SCREENING OF FUNGAL STRAINS PRODUCING CELLULASES BY SOLID FERMENTATION

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ABSTRACT

Cellulosic substrates are represented mainly by wood but also by recyclable waste products such as bran and wheat straw, peanut shell, sawdust and olive cake. Adding value to agricultural and industrial by-products by fermentation, such as cellulase production, is an attractive biotechnological option. Fungi are the primary source of the enzymes required to convert plant biomass into sugars, bio ethanol and other fermentation products and for improving the digestibility of feed. Isolates of cellulolytic strains from various samples (soil, plant debris, decaying wood) of the Yakouren forest litter (wilaya of Tizi-Ouzou-Algeria) were grown on selective agar media containing cellulose as sole carbon source. Screening of selected strains was carried out on Mandel's agar medium containing either amorphous or crystalline cellulose. Strain BY had a zone of hydrolysis of 25 mm diameter after 7 days at 30 °C compared to 30 mm for *Trichoderma reesei* reference strain used. In addition, strain BY was the only strain that hydrolyzed crystalline cellulose. Solid substrate fermentation studies were conducted using strain BY. Cultures were incubated at 30 ° C for 8 days. Filter paper activity of about 46 U/g dry matter was observed after 5 days of fermentation. Dry matter increased during the fermentation from 47.7 mg to 59.0 mg. In contrast, the pH decreased gradually from 6.4 to 6.14. Further research is required to identify other enzymes such as CMCase, the ß-glucosidases and xylanases, and to study the degradation of other substrates such as wheat straw, sawdust, wood and cornbran.

Keywords: Screening; Fungal cellulases; Wheat bran; Solid-state fermentation; Filter paper activity.

INTRODUCTION

Lignocellulosic biomass consists mainly of 40-60% cellulose, 15-30% hemicellulose and 10-30% lignin. There is growing global interest in the environmental impact of solid lignocellulosic waste generated by different agricultural activities. Industrial techniques are being used to find ways to reduce and, if possible, add value to these cellulose-rich wastes. Cellulose is the basic structural material of plant cell walls and the macromolecule is the most abundant and widely synthesized in the plant world. It is a renewable energy source virtually inexhaustible and untapped to date.

The solid state fermentation process is one of the most applied in industry with low investment and operation costs. This has made it an attractive alternative for the production of cellulases, the enzymes that break down cellulose, which are widely used in industrial processes in the food and pharmaceutical industry. However, the most promising use in the current economic climate seems to be the production of alternative fuels. Cellulosic residues hydrolyzed

by cellulases, provide a mixture of sugars. Fermentation of these residues can lead to products such as acetone, butanol, ethanol or methane.

Filamentous fungi that produce cellulases are usually grown on solid substrates in an environment that is similar to their natural environment. Their vegetative growth consists of aerial branched hyphae that colonize the porous solid matrix. In addition, filamentous fungi can grow at humidity levels as low as 12% and they tolerate high osmotic pressure, characteristics that are not usually found in yeasts and bacteria.

The exploitation of agricultural by-products such as wheat straw, peanut shell, and sawdust, by fermentation is a very interesting biotechnological approach for the production of cellulases due to their high cellulose content. This paper describes screening fungal strains for their cellulolytic activity and then testing one strain for its ability to produce cellulases in a solid substrate fermentation process.

MATERIALS AND METHODS

Fungal strains. Isolates of cellulolytic fungal strains were obtained from various samples (soil, plant debris, decaying wood) of the Yakouren forest litter (wilaya of Tizi-Ouzou, Algeria) using a selective medium containing cellulose as the sole carbon source.

Preparation of amorphous cellulose[1] Micro crystalline cellulose (Avicel) (10 g) was suspended in concentrated phosphoric acid (85%; 200 ml) with occasional stirring and constant cooling. The swollen microcrystalline cellulose was washed several times with cold deionized water. After incubating overnight with 3% (w/v) Na_2CO_3, the thick suspension of swollen cellulose was washed several times with cold deionized water. The amorphous cellulose was drained and stored at 4°C.

Screening of fungal strains for cellulase activity.
The isolated strains were screened for cellulase activity on an agar medium containing either amorphous or crystalline cellulose. The screening medium used is that of Mandel [2] containing in g/L: urea: 0.3; KH_2PO_4: 2; $(NH_4)_2SO_4$: 1.4; $MgSO_4$, 7 H_2O: 0.3; soy peptone: 0.75; yeast extract: 0.5; $CaCl_2$: 0.4; amorphous cellulose or crystalline cellulose: 10; agar: 20 and in mg/L: $FeSO_4$, 7 H_2O: 5; $MnSO_4$, 7 H_2O: 1.08; $ZnSO_4$, 7 H_2O: 1.4; $CoCl_2$: 2. The Mandel's medium (containing either amorphous or crystalline cellulose) was poured into sterile dishes (22 x 22 cm) and cooled. An agar plug of each strain was inserted into a corresponding hole in the medium. The plates were incubated at 30 °C until the appearance of transparent zones around the holes corresponding to the zones of hydrolysis of cellulose. *Trichoderma reesei* strain was used as a reference organism.

Production of cellulases from strain BY by solid fermentation
Subculture of the strain. Strain BY was sub cultured on Petri dishes containing malt medium and incubated at 28 °C for 10 days.
Preparation of substrate. Wheat bran (20 g) was moistened to 50% and placed into 500 ml Erlenmeyer and then autoclaved at 121 °C for 20 min.
Inoculum obtention**.** Ten mls of a sterile solution of 0.2%, Tween 80 was added to a Petri dish containing the culture. The mycelium was scraped from each Petri dish and the mycelial suspension was recovered into a sterile vial using a sterile pipette.
Substrate inoculation. Three mls of mycelial inoculum was used to inoculate the substrate (8.25 mg / g substrate). The substrate was inoculated and incubated at 30 °C for 7 days.

Enzyme extraction. Two hundred mls of distilled water were mixed with 10 g of the fermented substrate, the pH of the mixture was measured and the mixture was centrifuged at 10,000 rpm at 4 °C for 15 min. The supernatant contained a crude enzyme extract.

Monitoring the growth of strain BY.

Measurement of dry matter. A test sample of from 0.5g to 1 g of fermented substrate was used to determine the % dry matter during the period of fermentation.

Monitoring changes in pH. The pH was measured during the period of fermentation

Determination of enzyme activity. The cellulase activity was measured using the filter paper activity (FPA) assay, expressed in filter paper units (FPU) according to the method of Ghose [3]**.** This method measures the release of reducing sugars produced in 60 min from a mixture of enzyme solution (0.25 ml) and of citrate buffer (0.05 M; pH 4.8; 0.5 ml) in the presence of 25 mg of Whatman N° 1 (1 x 3 cm strip) and incubated at 50°C. The released sugars were analyzed by a nitrosalicylic acid method [4]. All simples were analyzed in triplicate and mean values were calculated. All activities were described in International Units (IU) where one unit of activity is defined as the amount of enzyme required to liberate 1 µmol of reducing sugars in 1 minute. Filter paper activity was expressed by U/ g of dry matter.

RESULTS AND DISCUSSION

Twelve fungal strains were isolated on cellulose agar medium and then sub cultured onto malt medium for further study (Table 1). Of the 12 strains screened, two *Trichoderma sp.* gave a clear zone of cellulose hydrolysis of 17 mm on the amorphous cellulose medium. Strain 4 (BY) gave a zone of 25 mm on amorphous cellulose as well as 15 mm on the crystalline cellulose medium. A 30 mm zone of hydrolysis was observed around the *Trichoderma reesei* on amorphous cellulose medium only (Table 1).

Table 1: Diameter of hydrolysis zones of the strains isolated

Strains	Hydrolysis zone (mm)	
	Amorphous Cellulose	Crystalline Cellulose
1	-	-
2	-	-
3	-	-
4 (BY)	25	15
5 (*Trichoderma sp*)	17	-
6 (*Trichoderma sp*)	17	-
7	-	-
8	-	-
9	-	-
10	-	-
11	-	-
12	-	-
Trichoderma reesi	30	-

In the solid substrate fermentation study, Figure 1 shows that the dry matter increased during fermentation and reached 59% in the seventh day of fermentation. The pH dropped during the fermentation from 6.4 to 6.05 rising again slightly to 6.14 (Figure 2). The cellulase activity was

measured using the filter paper activity (FPA) assay and expressed in filter paper units (FPU). The maximum activity was found after 5 days of fermentation with 46 U / g dry matter (Figure 3).

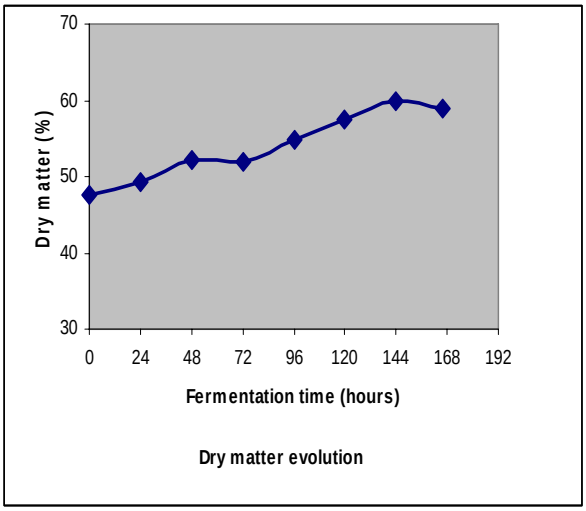

Figure 1: Dry matter evolution

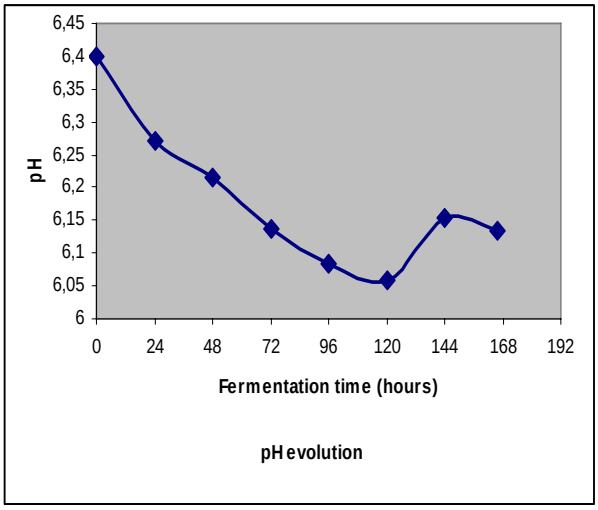

Figure 2: pH change during fermentation

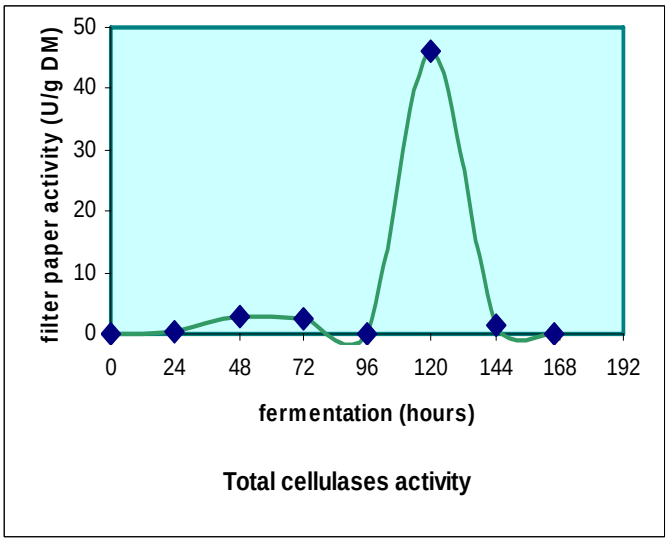

Figure 3: total cellulases activity

CONCLUSION

The present study identified an interesting cellulolytic strain – Strain BY. It has a zone of hydrolysis of 25 mm on amorphous cellulose and 15 mm of crystalline cellulose. However, a clear zone of 30 mm in diameter was observed for *Trichoderma reesei* on amorphous cellulose. These results are preliminary and further investigations will include assays for CMCase, B-glucosidases and xylanases. Other substrates such as wheat straw, sawdust, wood and corn bran will also be investigated.

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