Notes on the physiology and morphology of *Thamnostylum piriforme* isolated for the first time in South Africa

A. Botha, 1 C. Roux, 2 A.J. Botha, 3 J.L.F. Kock 4 and C.H. Pohl 5

1Department of Microbiology and Biochemistry, University of the Orange Free State, P.O. Box 339, Bloemfontein, 9300 South Africa  
e-mail: bothaa@micr.mw.uovs.ac.za  
2National Collection of Fungi, Biosystematics Division, PPRI, Private Bag X134, Pretoria, 0001 South Africa  
3Electron Microscope Unit, University of Pretoria, Pretoria, 0001 South Africa

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The morphology and physiology of two fungal isolates, which were the first record of the species *Thamnostylum piriforme* in South Africa, were studied. In both isolates, which were morphologically typical representatives of their species, sporulation was stimulated by UV light. Optimum growth and sporulation in the dark was found to occur at 25°C. The isolates could utilize a wide variety of carbon sources aerobically. These carbon sources included pentoses, hexoses, disaccharides, triaccharides, polysaccharides, glycosides, alcohols and organic acids. D-galactose, D-glucose and maltose could be fermented. The isolates were able to grow in a medium without vitamins and were tolerant to cyclohexamidine at a concentration of 0.01% w/v. The isolates tested positive for urease activity on Christensen’s urea agar, while extracellular enzymatic activity was indicated by the ability to liquefy gelatin. The most biomass-containing high value dietetic oil was obtained with acetic acid as carbon source. Consequently, it is possible to utilize this fungus for the production of bioprotein and high value dietetic oil on industrial effluents containing acetic acid.

Keywords: Carbon sources, lipids, physiology, *Thamnostylum piriforme*

*To whom correspondence should be addressed.*

Introduction

Recently it was discovered that some mucoralean fungi can grow and produce significant quantities of gamma-linolenic acid on petrochemical effluents that contain high concentrations of acetic acid (Kock & Botha 1995). In a subsequent search for additional acetate tolerant mucoralean fungi, it was discovered that *Thamnostylum piriforme* (Bain.) Arx & Upadhyay (Van Arx 1970), present in the leaf litter from the coastal forests and thornveld of Northern KwaZulu-Natal (Acocks 1988), could utilize acetic acid as sole carbon source, while producing oil containing gamma-linolenic acid (Botha et al. 1995). This was the first report of this fungal species, able to produce a dietetically important oil, in South Africa. Since knowledge of the morphology and physiology of a fungus is essential to assess its biotechnological potential, these characteristics of the isolates representing T. *piriforme* were investigated in this study.

Materials and Methods

Isolates used

*T. piriforme* isolates PPRI 5473 and PPRI 5534 from the National Collection of Fungi, Pretoria, were used. Both strains were isolated from leaf litter, obtained from the coastal forest and thornveld (Acocks 1988) of the tropical savannah region of northern KwaZulu-Natal, South Africa (Botha et al. 1995).

Morphology

Preparation of cultures. Pure cultures were grown on *Mucor*-synthetic medium (SMA) and Potato carrot agar (PCA) (Booth 1971) at 21 ± 5°C in natural light on a window sill.

Optical microscopy. Material for optical microscopy was mounted in clear lactophenol and studied using bright field illumination.

Scanning electron microscopy. Material for scanning electron microscopy was prepared according to two regimes: a) blocks of agar containing the fruiting structures were exposed overnight to osmium tetroxide vapour and then washed in 0.2 M Na cacodylate buffer, dried in a desiccator and sputter-coated with gold; b) blocks of agar with fruiting structures were submerged in 0.075 M phosphate buffer, pH 7.4–7.6 fixed in 0.25% osmium tetroxide and then dehydrated in acetone. The blocks were mounted on SEM stubs and sputter-coated with gold.

Electron micrographs were taken at 5 kV using a Jeol 840 scanning electron microscope.

Influence of temperature on growth and sporulation. Petri dishes containing SMA were inoculated with mycelia of the isolates and incubated in the dark at 10°C, 15°C, 20°C, 25°C, 30°C and 37°C. After six days, the cultures were examined for growth and sporulation.

Influence of UV light on sporulation. Petri dishes containing SMA were inoculated in duplicate with mycelia of the isolates. One culture of each duplicate was incubated at 25°C under UV irradiation. The other culture was incubated under identical conditions, except that it was incubated in the dark. Growth and sporulation were examined daily for one week.

Physiological properties

Preparation of inocula. Cultures of *T. piriforme* PPRI 5473 and PPRI 5534 were incubated for two weeks at 25°C under UV light on malt extract agar (Difco). A sterile, wet inoculating loop was used to transfer the sporangiospores from the hyphal growth of each culture to 5 ml sterile distilled water. The resulting suspensions (approx. 1.3 × 10^7 spores ml⁻¹) were used as inocula.

Carbon assimilation tests. The ability of the isolates to assimilate a series of carbon sources in synthetic media aerobically was determined according to the standard methods for yeast identification as described by Van der Walt & Yarrow (1984). Test tubes, each containing 7.6 g l⁻¹ Yeast nitrogen base (YNB Difco) and a different carbon source (Table 1) were inoculated with 40 μl of spore suspension per tube. Only 150 × 12-mm test tubes were used throughout this study, and the carbon sources, obtained from Sigma, Merck and
Figures 1–6. 1. Sporangium after deliquescence, and clusters of sporangioles of T. piriforme. Bar = 100 μm. 2. Sporangioles developing along the axis of a sporangiophore. Bar = 10 μm. 3. A developing cluster of sporangioles with characteristic circinate stipes. Bar = 10 μm. 4. A terminal cluster of sporangioles. Bar = 10 μm. 5. Verticillately branched structure of sporangiolar cluster. Bar = 10 μm. 6. A single, smooth sporangiole showing the circinate nature of the stipe. Bar = 10 μm.
indicating lipase activity, in the pH due to ammonia in the medium. Splitting of arbutin by p-glucosidase. The standard method was used to test the ability of a yeast to ferment a series of carbohydrates (Van der Walt & Yarrow 1984). Six milliliters of liquid medium, consisting of 0.5% w/v yeast extract (Difco) and 2.0% w/v of a different fermentable carbohydrate (Table 1), was inoculated with 40 μl spore suspension. The inoculated cultures in test tubes were incubated in an upright position at 25°C and monitored for gas production (i.e. fermentation) over a period of 14 days. Gas production was observed as bubbles accumulating in a Durham tube submerged in the medium (Van der Walt & Yarrow 1984).

Fermentation of carbohydrates. The standard method was used to test the ability of a yeast to ferment a series of carbohydrates (Van der Walt & Yarrow 1984). Six milliliters of liquid medium, consisting of 0.5% w/v yeast extract (Difco) and 2.0% w/v of a different fermentable carbohydrate (Table 1), was inoculated with 40 μl spore suspension. The inoculated cultures in test tubes were incubated in an upright position at 25°C and monitored for gas production (i.e. fermentation) over a period of 14 days. Gas production was observed as bubbles accumulating in a Durham tube submerged in the medium (Van der Walt & Yarrow 1984).

Growth in vitamin-free medium. The ability of the fungal spores to germinate and grow in a synthetic medium without vitamins was analysed according to the method described by Van der Walt & Yarrow (1984). The spore suspension (40 μl) was used to inoculate 5 ml liquid medium consisting of 6.7 g 1-1 Yeast nitrogen base (Difco), 5.0 g 1-1 glucose and 0.1 g 1-1 cycloheximide in test tubes. The inoculated media were incubated at 25°C on a roller drum rotating at 100 rpm. Growth was visually determined by comparing it with the fungal growth of a blank without a carbon source after six, eight and 10 days of incubation. The biomass was harvested by filtration (Whatman GF/A), freeze-dried and weighed after each period of incubation.

Cycloheximide resistance. The influence of 0.01% w/v and 0.10% w/v cycloheximide on fungal growth was examined according to the method described by Van der Walt & Yarrow (1984). The spore suspension (40 μl) was used to inoculate 5 ml liquid medium consisting of 6.7 g 1-1 Yeast nitrogen base (Difco), 5.0 g 1-1 glucose and 0.1 g 1-1 cycloheximide in test tubes. The inoculated cultures were incubated at 25°C on a roller drum rotating at 100 rpm. Growth was monitored after six, eight and 10 days of incubation.

Gelatin liquefaction. The ability of the fungal isolates to liquefy gelatin was analysed according to the method described by Van der Walt & Yarrow (1984). The surface of a solidified medium consisting of 100 g l-1 gelatin, 5 g l-1 glucose and 11.7 g l-1 Yeast carbon base (Difco) was inoculated with 40 μl of the spore suspension. The inoculated culture was incubated in an upright test tube at 25°C for seven days, whereafter the depth of the liquid layer was measured.

Splitting of arbutin. A slant consisting of 5 g l-1 arbutin, 5 g l-1 yeast extract (Difco), 12 mg l-1 ferric ammonium citrate and 20 g l-1 agar was inoculated with 40 μl of the spore suspension and incubated at 25°C (Van der Walt & Yarrow 1984). The slope was examined after seven days for the presence of a dark brown colour, characteristic of the complexes formed between ferric salts and hydroxyquinone due to the splitting of arbutin by β-glucosidase.

Urease activity. The ability of the fungal isolates to produce ammonia from urea, indicating the presence of urease, was tested as described by Van der Walt & Yarrow (1984). A slope of 0.5% w/v urea agar was inoculated with 40 μl of the spore suspension and incubated at 25°C. After seven days, the slope was examined for the presence of a deep pink colour, as the phenol red indicated a rise in the pH due to ammonia in the medium.

Lipase activity. The ability to produce lipases was determined as described by Kouker & Jaeger (1987). A loopful of the fungal growth was streaked out on a plate containing nutrient agar supplemented with 2.5% (w/v) olive oil and 0.001% (w/v) of the fluorescent dye, rhodamine B. The plate was incubated at 25°C for seven days, after which it was examined under UV irradiation for an orange fluorescent halo around the fungal colony - indicating lipase activity.

Results
Morphology
At 21 ± 5°C in natural light, T. piriforme produced only sporangioles on SMA after 7 days, but occasionally, and especially on PCA, terminal sporangia also formed (Figure 1). Some sporangioles produced only terminal sporangia. Sporangia deliquesced before the sporangioles ripened (Figure 1): the outer membrane, which consisted of cells bearing spinules, disintegrated and released the sporangiospores. A basal frill remained at the apex of the apophysis. Lateral clusters of sporangioles of varying sizes occurred along the axis of the sporangiophore (Figure 2). The sporangioles developed characteristic circinate stipes (Figure 3) similar to those in members of the genus Circinella. The largest groups of sporangioles were usually terminal (Figure 4) and arranged on a verticillately branched structure (Figure 5).

To ensure correct identification, cultures must, therefore, be grown under conditions that encourage the development of both states of this fungus. As in Circinella, the sporangioles did not deliquesc: the spores were released by disintegration of either the sporangioles themselves or by the severing of their stipes. Each individual, smooth sporangiole (Figure 6) contained a small number of spores. All sporangioles were black in colour when mature, but they did not develop at the same rate. They measured 15 μm in diameter. The columnellae were ovoid and small spinules on the outer surface were lost at maturity.

Influence of temperature on growth and sporulation. The results obtained are depicted in Figure 7. Maximum growth and sporulation took place at 25°C.

Figure 7. The colony diameter and vegetative reproductive structures produced by T. piriforme PPRI 5473 and PPRI 5534 on Mucor-synthetic agar after six days of incubation in the dark at various temperatures. The key to the different structures is given on the right side of the graph. a, Aerial hyphae; b, sporangiophore bearing restricted clusters of sporangioles; c, sporangiophore bearing well-developed clusters of sporangioles; d, sporangiophore bearing well-developed clusters of sporangioles and a terminal sporangium; e, sporangiophore bearing a terminal sporangium.
Influence of UV light on sporulation. At 25°C on SMA, UV irradiation enhances sporangiole formation and especially sporangium formation (not illustrated).

Physiological properties
The results of the physiological tests are summarized in Table 1.

Sporangiospores of *T. piriforme* PPRI 5473 and PPRI 5534 germinate and grow in aerobic conditions on a wide diversity of carbon sources. When comparing the pentose and hexose assimilation, as summarized in Table 1, it is interesting to note that the assimilable aldoses (L-arabinose, D-xylene, D-galactose and D-glucose) all show the same configuration at the asymmetric carbon atoms next to the carbonyl carbon atom (carbon atoms numbers two and three). The ketose, L-sorbose, could not be assimilated.

The disaccharides consisting of two D-glucopyranosyl moieties (i.e. celllobiose, maltose and trehalose), or a D-glucopyranosyl and a D-galactopyranosyl moiety bound in an α(1 → 6) linkage (i.e. melibiose) were assimilated (Table 1). The trisaccharide melitotose (O-α-D-glucopyranosyl-(1 → 3)-O-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside) and starch, a polysaccharide consisting of D-glucopyranosyl monomers, were also assimilated.

Only two of the nine alcohols tested as sole carbon source could support growth: the polyols D-mannitol and sorbitol (Table 1). Regarding the assimilation of organic acids, short-chain fatty acids with an even number of carbon atoms (i.e. acetic acid and butanoic acid) were utilized. Short-chain fatty acids with an odd number of carbon atoms (formic acid and propanoic acid), however, were not utilized. One of the complex organic acids (i.e. lactic acid) was utilized, while two (i.e. citric acid and gluconic acid) were not. It is interesting to note that in the stationary phase under aerobic conditions (Figure 8) a significantly higher amount

**Table 1** Physiological characteristics of *Thamnostylum piriforme* PPRI 5473 and PPRI 5473

| Assimilation of carbon sources | Pentoses | Polysaccharides |
|-------------------------------|----------|-----------------|
| D-arabinose                   | -        | soluble starch  |
| L-arabinose                   | +        | Glycoside       |
| D-ribose                      | -        | salicin         |
| D-xylene                      | +        | Alcohols        |
| Hexoses                       | erythritol |         |
| D-galactose                   | +        | ethanol         |
| D-glucose                     | +        | galactitol      |
| D-glucose                     | +        | glycerol        |
| L-rhamnose                    | -        | inositol        |
| L-sorbos                      | -        |                 |
| Disaccharides                 | D-mannitol |             |
| celllobiose                   | +        | methanol        |
| lactose                       | -        | ribitol         |
| maltose                       | +        | sorbitol        |
| melibiose                     | +        | Organic acids   |
| sucrose                       | -        | acetic acid     |
| trehalose                     | +        | butanoic acid   |
| Trisaccharides                | citric acid |             |
| meleztosose                   | +        | formic acid     |
| raffinose                     | -        | glucronic acid  |
| Fermentation of carbohydrates |          |                 |
| Pentoses                      |          |                 |
| D-arabinose                   | -        |                 |
| L-arabinose                   | -        |                 |
| D-ribose                      | -        |                 |
| D-xylene                      | -        |                 |
| Hexoses                       |          |                 |
| D-galactose                   | +        |                 |
| D-glucose                     | +        |                 |
| Disaccharides                 |          |                 |
| maltose                       | +        |                 |
| sucrose                       | -        |                 |
| Trisaccharides                |          |                 |
| raffinose                     | -        |                 |
| Growth without vitamins       | +        |                 |
| Growth in the presence of 0.01% cycloheximide | - | |
| Growth in the presence of 0.1% cycloheximide | - | |
| Gelatin liquefaction          | +        |                 |
| Splitting of arbutin          | +        |                 |
| Urease activity               | +        |                 |
| Lipase activity               | -        |                 |

*Tests were carried out in triplicate; symbols: +, positive result; -, negative result.
of biomass was obtained with acetic acid as carbon source than from the other carbon sources.

Other tests showed that only D-glucose, D-galactose and maltose could be fermented, the sporangiospores could germinate and grow in a synthetic medium devoid of vitamins, and that the fungus was sensitive to the antifungal drug cycloheximide at a concentration of 0.01% w/v.

Discussion

Morphology, growth and sporulation

The isolates of Thanassostylum piriforme produced morphologically similar spores from sporangia and sporangioles. These spores, however, were released in different ways. The sporangia deliquesced whereas the sporangioles disintegrated. Ultra-violet light stimulated sporangiole formation and especially promoted sporangium formation. Optimum growth and sporulation in the dark was found to occur at 25°C.

Physiology

The two strains of T. piriforme assimilated similar carbon sources to those assimilated by another mucoralean strain, representing Mucor circinelloides, tested under identical conditions (Botha et al. 1996). However, in contrast to the strain representing M. circinelloides, the strains of T. piriforme could not assimilate D-ribose, ethanol, ribitol or gluconic acid. On the other hand, the strains representing T. piriforme were able to utilize melibiose, while the strain representing M. circinelloides did not utilize this disaccharide. Before carbon assimilation patterns can be used in the taxonomy of these fungi, as is evident in the yeast domain (Van der Walt & Yarrow 1984), many more strains should be tested under identical conditions.

It was found that the strains representing T. piriforme produce the most biomass with acetic acid as carbon source. This is not surprising, since these strains were originally isolated on a medium containing this organic acid as carbon source (Botha et al. 1995). The results therefore indicate that as in Geotrichum candidum (Botha & Kock 1993), the fungal strains in this study should also be investigated for the production of bioproducts from an acetic acid-rich petrochemical effluent. In addition, gammanolinic acid can simultaneously be produced by the strains of T. piriforme during the same process. The ability of the strains to grow in a medium devoid of vitamins, may also be of biotechnological value, since the addition of these growth factors is critical in utilizing fungi in these processes. Furthermore, it is important to investigate the ability of fungi to assimilate aerobically and ferment different carbon sources, produce extracellular enzymes, grow and sporulate under various conditions. Other mucoralean fungi of potential biotechnological importance are currently being investigated in this manner.

Concluding remarks

The fungal strains representing T. piriforme in this study were able to grow in a medium devoid of vitamins. This ability, together with the fact that the strains produced the most biomass on acetic acid as carbon source, indicate that the fungus should be further investigated for economic bioprotein production from an acetic acid-rich petrochemical effluent.

Before planning a biotechnological process utilizing a fungus, as many characteristics as possible thereof must be known. Therefore, knowledge of the mode of sporulation, optimum physiological conditions and the utilization of carbon sources are critically important in utilizing fungi in these processes. Consequently, it is important to investigate the ability of fungi to assimilate aerobically and ferment different carbon sources, produce extracellular enzymes, grow and sporulate under various conditions. Other mucoralean fungi of potential biotechnological importance are currently being investigated in this manner.

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