Pertanika 15(1), 45-50 (1992)

Effect of Carbon and Nitrogen Sources on the Growth and Production of Cellulase Enzymes of a Newly Isolated *Aspergillus* sp.

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ABSTRAK

Filtrat kultur yang didapati dari Aspergillus *sp. yang baru dipencil menunjukkan aktiviti yang baik terhadap kertas turas sebagai substrat. Suhu dan pH optimum bagi pertubuhan organisma ini masing-masing adalah pada 37°C dan 6.5. Walau bagaimanapun, suhu dan pH optimum bagi aktiviti enzim selulase masing-masing adalah 44°C dan 4.5 untuk masa pengeraman 48 jam. Penghasilan enzim selulase dalam medium cair mencapai tahap maksimum pada hari ke 15 daripada keseluruhan tempoh pengeraman sepanjang 21 hari. Dari beberapa sumber karbon dan nitrogen yang diuji, karboksimetil-selulosa (kelikatan medium) dan $(NH_4)_2 Fe(S)_4)_2$. $6H_20$ merupakan sumber terbaik bagi penghasilan enzim manakala serbuk selulosa dan NH_4NO_3 merupakan sumber yang terbaik bagi penghasilan miselia.*

ABSTRACT

The culture filtrate obtained from a newly isolated Aspergillus *sp. exhibited good activities against the filter paper as substrate. The optimum temperature and pH values for growth were 37°C and 6.5, respectively. However, the optimum temperature and pH for the activities of the cellulase enzymes were recorded as 44°C and 4.5, respectively. Production of the enzymes in liquid medium reached its maximum level (9 units/ml) at the 15th day of incubation with an incubation period of up to 21 days. From the various carbon and nitrogen sources tested, carboxymethyl-cellulose (medium viscosity) and $(NH_4)_2 Fe(SO_4)_2$. $6H_20$ were found to be the best for cellulase enzyme production, whereas cellulose powder and NH_4NO_3 enhanced mycelial growth.*

INTRODUCTION

The production and utilization of cellulolytic enzymes is a topic of great interest in the world searching for renewable resources. The lack of an economical process for saccharification of waste cellulose by microbial enzymes is one of the major problems yet to be solved by fermentation technology. Reduction of costs will involve the proper selection of strains yielding high levels of the enzymes. Thus, the search for new and different cellulose degrading microorganisms has increased and various new species have been isolated and characterized as regards to their capacities to produce cellulases (Tanaka *et al.* 1980; Tong *et al.* 1980; Hudson *et al.* 1990)

In an attempt to isolate cellulose-degrading fungi from various natural habitats, samples from compost heaps, decomposed wood and rubbish dumping grounds were collected. Of the forty-eight isolates tested, the most active organism identified was *Apergillus* sp. obtained from compost heaps of oil palm waste. Our preliminary studies indicated that this newly isolated species had the ability to degrade quickly a wide variety of cellulosic materials. In this paper, the effect of carbon and nitrogen sources on the growth and cellulase production by this organism is reported.

MATERIALS AND METHODS

Whatman No. 1 filter paper and Whatman chromatography paper were obtained from Whatman Ltd. Palm-press-fibres were kindly supplied by a palm oil factory in Bukit Rajah, Klang. Carboxymethyl-cellulose and cellulose powder were purchased from Sigma Chemical Company, St. Louis USA. Potato dextrose agar was a product of Difco Laboratories, Detroit USA. Xylan from oat-spelt was obtained from Fluka, Switzerland and peptone was purchased from Topley House, England.

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Source of Microorganism

Various isolates of fungi (48 isolates) were obtained from soil samples collected around the vicinity of Kuala Lumpur. These samples included compost, wood chips, decomposed tree trunks and leaves. The most active cellulolytic organism from these isolates was identified to be an *Aspergillus* sp. isolated from the oil palm compost heap. It was then selected for the following studies.

Buffers

The citrate-phosphate buffer from pH 3.0 to 8.0 was prepared using 0.1 M-citric acid and 0.2 M-dibasic sodium phosphate. The buffer 0.2M tris/HCI was used for pH 8.0 and 9.0.

Preparation of Culture Filtrate

Aspergillus sp. was subcultured on PDA and grown at 37°C for 48 h. Three agar-mycelium discs (8.0 mm in diameter) were transferred to a Wheaton medical flask (C-16, 500 ml) containing 60 ml of Fergus (1969) medium with 3.3 g filter paper as carbon source. After 15 days of incubation at the above mentioned temperature, the liquid medium was filtered by suction through a Whatman GF/C filter to remove hyphal fragments and any isoluble cellulose residues. The clear culture filtrate obtained was stored in deep freeze, for later use if necessary.

The inoculum was taken from the culture grown on PDA for 48 h at 37°C. An agar-mycelium disc (8.0 mm in diameter) was cut from the perimeter of the colony with a sterile cork borer, inverted and placed at the centre of a 9 cm diameter petri dish containing agar medium.

For seeding liquid media, three agar-mycelium discs (8.0 mm in diameter) were placed into each medical flask containing 60 ml of Fergus (1969) medium.

Maintenance of Stock Culture

The organism was cultured onto PDA slopes and incubated for two days at its optimum temperature and stored at 4°C for not more than three months prior to further subculturing.

Determination of Opitmum Temperature for Growth

The optimum growth temperature on solid agar was ascertained by measuring the diameters of colony on PDA in 9 cm diameter petri dishes containing approximately 30 ml of agar medium. Each agar plate was inoculated with one agar-mycelium disc as described earlier and incubated for 48 h at the following temperatures, that is, 20°C, 28°C, 37°C, 44°C, 50°C and 60°C. To reduce desiccation of the agar medium at high temperatures, a beaker of distilled water was placed in each incubator.

Determination of Optimum pH for Growth

The optimum pH on solid agar was determined by measuring the diameters of colony on PDA medium in a 9 cm petri dish. Medium with different pH, that is, 4.5, 5.5, 6.5, 7.5, 9.0 were each inoculated with an agar-mycelium disc (8.0 mm in diameter) and incubated for 48 h at the optimum temperature for growth.

Effect of Incubation Period on the Production of Enzymes

Medical flasks containing 60 ml of Fergus medium were inoculated and incubated at 37°C for different times. The contents of two flasks from each culture were harvested at intervals of two to three days up to 21 days by suction through a Whatman Gf/C filter. The culture filtrates were then assayed for cellulase activity on filter paper determined by estimating the reducing sugars formed using the Nelson-Somogyi method (Nelson 1944; Somogyi 1952) as described below.

Determination of Optimum Temperature and pH for the Production of Cellulase Enzymes

The optimum temperature and pH for the production of cellulase enzyme in liquid medium were determined using a range of temperatures (28°C, 37°C, 44°C, 50°C, 60°C, 70°C and 80°C) and pH (3.0, 4.5, 5.5, 6.5, 7.5, 8.0 and 9.0) with an incubation period of 15 days. The culture filtrate was then assayed for cellulase activities as described below.

Effect of Different Carbon Sources on Mycelial Growth and Cellulase Enzyme Production

To test the effects of different types of carbohydrate as the sole carbon source on the mycelial growth and cellulase enzyme production, ten different carbon sources were used and incorporated separately into the Fergus (1969) medium incubated at optimum conditions.

A rough estimation of the amount of mycelial growth was done by making visual observation of the mycelium in the medical flasks whereas the amount of cellulase enzymes produced was determined by the method described below.

Effect of Different Nitrogen Sources on Mycelial Growth and Production of Cellulase Enzymes

The effect of nine nitrogenous compounds on the mycelial growth and enzyme production in Fergus (1969) medium was determined under optimum conditions. The concentration of each nitrogen source in the medium was 0.1% (w/v). In all cases, the pH of the medium was adjusted to 6.5. Upon incubation for 15 days, the dry weight of mycelia was recorded and the amount of cellulase enzymes produced was estimated by the method described below.

Determination of Reducing Sugars

The number of reducing sugar groups created by hydrolysis of the cellulosic substrates was measured spectrophometrically by using the Nelson-Somogyi procedure (Nelson 1944; Somogyi 1952).

Assay of Cellulolytic Activity

An indication of total cellulolytic activity was obtained by the determination of filter paper degrading activity. The standard reaction mixture contains 20 mg of filter paper (Whatman No. 1), 0.5 ml of citrate-phosphate buffer, pH 5.0, 0.5 ml of enzyme solution (culture filtrate) of appropriate dilution (if necessary) and one drop (10µl) of toluene. After incubation at 44°C for three days, 0.5 ml of the reaction mixture was withdrawn and assayed for reducing sugars using the method mentioned above.

An absolute definition of a unit of cellulase activity is difficult. There is little to be gained by expressing the activity in terms of glucose equivalents, since glucose is not the only product of the enzyme reaction (Shepherd *et al.* 1988). A unit of cellulase activity is defined as that amount of enzyme that produces an increase in absorbance of 0.10 at 560 nm under the conditions defined. A change in absorbance of 0.10 is equivalent to 30 µg of glucose under the conditions given, and thus cellulase preparations with units quoted in glucose equivalents can be compared.

RESULTS AND DISCUSSION

Forty eight fungal isolates were tested for their cellulolytic activity towards filter paper as carbon source. It was found that of all the fungi tested, one showed a high cellulase activity towards filter paper and other cellulose substrates. This active cellulolytic fungus was identified as an *Aspergillus* sp.

Degradation of the filter paper in the Fergus (1969) liquid medium began after three days of incubation at 37°C. By the 15th day, the integrity of the filter paper was lost completely. The optimum temperature for growth on PDA was 37°C and the colony reached its maximum size of 9.0 cm in diameter upon incubation for 48 h. No growth was observed at 20°C and 60°C. At 37°C, the optimum pH value for growth was 6.5 with an average diameter of about 8.1 cm for an incubation period of 48 h.

Effect of Incubation Period on the Production of Cellulase Enzymes

Fig. 1 demonstrates the production of cellulase enzymes over a period of 21 days as measured by the ability of the culture filtrate to degrade filter paper. Initially, the activity was low and fluctuated until the 12th day, after which the activity increased sharply up to the 15th day. By this time, the integrity of the filter paper in the medium was lost almost completely and a thin slurry was formed. Futher incubation resulted in a decline in the activity.

Effect of Temperature on Cellulase Activity towards Filter Paper

In general, cellulases characteristically have high optimum temperatures compared with other enzyme systems. The cellulases described in this work have an optimum temperature of 44°C (*Fig. 2*). At 60°C, the activity decreased to about 60% of the maximum activity.

At temperatures higher than 70°C, the cellulase activity was denatured almost completely. At lower temperatures, a sharp decrease in the activity was observed at temperatures below 37°C,

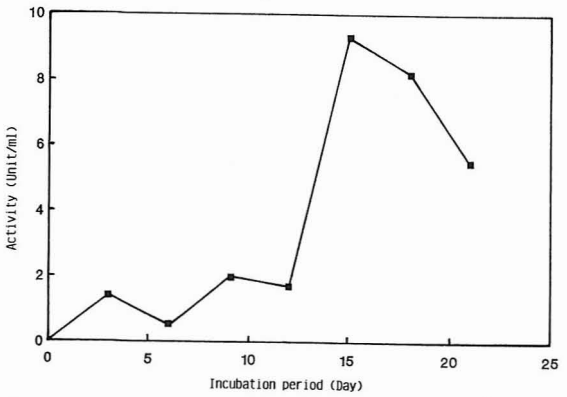

Fig. 1: Effect of incubation period on the production of cellulase activities. Point represents an average of duplicates.

and at room temperature (28°C), the cellulase activity was only about 20% of the maximum.

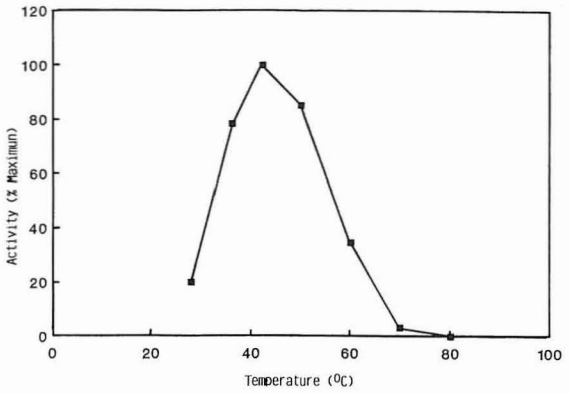

Fig. 2: Temperature optimun of cellulase activities. Point represents an average of duplicates.

Effect of pH on Cellulase Activity towards Filter Paper

Basically, fungal cellulases are stable from pH 3.0 to 8.0 at 30°C, active at pH 3.7 to 7.0 (Tong & Cole 1982). The optimal pH observed for cellulases produced by *Aspergillus* sp. occurred at pH 4.5 (*Fig. 3*). At a lower pH of 3.0, there was a sharp decrease in the cellulase activity to about 35% of the maximum. At a pH higher than 4.5, a gradual decrease in the activity was observed with only 9% of the maximum activity remaining at pH 8.0 and 9.0. In some fungal species, such as *Trichoderma koningii* (Wood 1969) and *Aspergillus* sp. (Khatijah *et al.* 1983) double pH optimum has been reported.

Effect of Different Carbon Sources on the Mycelial Growth and Production of Cellulase Enzymes

To test the effect of different types of carbohydrate as the sole carbon source on mycelial growth and on the production of extracellur cellulolytic enzymes, culture was inoculated into Fergus (1969) medium and incubated for 15 days at 37°C.

The results are shown in Table 1 and *Fig. 4*. There were some difficulties experienced in measuring the amount of mycelial dry weight in some of the substrates used. This was due to the fact that some of the mycelia became adsorbed onto the substrate thereby making its separation difficult. Therefore, a visual obervation of the mycelial growth in the liquid medium was adopted. Cotton, cellobiose, filter paper and particularly cellulose powder were found to be good sources for the mycelial growth, whereas xylan, citric acid and mannitol were less suitable for mycelial growth.

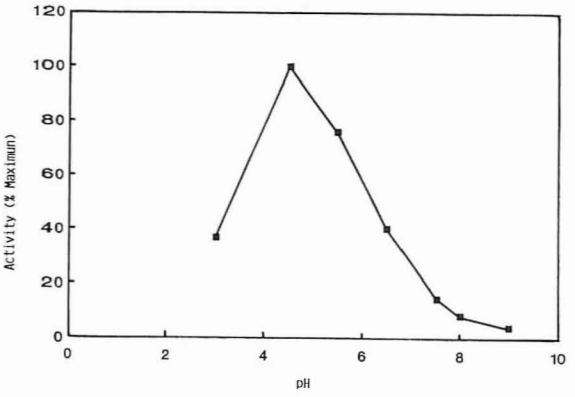

Fig. 3: pH optimun of cellulase activities. Point represents an average of duplicates.

The amount of extracellular cellulolytic enzymes in the crude filtrates was subsequently determined. It is interesting to note that there is no correlation between the amount of mycelial growth and the amount of cellulase enzymes produced. Xylan and CMC which did not promote much mycelial growth turned out to be good carbon sources in enhancing the production of cellulase enzymes. Other carbon sources which yielded good cellulase activity included cellobiose and cellulose powder. This supported the findings of Reese and Mandels (1971) that cellulase enzymes

TABLE 1. Effect of different carbon sources on the mycelial growth.

Carbon source	Arbitrary scale
Xylan	+
Citric acid	+
Mannitol	++
CMC	++
Yeast	+++
Cotton	+++
Cellobiose	++++
Filter paper	++++
Cellulose powder	+++++
	+++++

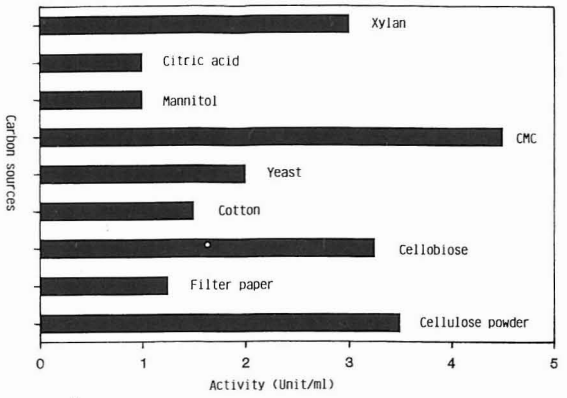

Fig. 4: Effect of different carbon sources on the production of cellulase activities. All values are average of duplicates.

were produced when cellulose was present as the carbon source in submerged cultures of fingal mycelia. Other carbon sources which are easily assimilated and have been shown to promote fungal growth may not induce the production of cellulolytic enzymes (Mandels and Reese 1965; Noviella, 1966). In addition, Rautela and King (1965), as well as Fan *et al.* (1982) have shown that different forms of cellulose vary from one another in their growth-supporting, enzyme-inducing and reacting capabilities. This may be due to the difference in the microstructures among the cellulose substrates (Wang 1982). The cellulase activity observed may not be due to the actual amount of enzymes secreted in the cultures. This is due to the fact that cellulases have an affinity for certain cellulose sources. The cellulase enzymes would then be adsorbed onto the cellulose substrate thereby reducing the amount of detectable cellulase enzymes in the culture filtrate (Halliwell 1961). The percentage of this adsorbed cellulolytic activity has been estimated to vary from 0 to 40% depending on the species of the fungus. Greaves (1971) reported that by successively washing and gently agitating the mycelium and cellulose several times, the cellulase enzymes can be released from cellulose. Similarly, certain cellulose substrates, for example, the non-absorbent cotton wool, have surfaces that are not suitable for the absorption of cellulolytic enzymes leaving a higher amount of enzymes detectable in the filtrates.

Effect of Nine Different Nitrogen Sources on the Mycelial Growth and Production of Cellulase Enzymes

The effect of nine different nitrogenous compounds on the mycelial growth of *Aspergillus* sp and production of cellulase enzymes in Fergus (1969) medium with filter paper as the carbon source was determined.

As shown in *Fig. 5*, sodium nitrate, sodium nitrite and ammonium nitrate were found to be the best nitrogen sources for mycelial growth. However, ferrum ammonium sulphate and ammonium molybdate were not suitable for the growth of *Aspergillus* sp. Surprisingly, peptone did not support good mycelial growth either.

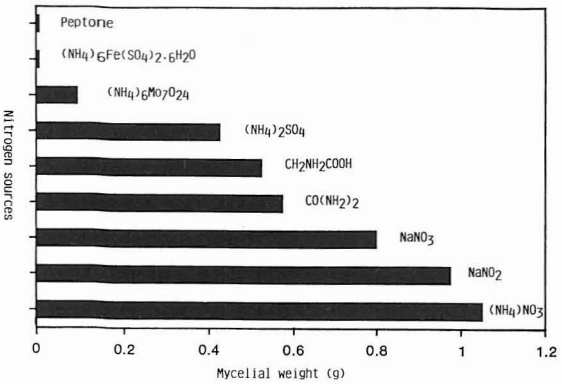

Fig. 5: Effect of different nitrogen sources on the mycelial growth. All values are average of duplicates.

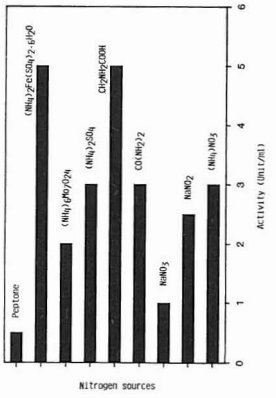

Fig. 6: Effect of different nitrogen sources on the production of cellulase activities. All values are average of duplicates.

With regard to the cellulase activity, again there was no correlation between the mycelial growth and cellulase production (*Fig. 6*). Ferrum ammonium sulphate heptahydrate and glycine stimulated maximum production of the cellulase enzymes followed by ammonium sulphate, urea, sodium nitrite and ammonium nitrate. Although ammonium nitrate supported the best mycelial growth, it did not stimulate as high a level of

cellulase production as that achieved by ferrum ammonium sulphate heptahydrate and glycine. This may be explained by the fact that ammonium ions were absorbed by the mycelium which resulted in a lowering of the pH that subsequently inhibited the production of cellulase enzymes (Wang 1982).

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(Received 29 November 1991)