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Full Length Research Paper

Effect of cultivation conditions on growth and antifungal activity of *Mycena leptocephala*

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Growth and production of antifungal agent by *Mycena leptocephala* was investigated in different culture media composition at various initial pH and temperatures. Maximum growth and activity was observed at the initial pH of 5.5 and 25°C. No detectable growth and activity was observed at pH of 3.5 and 7.5. Growth of the fungus and antifungal activity were also very low at 37 °C and 20°C. The organism grew better and culture extract showed higher level of antifungal activity when malt extract plus glucose were used as carbon source and yeast extract was used as nitrogen source. Meanwhile the lowest level of growth and antifungal activity were observed when starch was used as carbon source for growth of the fungus.

Key words: Culture conditions, basidiomycetes, bioactive compounds, carbon source, nitrogen source and temperature.

INTRODUCTION

Mycenas are a large group of basidiomycetes, in which the bioactivity of many species of this genus has been reported, and the structures of many of these bioactive metabolites have been identified (Anke et al., 1979; Baurele and Anke, 1980; Hutzel et al., 1990). Different species of this genus are capable of producing bioactive compounds such as antimicrobial and cytotoxic metabolites (Jenete et al., 1985). Some of these antibiotics have been identified as having different structures, such as polyacetylenic, terpenoids, and methoxy acrylates (Baurele et al., 1982). Effects of culture composition, pH and temperature on growth and biomass concentration of different basidiomycetes have been reported (Song, 1987). However, study on the effects of culture condition and production of active metabolites of such fungi remains poorly exploited.

One reason could be that little is known about their physiology because of the little amount of work done on basidiomycetes, which is due to the difficulty of growing such fungi. In this article, the effect of different cultivation conditions on growth and antifungal activity by this organism is investigated.

MATERIALS AND METHODS

The organism and growth conditions

M. leptocephala was collected from the west of Scotland and identified kindly by Prof. R. Watling of the Edinbrough Botanic Garden. Mycelial cultures of the fungus were obtained from tissue plugs of fruiting bodies as explained by Hale and Savory (1976). The culture was grown on malt extract agar and maintained at 4°C perior to use. The basal medium, for growth and production of antifungal agent, used was composed of 0.5 g/L KH_2PO_4, 0.5 g/L Mg SO_4 .$7H_2O$, 5 µg/L $FeCl_3$ and Thiamin 50 µg/L.

To study the effect of different nitrogen source, the medium containing 1% glucose was supplemented with 1% (w/v) organic and inorganic nitrogen sources. Growth and productivity in the presence of different carbon sources was studied using the basal

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Table 1. Efffect of nitrogen sources on growth and activity of *M.leptocephata* at pH 5.5, temperature 25^{o}C (glucose was used as carbon source).

Nitrogen sources	Final pH	Growth (g/L)	Activity (mm)*
Peptone	4.7	2	12.3
Tryptophane	4.9	1.9	11.2
Yeast extract	4.8	2.1	14.8
NH_4CL	5	1.2	8.2
Urea	4.9	1.4	10.2
$NaNo_3$	5.2	1.1	7.6

*mm zone of inhibition (n=3).

Table 2. Effect carbon source on growth and activity of *M. leptocephala* at pH 5.5 and 25^{o}C temperature. Yeast extract was used as nitrogen source.

Carbon sources	Final pH	Growth (g/L)	Activity (mm)
Glucose	4.7	2	15.2
Fructose	5.1	1.7	13.4
Maltose	4.9	1.7	13.4
Sucrose	5.2	1	8.1
Starch	5.3	0.8	8.1
Malt extract	4.8	1.5	14
Molasses	4.7	1.6	13.1

medium, yeast extract (0.1%, w/v) and different carbon sources (1% w/v).

To study the effects of incubation temperature and initial medium pH on growth and production of antifungal agents, the basal medium containing glucose and malt extract (1% w/v) and yeast extract (0.1%, w/v) was used.

In all cases, 100 ml medium in 500 ml flasks was inoculated with 5 ml of mycelium suspension of the organism (approx. 1 g/L dry weight). The culture was incubated in an incubator shaker at a 100 rpm After 10 days the culture was harvested and the mycelium free fluid was used as the bioactive compounds source. 50 ml of culture filtrate of each flask (3 flask for each condition, n= 3) was separately extracted with ethyl acetate. Each extract was concentrated to dryness under reduced pressure. The obtained products were weighed and kept at 4 C^{o} perior to test. Growth was determined by measuring dry weight of mycelium using pre dried and weighed 4.25 cm diameter Wattman GF/C filter circle and a 4.25 cm Gelman filtration unit.

Antifungal activity assay

To determine antifungal activity of the culture extracts, paper disc agar diffusion technique was employed as assay technique.

A series of 90 mm Petri dishes containing malt extract agar for growth of yeast-like fungus, *Candida lipolytica* (ATTC 825) were prepared. Cell suspension was prepared with normal salin containing 1×10^{6} organisms per ml and gave a turbidity comparable to that of McFarland standard tube No. 0.5. Each plate was separately inoculated with the tested fungus by swabbing aseptically on the whole surface of the agar with cotton wool. A 6 mm diameter filter paper disc was impregnated with 20 µl of the extracts in absolute ethanol (25 mg ml^{-1} culture extract). The discs were air dried and placed aseptically at the center of the plates. The plates were left undisturbed for 1 h to allow the extract to diffuse into the agar. Nystatin (0.025 mg) in absolute ethanol impregnated onto the disc and air dried, were used as positive control. The plates were incubated at desired temperature. The growth inhibition which was indicated by areas of clear zone was measured by vernier callipers. The evaluation of inhibitory properties was carried out in triplicates.

Statistical analysis

A classical method of experimentation with one factor at a time changed while all others are held constant was carried out as explained by Strobel and Sullivan (1999).

RESULTS

Effect of nitrogen sources

A high level of antifugal activity was observed when yeast extract was used as nitrogen source (Table 1). Compared to this nitrogen source, growth and activity was lower in media containing NH_4Cl and NaNO3 .

Effect of carbon sources

Good growth and antifungal activity were observed when glucose was used as carbon source (Table 2). Growth and antifungal activity were also good in media containing fructose, maltose, malt extract and molasses. In contrast activity and growth of the fungus was very low when sucrose and starch were used as carbon source.

Table 3. Effect of glucose and complex carbon source (1%w/v)on growth and activity of *Mycena leptocephala* at 25 and pH 5.5.

Carbon source	Final pH	Growth (g/L)	Activity (mm)
Malt extract +glucose	4.8	2.3	21
Molasses + glucose	4.7	2.1	20.6
Starch + glucose	4.9	1.9	15

Table 4. Effect of temperature on growth and activity of *Mycena leptocephala* at pH 5.5. Malt extract and yeast extract were used as carbon and nitrogen sources.

Temperature (^{O}C)	Final pH	Growth (g/L)	Activity (mm)
20	5.1	1.6	12
25	4.7	2.1	20.6
30	4.9	1.8	17.2
37	5.2	1.1	8.9

Table 5. Effect of initial pH medium on growth and activity of *M. leptocephala* at 25^{O}C and 30^{O}C temperature. Glucose and yeast extract was used as carbon and nitrogen sources.

Initial pH	Final pH	Growth (g/L) 25^{O}C	Activity (mm)	Final pH	Growth (g l^{-1}) 30^{O}C	Activity (mm)
3.5	3.4	0.3	-	3.3	0.4	-
4.5	4.2	1.6	10	4.1	1.5	9.6
5	4.3	2	19.7	4.2	1.9	18.7
5.5	4.6	2.1	21	4.5	2	20.2
6	5.7	1.8	19.5	5.6	1.6	18.7
7	6.8	0.8	7	6.6	0.7	0
7.5	7.3	0.5	0	7.3	0.3	0

Effect of glucose plus complex carbon source

As the results in Table 3 shows, growth of the fungus did not increase significantly in the different glucose media. However activity was increased when malt extract was added in the medium at concentration of 1% (w/w).

Effect of temperature

Growth and antifungal activity of *M. leptocephala* were also investigated at an initial pH of 5.5 and different temperature. Growth and antifugal activity at 25^{O}C were high (Table 4), at 20 ^{O}C and 30^{O}C a little reduction in activity was observed. Growth and antifugal activity were very low at 37^{O}C .

Effect of initial pH

Initial pH of the medium on growth and antifungal activity was investigated at 25^{O}C and 30^{O}C. In both cases, maximum growth and antifungal activity were observed at initial pH of 5.5 and only a very small growth was recorded at pH 3.5, 4 and 7 and 7.5 (Table 5).

DISCUSSION

The fermentation medium designed for the initial production of an antibiotic usually does not have to be developed very skillfully since the potential for antibiotic is quite low with wildt -ype strains (Miller and Churchill, 1986). Therefore a series of experiments based on single-dimensional optimization programme were carried out.

Good growth and antifungal activity were observed when complex nitrogen sources were employed. This may provide a long period during which conditions are suitable for optimal growth and production of active compounds, as earlier reported by Calam (1986). There are few reports on the effect of nitrogen in the production of bioactive compounds from basidiomycetes, even though most researchers have used organic nitrogen source for the production of such compounds (Stadler et al., 1994; Mandle and Vodickova, 1994).

Increased antifungal activity of the fungus in media containing a simple sugar, like glucose plus a slow releasing carbon source, like malt extract can be explained by the high production rate of secondary metabolites when their producing organisms grow in complex media (Martin and Demain, 1986). These

authors showed that in media containing glucose plus a more slowly utilized carbon source, glucose is usually used first and after glucose has become depleted, the second carbon source is used for antibiotic biosynthesis. Increasing activity of the culture extract of the medium containing glucose plus malt extract may support this observation. Use of glucose resulted in good growth of the fungus, but it should be noted that in many fermentations processes increasing the concentration of glucose has a suppressive effect on production of bioactive compounds (Hutter, 1982).

The culture pH and temperature affect the antifungal activity and growth of the fungus. Most basidiomycetes grow over a wide pH and temperature range (Litchfield 1967; Brown 1988), although many bioactive compounds are only stable within a narrow range of pH.

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