Production of lipase and toxic metabolites by *Cladosporium cladosporioides* under varied conditions

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Abstract. *Cladosporium cladosporioides* was investigated for the production of lipase and toxic metabolites under varied conditions. Shea butter and groundnut oil promoted lipase elaboration. As the species ages in broth, lipase production increases and a considerable growth accompanied the lowering of pH to 4.0. Also some antifungal metabolites were produced, although they did not have any effect on *Mucor hiemalis* and *Aspergillus flavus.* Active charcoal was an effective adsorbent of the antimicrobial secreted into the broth.

Key words: *Cladosporium cladosporioides,* lipolytic activity, toxigenic fungi

Introduction

Cladosporium cladosporioides (Fresen.) G.A. de Vries is a dark green fungus when observed on the Potato Dextrose Agar plates, with a black coloration on the reverse. It is slow-growing and velvety with conidia that are smooth, unicellular, and ellipsoidal in shape. Because of the delicate arrangement of the conidia, it is difficult to mount and retain whole structure on microscope as they disperse easily. This dermatiaceous fungus is of high agricultural and medical significance (Pritchhard & Muir 1987; Bocklisch & Otto 2000; De Hoog *et al.* 2000). This organism, like most species of the genus, does not grow at temperatures above 35 °C. Due to safety, they are carefully handled in microbial safety hoods in the laboratory as they can incite diseases like skin lesion, sinusitis, pulmonary invasions, and other human mycoses (Dixon & Polak-Wyss 1991).

It is a frequently found genus of fungi in outdoor air and can be isolated from soil and plant litter (Domsch *et al.* 1980). Several metabolites have been isolated from *Cladosporium herbarum* (Pers. : Fr.) Link by Jadulco *et al.* (2002) and in the same year, some biologically active polyketide metabolites were confirmed as products of a strain resembling *Cladosporium* (Holler *et al.* 2002). The production of enzyme lipase and antifungal metabolites had been reported in fungal studies with fairly sparse information on *Cladosporium cladosporioides.* Although there is no certainty for lipase production by *Cladosporium* (Barbosa *et al.* 2001), this organism was investigated in the present study for the production of this enzyme and other secondary metabolites with a view to determining the best cultural conditions for their secretion. As a suitable candidate for lipase production, the application of *C. cladosporioides* lipase in industrial and domestic settings will be more acceptable. Also the exploitation of its amtifungals in biocontrol can be further intensified.

Materials and Methods

Cladosporium cladosporioides (IMI 301 004) was maintained on Potato Dextrose Agar in the Culture collection unit of the Department of Basic and Applied Sciences (Babcock University, Ilishan Remo, Nigeria) for further studies. The mycelial discs were obtained from the periphery of 5-day-old cultures, grown and maintained on malt extract agar plates. Modified Mineral Salt broth (MSB) of Gill & Arora (2003) was used in the course of the project. It contained KH_2PO_4 2 g, $MgSO_4.7H_2O$ 0.6 g, $CaCl_2.2H_2O$ 2 g, Sodium acetate 20 mM, thiamine HCl 1mg, Ammonium tartrate 0.2 g, distilled water 1 liter.

Effect of plant fats on growth and lipolytic activity

The basal medium contained 3.0 % soybean meal, 1.0 % peptone, 1.5 % soluble starch, 0.2 %, K_2HPO_4, 0.15 % $MgSO_4.7H_2O$ and 0.25 % $CaCO_3$. The following fats: groundnut oil, palm oil, soybean oil, and shea butter served as treatment and were added as 0.2 % w/v. One hundred ml aliquots of the medium was inoculated using two, 5 mm agar discs of 7-day-old isolates and the conical flasks were incubated at 30 °C on a rotary shaker at 110 rpm for 5 days. The cultures were filtered using Whatman no 1 FP and supernatant assayed for lipase production, a unit of activity was defined as the amount that liberated 1 micromol of free fatty acids per minute under test condition (Sztager *et al.* 1988) while dry (oven dried at 60 °C to constant weight) mycelial weight in g/100 ml was taken. Control flasks had no fat source while results are means of 3 replicates.

Tributyrin agar method

This was carried out by determining the extent of zone of clearing/enzyme diffusion zone by lipase through qualitative assessment after 48, 96, 144, 192, 240 hr. The MSB earlier described was the basal medium and supernatant were collected from 2, 4, 6, 8, and 10-day cultures respectively and administered accordingly.

Antifungal activities of the metabolic products

The antifungal effect of the test fungus was investigated as follows. One loopful of the conidia of *Cladosporium cladosporioides,* taken from the periphery of malt extract agar cultures, was inoculated into 100 ml medium containing sucrose 3.5 %, polypeptone 2 %, KH_2PO_4 0.5 %, $MgSO_4$ $7H_2O$ 0.25 %, pH 5.6 (Uozumi & Arima 1974) and incubated for 10 days. Harvesting was conducted using sterile cotton wool and the filtrates were assumed as metabolic solutions, believed to contain the metabolites. The chloroform extracts were prepared as earlier described (Asao *et al.* 1963; Denault & Underkofler 1967) and they served as the test solutions. The test organisms, previously isolated from agricultural soil samples, were *Mucor hiemalis* Wehmer, *Rhizopus stolonifer* (Ehrenb. : Fr.) Vuill. (*R. nigricans* Ehrenb.), and *Aspergillus flavus* Link. : Fr. All the organisms were identified by standard morphological methods (Domsch *et al.* 1980; Samson *et al.* 1995). Ten ml spore suspensions (10×10^4/ml of each test fungus) were inoculated on MEA and 5 mm FP disc saturated with the chloroform extract, roto-evaporated, was centrally placed and zone of inhibition measured in mm and presented in factors after 7 days of incubation at 30 °C.

The crude filtrate of a 3 week old culture of *Cladosporium cladosporioides* was similarly investigated for its fungicidal activity on the 3 fungi by the method of Nespiak *et al.* (1961) as follows:

A = 50 ml filtrate +10 g agar
B = 50 ml filtrate + 50 ml corn extract broth + 10 g agar
C = 50 ml filtrate, passed through 20 g active charcoal + 10 g agar
D = 50 ml filtrate, passed through 20 g active charcoal + 50 ml corn extract broth +10 g agar
E = 5 g dried mycelium suspended in 200 ml distilled water + 10 g agar
F = 50 ml corn extract broth + 10 g agar

The mycelial growth was monitored, measured, and recorded in factors representing "insignificant", "average" or "normal growth". All readings were means of 3 replicates.

Impact of sulphur stress on growth

The standard culture medium (control) contained: KNO_3 3.5 g; KH_2PO_4 1.75 g; KCl 0.5 g; $FeSO_4$ 0.5 g; $MgSO_4$ $7H_2O$ 0.75 g, distilled water 1 liter. All sulphates were replaced by their corresponding chlorides and two, 5 mm agar discs served as the inoculum in each of the 250 ml flasks containing 50 ml of the medium. Five replicates were made for the fungus, incubation was at 35 °C and observations were carried out at 4 day intervals for 16 days. The pH of the medium was monitored throughout the period of incubation setting the initial at 6.5. Mycelial weight (g/50 ml) was taken as earlier described (Fapohunda & Ogundero 1990).

Table 1. Effect of metabolic products of *Cladosporium cladosporioides* on the growth of other fungi. Incubation of 30 °C took place for 7 days. Results are means of 3 replicates.

	Metabolic products/ Crude filtrates						Metabolite
	A	**B**	**C**	**D**	**E**	**F**	**Chloroform extract**
Mucor hiemalis	++	+	–	+	+	–	–
Rhizopus stolonifer	+++	+	–	+	+++	+	++
Aspergillus flavus	+	–	+	+	++	–	++

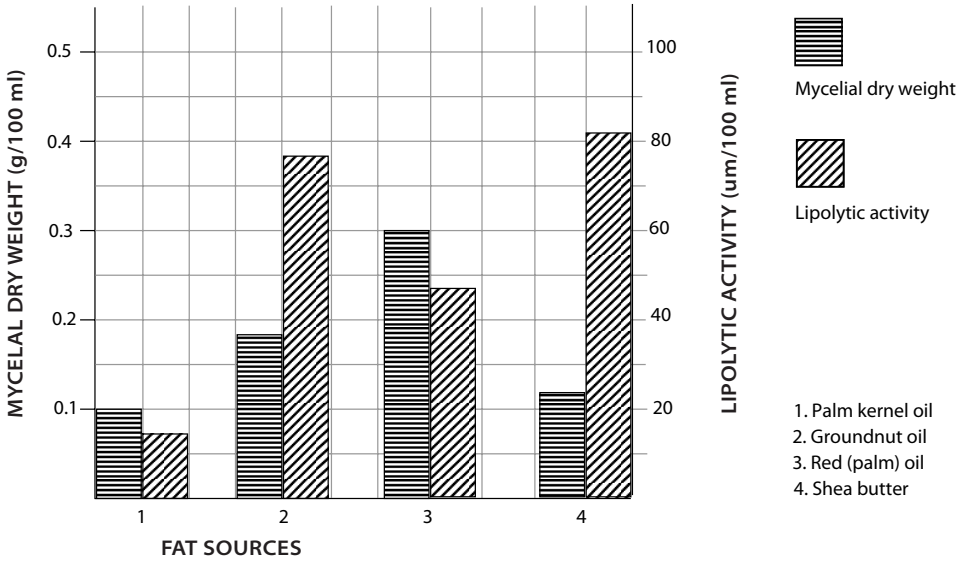

Fig. 1. Effect of plant fats on growth and lipolytic activity

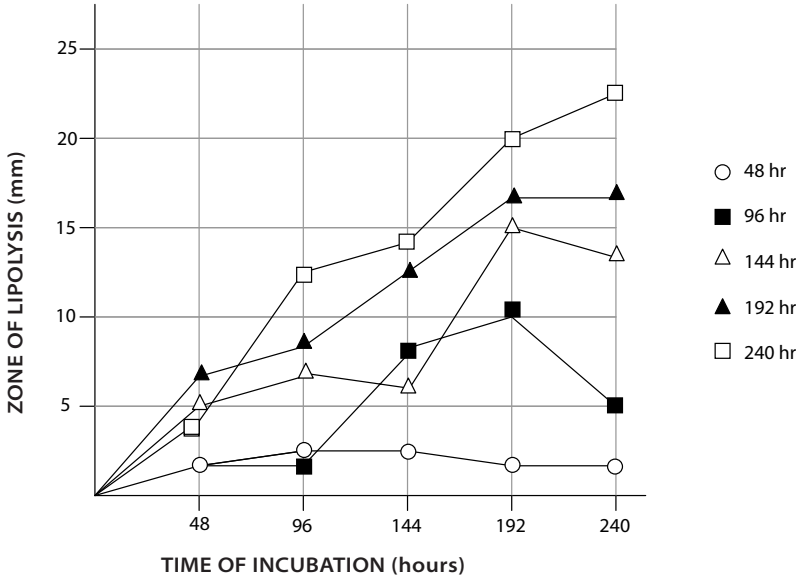

Fig. 2. Tributyrin agar method: influence of time of incubation on the lipolytic activity of *Cladosporium cladosporioides*

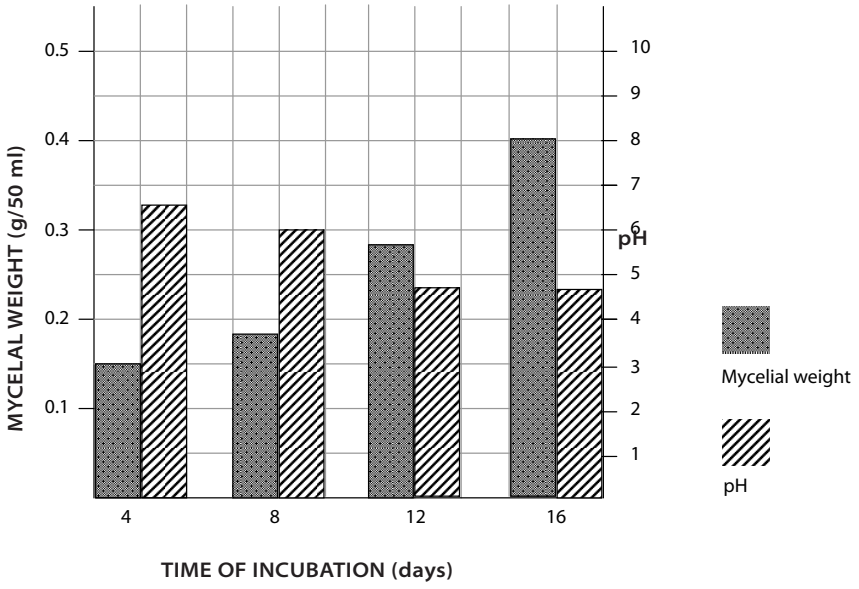

Fig. 3. Impact of sulphur stress on the growth of *Cladosporium cladosporioides*

Results and Discussion

The choice of the fat sources in Fig. 1 was informed by the belief that water soluble substances are not always good compounds for lipase activity (Kouker & Jaeger 1987; Rapp & Backhaus 1992). Of the fat sources, shea butter and ground nut oil showed a higher lipolytic activity than others. This study confirmed lipase production by this species, which interestingly was only treated as a suspect lipolytic organism (Barbosa *et al.* 2001), even when its cellulolytic capacity was not in doubt (Karomasena *et al.* 2001). This may not be surprising, because lipidic carbon sources have been recorded as enhancing lipase production, although even when it is affected by factors like pH and dissolved oxygen concentration (Ellibol & Uzer 2001). Also the efficacy of lipase synthesis in this organism may not be compared favourably to moulds like *Penicillium kloeckeri* Pitt (*P. wortmanii* Klöcker), noted for the highest production among 56 fungal strains screened by Costa & Peralta (1999). Generally, lipase production and activity vary with fungal species and substrate, while rapeseed and corn oil were the most suitable substrates for cell growth and lipase production by *Rhizopus oryzae* Went & Prins. Geerl., maximum activity was got from olive oil when *Penicillium expansum* Link was investigated (Essanri *et al.* 1998). Figure 2, which expresses the zone of lipolysis against time of incubation, shows that lipase action increases as the duration of the culture supernatant increases. The supernatant of a 240 hr (10-day) culture of *Cladosporium cladosporioides* gave the highest enzyme production and this still increased as the assay period on tributyrin increased. In other words, as the culture ages in broth, the production of lipase increases. Being a contaminant of food, the lowering of the pH to 4.0 with considerable growth is significant because this pH is explored based on its broader application to real food systems (Laverrnicocca *et al.* 2003).

The antagonistic effect of this fungus on the test fungi suggested the occurrence of potent metabolites which can either be volatile (Dennis & Webster 1997b) or nonvolatile (Dennis & Webster 1997a). The seeming resistance of *Mucor hiemalis* and *Aspergillus flavus* in the presence of corn extract to the antagonism as expressed in the growth level cannot be explained. However, it might be due to either the test procedure adopted or the genetic make up of the moulds (Lundberg & Ulrestan 1980). Although this genus *Cladosporium* is known to produce, **cladosporin** and **emodin** both of which are lethal on mice, only conidial allergies, not death on man by this cosmopolitan fungus is on record (Masclaux *et al.* 1995) and the average impact the chloroform extract had on growth of *Rhizopus* and *Aspergillus* strains (Table 1) makes further works in this direction attractive. The work also revealed that active charcoal is a good adsorbent of toxic compounds as the solution had no inhibitory effect on the 3 test fungi.

The fungus increased in growth (highest = 0.4 g/100 ml) in spite of the sulphur stress although pH tended toward acidity as the duration of incubation increased and up to the 16th day) (Fig. 3). A sharp drop in pH was recorded in the 12th day (from 6.0 to 3.9) and in spite of this however, mycelial growth continued to increase. It is significant that low pH enhances the incitement of diseases in plant materials characterized by acidity (Barbosa *et al.* 2001). The dearth of recorded notes of death due to *Cladosporium* 'poisoning' is not enough to discount the toxic danger inherent in cumulative assemblage in human bodies from 'sick' and 'unkempt' buildings and in-door environments (Croft *et al.* 1986; Gravesen *et al.* 1999; Gravesen 2000; Kuhn & Ghamoum 2003). Research on the purification and characterization of the antifungals from this species is on-going in our laboratory.

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