the biosphere and comprises about 40% of the dry weight of the most plants [3]. Cellulose-rich plant biomass is one of the foreseeable sources of fuels, single cell protein and feed stock to chemical synthesis due to its continuous synthesis from CO₂ and solar energy at annual rate of 72×10^{10} tons through photosynthesis by plants [12]. Ability to utilize cellulose is restricted to certain bacteria and fungi [1,2,18] due to liberation of cellulolytic enzymes. Cellulose utilizing microorganisms present in the soil and water bodies is responsible for one of the largest material flows (carbon flux) in the biosphere at both local global scales. Cellulolytic enzymes include cellobiohydrolases or exoglucanases (E.C. 3.2.1.91), endoglucanases or CMCases (E.C. 3.2.1.4) and β -glucosidases (E.C. 3.2.1.21), which act synergistically to convert lignocellulosic biomass [10] in to glucose in saccharification which can be subsequently utilized for different

purposes [5]. However, high cost of production of cellulolytic enzymes limit their industrial use in production of soluble sugars from cellulosic materials [9]. One of the approaches in improving saccharification is to search for highly efficient cellulolytic microorganisms with production of cellulase in copious amounts and to explore on cultural practices these organisms for enhanced rate of enzyme production. Hence, kinetic parameters of production of cellulase with a local potential isolate of *Aspergillus niger* by SF and SSF methods was compared in the present laboratory investigation.

Materials and Methods

Microorganism and preparation of inoculum

Aspergillus niger was isolated from the soils contaminated with the effluents of Cotton ginning mills, Nandyal, Andhra Pradesh [15]. It was maintained on Czapek Dox medium, its spore suspension was prepared by adding 2 ml of sterile distilled water to 7 day old slants.

Fermentation method in both SF and SSF

The spore suspension was used to inoculate at the spore density of 2×10^6 spores/ flask, in 50 ml Czapek Dox broth

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amended with 0.5% of cellulose in 250 ml Erlenmeyer flasks, which were incubated (30°C, 200 rpm). Ten grams of rice bran in 250 ml Erlenmeyer flask was used as solid support. Ten milliliters of the Czapek Dox broth amended with 0.5% cellulose was only once added to all matrices in the flasks, moistened the rice bran for growth in solid state fermentation (SSF). The above spore suspension was also added to solid medium at the spore density of 2×10^6 spores/ flask. At desired intervals, 250 ml Erlenmeyer flasks with growing culture of *Aspergillus niger* of both fermentations were withdrawn during the course of experiments. In SSF, fungal mass was mixed with 20 ml of acetate buffer (0.2 M, pH 5.0), the slurry was filtered through nylon cloth and the filtrate centrifuged (10,000 rpm, 20 min) at 4°C [19]. The 50 ml of broth derived from submerged fermentation (SF) was filtered on Whatman No.1 filter paper. The filtrate was used for enzyme assays. Each sample was monitored for pH, protein, Filter paperase (FPase), Carboxymethyl cellulase (CMCase) and β -Glucosidase activity.

Estimation of protein

Aliquots of *Aspergillus niger* culture filtrates with appropriate dilution were used for estimation of soluble protein content according to the method of Lowry *et al.* [11].

Enzyme assays

Filter paper assay method [13] was employed to measure total cellulase activity in the culture filtrate derived from SF and SSF. Activity of cellulase was expressed in filter paper units (FPU). One unit is the amount of enzyme that released 1 μ mole of reducing sugar from filter paper per min. Activity of endoglucanase in the culture filtrate was quantified by Carboxymethyl cellulase method [4]. One unit of endoglucanase activity is defined as the amount of enzyme releasing 1 μ mole of reducing sugar from Carboxymethyl cellulose per minute. β -Glucosidase activity in the culture filtrate was determined according to the method of Herr [8]. One unit of β -glucosidase activity is defined as the amount of enzyme liberating 1 μ mole of *p*-nitrophenol per minute.

Results

Production of filter paperase in SSF and SF

The culture of the local isolate of *A.niger* was grown in

a time-course study to compare production of cellulolytic enzymes and production of extracellular protein by both fermentation methods. The SSF yielded maximum titre of FPase with 0.396 FPU/ml within 24 hours incubation, whereas SF gave maximum titre of 0.127 FPU/ml at the end of 1st week (Fig. 1).

Production of carboxymethyl cellulase in SSF and SF

Maximum titre of carboxymethyl cellulase with 0.620 U/ml was recovered from the SSF at second day, as against 0.06 U/ml in SF at fourth week (Fig. 2).

Production of β -glucosidase in SSF and SF

Higher production of β -glucosidase in SSF was recorded in the second day filtrate with 0.01288 U/ml, whereas in

Fig. 1. Production of Filter paperase* by *A. niger*. *Values in the figure are means of 3 replicates $\pm$ standard deviation.

Fig. 2. Production of Carboxymethyl cellulase* by *A. niger*. *Values in the figure are means of 3 replicates $\pm$ standard deviation.

Fig. 3. Production of β -Glucosidase* by *A. niger*. *Values in the figure are means of 3 replicates $\pm$ standard deviation.

Fig. 4. Production of extracellular protein* by *A. niger*. *Values in the figure are means of 3 replicates $\pm$ standard deviation.

SF, maximal activity of β -glucosidase in the filtrate at 3rd week, didn't exceed 0.00124 U/ml (Fig. 3).

Protein determination in SSF and SF

Protein content was more in the filtrate of 1st day SSF with 6.6 mg/ml and there onwards declined (Fig. 4). In contrast, the reverse trend was noticed in SF with recovery of maximum protein content (1.5 mg/ml), at 4th week.

Higher production of enzymes by *Aspergillus niger* was correlated to production of protein. During the course of growth in both fermentation, pH changes occurred in the media within a range of 3.28 to 7.20 in SF whereas in SSF, it was 4.48 to 5.28.

Discussion

Though there are many publications for production of

cellulolytic enzymes using SF, very few reports are available on SSF. *Aspergillus niger* culture exhibited all three enzyme activities in the present study. Different cellulolytic enzymes reached peak production at different time intervals. Titres of all recovered enzymes at peak production interval in SSF were higher than that of respective enzymes at peak production interval in SF. Similarly, activities of cellulolytic enzymes were demonstrated with number of fungal cultures in different studies [6]. Majority of these studies on production of cellulolytic enzymes were conducted with either SSF or SF. The present study compared both methods. In SSF the higher production of cellulolytic enzymes occurred within 3 days of incubation. Whereas in case of SF maximum production of cellulolytic enzymes occurred longer incubation (weeks) time than SSF. From this study, it is clear that SSF has a distinct advantage than SF for the production of cellulolytic enzymes because of its increased yield and if the process is optimized it could be of great significance for the production of cellulolytic enzymes at a commercial scale. SSF culture conditions are reportedly more suitable than liquid for the growth of filamentous fungi [16] and might be the reason behind our findings of higher enzyme production in SSF than SF. However, the yields of cellulase production in the present study were low in comparison to the results reported by others [14,17,20-22], but were higher than titres obtained in a most recent study with *A. niger* [7]. Difference in titres of production of cellulolytic enzymes in different studies could be attributed to a variety of cultural patterns of organisms and of raw materials used in the studies.

Acknowledgment

This study was supported by the Dong-A University research fund.

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초록 : 액체와 고체 발효 조건에서 *Aspergillus niger*의 셀룰로오스계 효소 생산

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*Aspergillus niger*가 액체발효(SF)와 고체발효의 시험 규모 조건에서의 셀룰로오스계 효소의 생산을 비교하였다. 액체배지는 0.5% 셀룰로오스가 함유된 Czapek Dox를 사용하였고, 고체 지지체로 사용한 쌀겨는 셀룰로오스로 적시고 SSF 발효를 위하여 Czapek Dox 배지를 첨가하였다. CMCase, 여지 paperase 그리고 β -Glucosidase의 생산을 경시적으로 측정하였다. SSF 배양에서의 3일간의 효소 생산량은 SF에서의 7일간 배양과 같았다. 따라서 SSF 조건이 SF 배양 조건보다 많은 효소를 생산함을 알 수 있었다. SSF 조건에서 FPase, CMCase 및 β -glucosidase의 최대 활성은 0.40, 0.62 및 0.013 U/ml 였으나, SF 조건에서는 0.13, 0.06 및 0.0013 U/ml의 활성을 나타내었다. 결론적으로 *Aspergillus niger*에 의해 생산되는 셀룰로오스계 효소의 생산을 위해서는 SSF 발효 조건의 선택이 유리하다는 것을 알 수 있었다.