SCREENING FOR EXTRACELLULAR ENZYMES AND PRODUCTION OF CELLULASE BY AN ENDOPHYTIC ASPERGILLUS sp, USING CAULIFLOWER STALK AS SUBSTRATE

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Abstract

Agricultural and industrial wastes are among the causes of environmental pollution. Their conversion to useful products may ameliorate the problems they cause. The present study was carried out to maximize economic benefits by microbial activity for waste management. Endophytic *Aspergillus sp* of *Adathoda beddomei* was screened for production of various hydrolytic enzymes. The production of cellulase using cauliflower stalk as substrate was reported. The saccharification of cauliflower stalk by endophytic *Aspergillus* was studied by CMC assay and FPU assay with potato pieces as control substrate. The effect of time on the cellulase activity indicated that the activity was at peak till 6 days, whereas it decreased from the 7^{th} day.

Key words: cellulase; waste management; CMC assay; Aspergillus; endophyte

I. INTRODUCTION

Of recent ,the potential use of microorganisms as biotechnological sources of industrially relevant enzymes have stimulated interest in exploration of extracellular enzymatic activity in several microorganisms . Endophytic fungi are those fungi which colonise plants internally without apparent adverse effect (1). Endophytic fungi are relatively unexplored producers of metabolites useful to pharmaceutical and agricultural industries. A single endophyte produce several bioactive metabolites. As a result, the role of endophytes in production of various natural products with greater bioactivity have received increased attention (2,3). Extracellular enzyme synthesis in endophytic fungi for penetrating the host plant cell wall, as well as biocontrol has been demonstrated previously (4,5). The confirmation of the potential for enzyme secretion by a species and the analysis of the conditions of production could lead to improvement of the environmental condition favouring the maximal exploration of this capacity.

Higher cost of refined refined substrates are the limiting factor in the economics of enzyme production. hence utilisation of relatively inexpensive, alternate substrates such as different agroindustrial by products,waste paper, saw dust,municipal solid waste and liquid waste as productive media for fungal natural products and enzymes were described(6).

In our present investigation,we screened the endophytic *Aspergillus sp* isolated from *Adathoda beddomei* for the ability to produce amylase,cellulase,protease and lipase. As part of the study we report the production of cellulase using cauliflower stalk waste as substrate. The effect of time on biomass production and cellulase activity was studied using potato pieces as control substrate.

II. MATERIALS AND METHODS

A. Isolation of Endophytes

Healthy leaves of *Adhathoda beddomei,* were collected from Siddha Institute, Chennai. Samples were cleaned under running tap water and then air dried. Surface sterilization was carried out by sequential washings in 70% ethanol for 1 min, 5% sodium hypochlorite solution for 5 min and sterile distilled water for 1min twice. The surface sterilized leaves were cut in to small pieces using a sterile blade and transferred to sterile Potato dextrose agar (PDA) plate supplemented with chloramphenicol (50 μ g/ml). The plates were incubated at 27°C for 7-14 days. The endophytic fungi grown on plates were transferred to sterile PDA slants to maintain culture purity. The fungi were identified by colony morphology and LPCB mount (7,8).

B. Screening endophytic fungi for enzyme production

The fungal endophytes were cultivated on potato dextrose broth by placing agar block of actively growing

pure culture in 250 ml Erlenmeyer flask containing 100ml of the medium. The flasks were incubated at 27°C for 7 days with periodical shaking at 150 rpm. After the incubation period, the cultures were taken out and filtered through sterile mesh cloth to remove mycelia mats. The crude filtrate was used as the sample for qualitative enzyme analysis as described by Kumaresan and Suryanarayanan (9).

Cellulase

Nutrient agar was prepared supplemented with 0.5% of Na-CMC. Wells were punched on the agar plates. 1% congo red for few minutes and destained with 1M Nacl several times. Clear zone around the wells indicated production of cellulase

Amylase

GYP medium (1g glucose + 0.1 g yeast extract + 0.5 g peptone, 16 g agar in 1000ml of distilled water) was supplemented with 2% starch. 50%l of filtrate was added to the wells on starch agar and incubated for 16-24 hrs. After the incubation period, plates were flooded with iodine stain. Clear zone around the wells indicates production of amylase.

Lipase

Nutrient agar supplemented with 1% of Tween. 50%l of filtrate added to the wells and incubated for 16-24 hrs.A clearing or precipitation around the wells indicates lipolytic activity .

Protease

GYP medium was supplemented with 0.4% gelatin.50%l of filtrate added to the wells on gelatin agar and incubated for 16-24 hrs. The plates were flooded with saturated ammonium sulphate. A zone of precipitin around the wells indicate production of protease.

Quantitative enzyme assay

The filtrate has been used for the standard quantitative assays of the hydrolytic enzymes.

Amylase

To 1ml of the filtrate , 1ml of starch (1% soluble starch on 0.02M disodium hydrogen phosphate and 0.006M sodium chloride at pH 6.9) was added and incubated at 45°C for 1 hour. To the above mixture, 3 ml of dinitro salicylic acid (DNSA) was added and kept in boiling for 5 minutes and cooled. The reddish brown colour was observed which was measured spectrometrically at 540 nm. Amount of sugars produced were read off from a standard curve obtained by recording the absorbance of concentration of maltose standard solution.

Protease

To 3 ml of culture filtrate,3ml of phosphate buffer and 3 ml of 1% casein was added and incubated at 35°C for 1 hour. To this 5 ml of 20% TCA was added and kept undisturbed for 1 hour . the solution was filtered with whatmann no 540. From the above filtrate, 1 ml was taken to which 2 ml of 20% sodium carbonate solution was added along with 1 ml of Folin Ciocalteu reagent and mixed thoroughly and incubated for 30 minutes. 6 ml of distilled water was added and the absorbance was measured spectrometrically at 660 nm. From the standard curve,the amount of enzyme liberated was calculated.

Cellulase

1ml of carboxmethyl cellulose (CMC) was added to 1ml of the filtrate and incubated for 50 minutes at room temperature . 2ml of Dinitrosalicylic acid (DNSA) was added and boiled at 90°C for 10 minutes and cooled. To this reaction mixture 1ml of sodium potassium tartarate solution was added and the absorbance was measured spectrometrically at 540 nm.

C. Inoculation and culture conditions for cellulase production

The strain maintained on PDA slants at 40_C for 6 days were prepared as conidial suspensions by washing slant cultures with 5 ml sterilized water. Spore suspension was counted at 10^6 spores/ml by Haemacytometer. All trials were carried out in 250 ml Erlenmeyer flasks containing 10 g of cauliflower stalk (cut into small pieces) and potato pieces (control substrate) mixed with 5 ml of PDB.

D. Cellulase assays

Filter paper activity for total cellulase activity was determined by standard method. Aliquots of appropriately diluted culture filtrate as enzyme source were added to Whatmann no 1 filter paper strip immersed in one ml of 0.05 M sodium citrate buffer of pH 5. After incubation at 50 ± 2°C

for 1hr, the reducing sugar released was estimated by DNS method. One unit of FPU activity was defined as the amount of enzyme releasing 1 μ mole of reducing sugar from filter paper per minute.

Endoglucanase activity was measured using a reaction mixture containing 1ml of 1% CMC in 0.5M citrate acetate buffer (pH 5.0) and aliquots of suitably diluted filtrate. The reaction mixture was incubated at 50 ± 2°C for 1hr, and the reducing sugar produced was determined by DNS method. One unit of endoglucanase was defined as the amount of enzyme that liberated 1 μ mol of glucose per ml per minute of reaction (10).

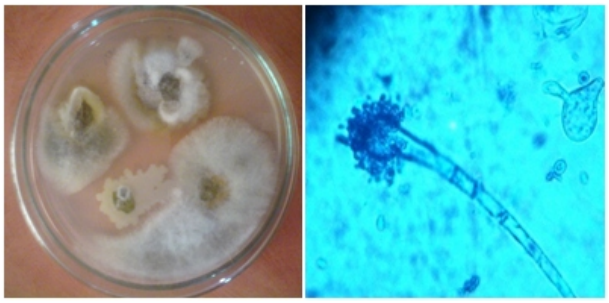

Fig. 1. Growth of endophytic fungi on PDA and LPCB mount showing the morphology of isolated fungi

Discovery of endophytic fungi in plant tissues opened up new possibilities in the search for metabolically active compounds. According to Dreyfuss and Chapela about 4000 secondary metabolites of fungal origin have been described as biologically active(11) .

Substrate utilization studies on fungal endophytes of various plants have conclusively demonstrated that most endophytes are able to utilize most substrates present in the cell walls of the hosts for their capacity to produce some extracellular enzymes. Foliar endophytes are also involved in litter decomposition (12,13).

In this study, endophytic fungi belonging to *Adhathoda beddomei* was isolated. The fungus was identified as *Aspergillus* sp by LPCB mount. As shown in Figure 1. The fungi isolated was screened for cellulase, amylase, protease and lipase production. The fungi was positive for all the enzymes screened as shown in Figure 2. Cellulases, amylases, proteases belong to the class of hydrolases which breakdown lignocellulosic material of the plants. Amylase production by endophytic fungi has been reported by Frohlich *et al (14)*. Since the endophyte lives within the senescent tissues, they are the initial colonizers of the dead tissue and utilize the readily available starch by production of amylase. Cellulases are consortia of hydrolytic enzymes converting cellulose to glucose units [3].The production of protease, cellulase and lipase indicates the capability of the fungi to degrade the cuticular wax of the leaf surface and ability to penetrate the cell wall. Moreover it indicates the pattern of plant-fungal relationship from mutualistic to latent pathogen (4).

The quantitative analysis indicated the level of enzyme production. 40 μ g/ml of anylase, 32 μ g/ml of protease and 60 μ g/ml of cellulase was produced respectively by the endophytic Aspergillus sp.

The production of hydrolytic enzymes on agro, industrial, municipal wastes have been demonstrated by various workers(15). Large quantities of cellulosic wastes, generated from agricultural residues, forests and agro-industrial practices generally accumulate in the environment and cause pollution problem (16). Biodegradation of cellulosic wastes is accomplished by cellulolytic enzymes and cellulase is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds. Active efforts are being made to convert waste cellulose resources into either glucose or alcohol and use this either as fuel or

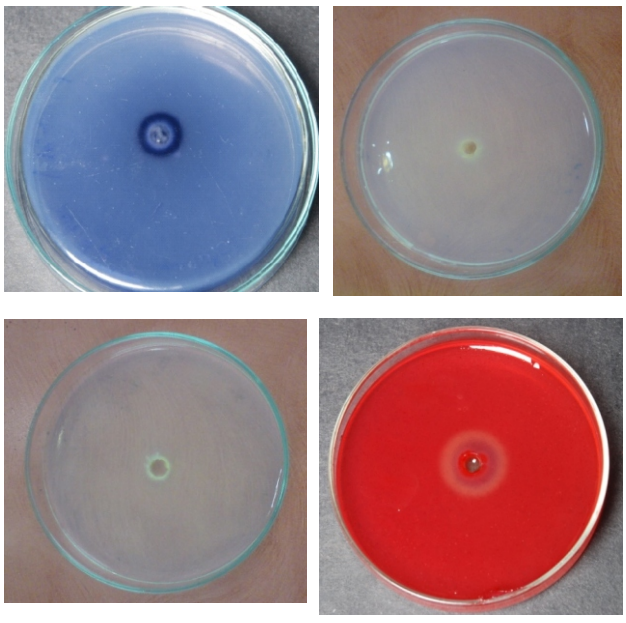

Fig. 2. Extracellular enzyme production by endophytic *Aspergillus sp*

as a valuable starting material for chemical synthesis. Large scale production of cellulase for commercial purposes requires the identification of high yielding fungal sources and optimization of process conditions (17). Different agroindustrial by products like sugarcane bagasse, corn bran,wheat bran,fenugreek straw as productive media for fungl natural products were described Mohamed Ashour et al (18). Banana fruit stalks have been confirmed to be good solid substrates for production of cellulase by *Neurospora sitophila* at optimized culture conditions(19).

In accordance with the above studies, the production of cellulase by endophytic Aspergillus sp on cauliflower stalk waste was accomplished by solid state fermentation.

Growth of the fungi on the solid state culture as well as cellulase production, by the isolated *Aspergillus* sp. were followed simultaneously for 7 days using Cauliflower stalk pieces (CFSP) and potato pieces (PP) as substrates. Results clearly indicated that PP supported growth comparatively more than CFSP and growth reached maximum on day 6 producing nearly 4.2 gm/10 gm of substrate used (Figure 3). This variation in the biomass production when different substrates were used is due to the fact that CFSP is a tough substrate to be acted upon by the fungal species compared to PP. Even Moumita Karmaker and Rina Rani Roy reported the differential rate of saccharification of sugar cane bagasse, water hyacinth and orange peel and attributed this difference to the nature and complexity of the substrate (17).

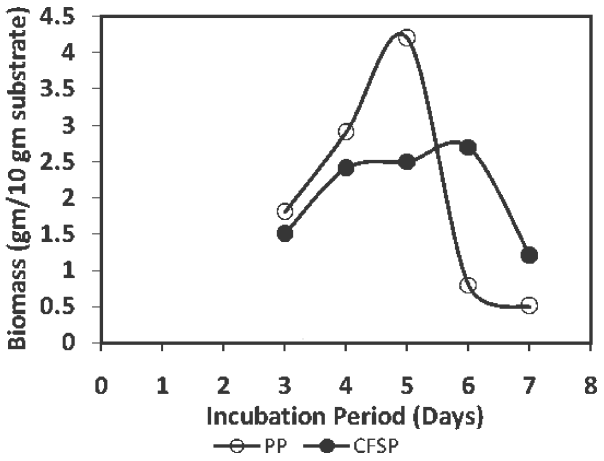

Fig. 3. Effect of time on biomass production

The CMC assay is used to measure the released Endo β glucanase during the degradation of cellulose. When PP were used as substrate cellulase activity was at its peak with 33.141 IU/ml on day 6 which decreased to 11.51 IU/ml on day 7. In case of CFSP cellulase activity was found out to be highest with 6.278 IU/ml on day 6 which also decreased on day 7 (Figure 4). The filter paper assay is a combined assay for endo and exo beta glucanase during the degradation of cellulose. The FPA activity of 32.69 IU/ml was noticed on day 6 when PP were used as substrate. CFSP gave rise to FPA activity of 18.21 IU/ml on day 6. In both cases activity decreased on day 7(Figure 5). The decrease in enzyme activity after day 6 may be attributed to cumulative effect of cellobiose (20). Nutritional and environmental factors such as type of carbon source, period of cultivation, agitation affect enzyme synthesis and production by fungi (21,22,23). Fermentation period is an important parameter for growth as well as for the production of enzymes. Thus optimization of these conditions could lead to the optimal enzyme production and an effective waste management.

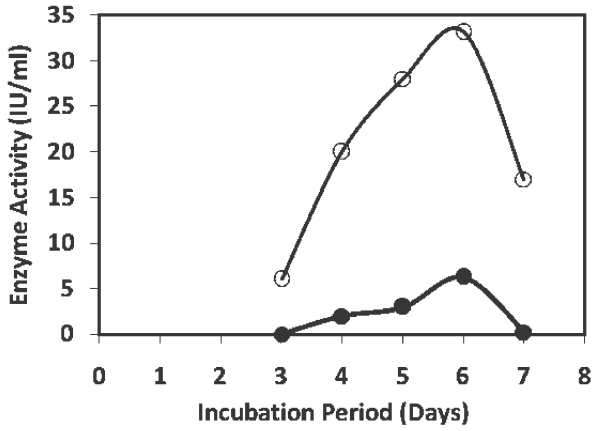

Fig. 4. Effect of time on enzyme activity given by CMC assay

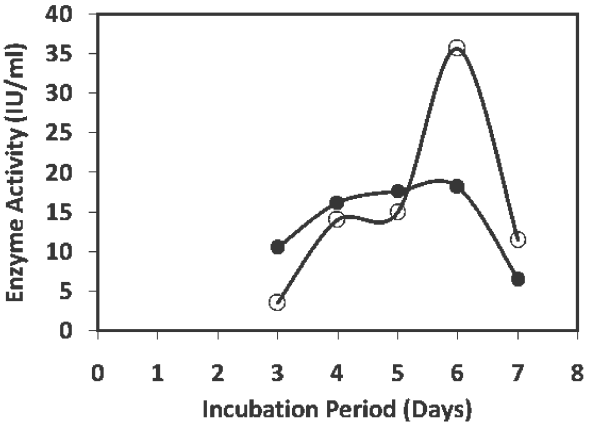

Fig. 5. Effect of time on enzyme activity given by FPA

CONCLUSIONS

In this investigation, the waste cauliflower stalk proved to be a good source for production of cellulase by endophytic *Aspergillus sp*. The production of cellulase could be achieved simultaneously with waste management. Further investigations are required to utilise the potential of the endophyte for cellulase production by employing genetic, biochemical and microbial engineering techniques.

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