Chapter 21

Detection and Enumeration of Heat-Resistant Molds

L. R. Beuchat and J. I. Pitt

21.1 INTRODUCTION

Spoilage of thermally processed fruits and fruit products by heat-resistant molds has been recognized in several countries.[5,8,9,10,12,15,22,24,29,30,31,39] *Byssochlamys fulva, B. nivea, Neosartorya fischeri, Talaromyces macrosporus, T. bacillisporus,* and *Eupenicillium brefeldianum* have been most frequently encountered.[10] *Byssochlamys* species have been recognized as spoilage molds in canned fruit since the early 1930s[12,13] and have been extensively studied.[2,5,14,21,29] Spoilage by other heat-resistant molds is a less serious problem recognized only in recent years. Consequently, less information is available concerning the behavior of these other genera in thermally processed fruit products.

Heat-resistant molds are characterized by the production of ascospores or similar structures with heat resistance, in some instances comparable to bacterial spores. This enables them to survive the thermal processes given to some fruit products (Table 1). Germination of ascospores may result in visible growth of mycelia on fruits and fruit products. Production of pectic enzymes by *Byssochlamys* can result in complete breakdown of texture in fruits[30] and also can result in off-flavor development.

Some *Byssochlamys* species produce patulin, byssotoxin A, and byssochlamic acid, all having toxic effects on laboratory animals.[5,20,27,32] *Neosartorya fischeri* is known to produce fumitremorgin A, B, and C, terrein, and verruculogen.[11,26] Heat-resistant molds, therefore, may constitute a public health hazard as well as a spoilage problem.

21.11 Distribution

Heat-resistant molds are widely distributed in the soil, particularly in vineyards, orchards, and fields in which fruits are grown.[8,12,13,29,36] Consequently, these molds may become contaminants on fruit and other vegetation upon contact with soil, before delivery to the processing plant. The number of ascospores on fruits is generally low, less than 1 per g.[20,36]

21.2 GENERAL CONSIDERATIONS

21.21 Samples

Because of their low incidence in fruit, ascospores are not likely to exceed 1 to 10 per 100 g or mL of processed products. Thus, for their effective detection, it is important that relatively large samples be analyzed. Centrifugation may be used to concentrate ascospores in liquid fruit products, the force and time necessary being influenced by volume, viscosity, and specific gravity of the sample.

Since the viability of ascospores is not appreciably affected by freezing and thawing, food samples can be stored frozen prior to analysis.

21.22 Enumeration Principles

A secondary but perhaps important point is that ascospores of heat-resistant molds may require heat activation before growth will occur.[3,16,18,20,38] The composition of the heating menstruum can influence the rate and extent of activation.[4,7,31,34] Maximal activation of *B. fulva* and *N. fischeri* var. *glaber* ascospores was obtained by heating at 70°C for 30 min in grape juice; in distilled water, 120 min were required for *B. fulva* while only 1% of the *N. fischeri* ascospores were activated.[37] Different strains within the same species may require different treatment times and temperatures to achieve maximal activation.

Detection and enumeration of heat-resistant ascospores rely on a selective heat treatment that inactivates vegetative cells of fungi and bacteria as well as less heat-resistant spores.

Heat-resistant molds are not fastidious in their nutrient requirements, and therefore the media listed below as well as many fruit juice agars will support germination of ascospores and subsequent vegetative growth. Since ascospores may be stressed by the heating process, highly acidic media are not recommended. However, when culturing low-acid foods that are heavily contaminated with bacterial spores, acidification or the addition of chloramphenicol to the plating medium may be required to inhibit the bacteria.

21.3 EQUIPMENT, MATERIALS, AND REAGENTS

Potato dextrose agar (PDA)
Malt extract agar (MEA)
Orange serum agar (OSA)
Czapek yeast autolysate (CYA) agar

Table 1. Tolerance of Heat-resistant Molds Isolated from Foods[a]

Mold	Heat-resistant Structure	Heating Medium	Heat Resistance
Byssochlamys fulva	Ascospores	Glucose-tartaric acid, pH 3.6	90°C, 51 min, 1000-fold[2]
		Grape juice, 26° Brix	85°C, 150 min, 100-fold[21]
Byssochlamys nivea	Ascospores	Grape juice	88°C, survived 60 min[19]
		Apple Juice	99°C, survived in juice[1]
Eupenicillium lapidosum	Ascospores	Blueberry juice	81°C, 10 min, survival; 81°C, 15 min, death[41] z = 10.3°F
	Cleistothecia	Blueberry juice	93.3°C, 9 min, growth; 93.3°C, 10 min, death[41] z = 10.6°F
Eupenicillium brefeldianum	Ascopores	Apple juice	90°C, 1 min, death[40] z = 7.2°C
	Cleistothecia	Apple juice	90°C, 220 min, death[40] z = 11.7°C
Talaromyces macrosporus	Ascospores	Apple juice	90°C, 2 min, death[40] z = 7.8°C
	(3 isolates)	Fruit-based fillings	D91°C = 2.9 to 5.4 min[3] z = 9.4 to 23.3°F
		Apple juice	D90.6°C = 1.4 min[3] z = 9.5°F
		Apple juice	D90.6°C = 2.2 min[33] z =5.2°C
	Cleistothecia	Apple juice	90°C, 80 min, death[40] z = 11.7°C
Monascus purpureus	Whole culture	Grape juice	Survival several min 100°C[9]
Humicola fuscoatra	Chlamydospores	Water	80°C, 101 min, 10-fold inactivation[23]
Phialophora sp.	Chlamydospores	Apple juice	80°C, 2.3 min, 10-fold inactivation[14]
Neosartorya fischeri	Ascospores	Water	100°C, 60 min, survival[17]
	(3 isolates)	Fruit-based fillings	D91°C = <2.0 min; D88°C = 4.2–16.2 min[3] z = 5.4 = 11°F
		Apple juice	87.8°C, 1.4 min[33] z = 5.6°C
Neosartorya fischeri var. *glaber*	Ascospores	Water	90°C, 60 min, survival[24]
		Grape juice	85°C, 10 min, 10% survival[37]
Thermoascus auranthiacum	Whole culture	Grape juice	88°C, 60 min, survival[19]

[a] Adapted from Splittstoesser and King.[35]

21.4 PROCEDURES

21.41 Petri Dish Method[25,36]

Fruits and products containing pieces of fruits must be blended or homogenized before analysis can proceed. To a tared sterile blender jar, add 100 g of fruit or fruit product plus 100 mL of sterile water and blend 5 min or until mixture appears homogeneous. After blending, enclose the jar in a polyethylene bag to safeguard against leakage through the bottom bushing and place the jar in a closed 75°C to 80°C waterbath for 1.5 hr. This holding time ensures that the homogenate will be at 75°C for at least 30 min.

Alternatively, samples (100 g plus 100 mL of sterile water) may be homogenized by a stomacher for 2 to 4 min (see Chapter 2). Two 50-mL portions of homogenate are then transferred to sterile 200 × 30 mm test tubes and placed in a closed waterbath at 75°C to 80°C for 30 min. The surface of the sample in the jars or tubes should be well below the surface of the water in the bath throughout the heat treatment.

After heating, duplicate 50-mL samples of thoroughly mixed homogenate in blender jars or the entire contents (50 mL) of duplicate tubes of sample that had been homogenized in a stomacher are combined with PDA or MEA in 150-mm diameter petri dishes. Each 50-mL sample is equally distributed in four dishes and thoroughly mixed with 10 mL of 1.5 strength agar. Petri dishes are loosely sealed in a plastic bag to prevent drying and incubated at 30°C for up to 30 days. Most viable ascospores will germinate and form visible colonies within 7 to 10 days; however, heat-injured and other debilitated ascospores may require additional time to form colonies. The 30-day incubation time also enables molds to mature and sporulate, thus aiding their identification.

Fruit juices (35° Brix or less) are analyzed in a manner identical to that described above. Fruit juice products (35° Brix or more) and fruit juice concentrates should be diluted (1:2) with sterile water and thoroughly mixed before heat treatment is applied to duplicate 50-mL samples. The general procedure is illustrated in Figure 1.

21.42 Direct Incubation Method[18,30]

The petri dish method is subject to error from aerial contamination (see Section 21.51). An alternative method that avoids this problem is described in this section. It is suitable for fruit pulps and homogenates.

Homogenized 50-mL samples are heated in flat-sided bottles such as 100-mL medicine flats. Bottles are heated in an upright position in a waterbath at 80°C for 30 min and then incubated on their sides, allowing as large a surface area as possible, at 30°C for up to 30 days. This procedure avoids the risk of contamination from the air and minimizes loss of moisture. Colonies develop on the surface of the homogenate.

Larger samples, such as 100-mL quantities in 200-mL bottles, can also be handled by this method. One apparent disadvantage is that colonies developing in the bottles must be picked and grown on suitable media for identification. However, cultivation on identification media is also recommended with the petri dish method.

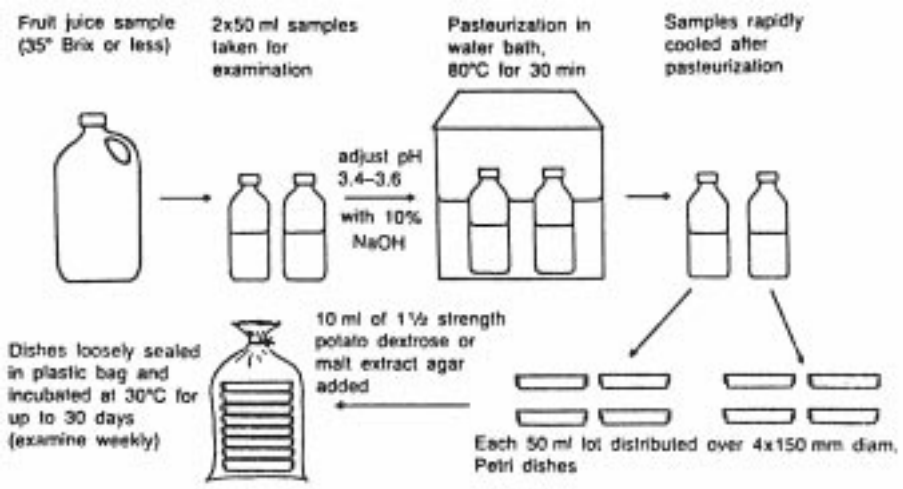

Figure 1. *Procedure for detection and enumeration of heat-resistant mold spores. (From Hocking and Pitt[10]).*

21.5 PRECAUTIONS

21.51 Air Contamination

With the petri dish method, aerial contamination during plating may be a problem. The appearance of green *Penicillium* colonies, or colonies of common *Aspergillus* species such as *A. flavus* and *A. niger* is a clear indication of contamination, as these molds are not heat resistant. To minimize this problem, pour plates in clean, still air or use a laminar flow hood, if possible. Alternatively, use the direct incubation method.

21.6 INTERPRETATION

Heat-resistant mold ascospores are not uncommon on fruit when it is received at the processing plant.[36] The acceptable level of contamination will depend on whether the fruit is a major or minor ingredient, whether the final product will contain a preservative such as sorbate or benzoate, and the thermal process to which the fruit is to be subjected. A count of 5 ascospores per 100 g (mL) of product at a stage just prior to the retort or heat exchanger indicates a serious problem. For ultra-high temperature (UHT) processed fruit juice blends that do not contain a preservative, even a lower level of contamination is unacceptable. One manufacturer of pasteurized fruit juices has issued a specification that calls for the absence of *Byssochlamys* from a 100-mL sample taken from each 200-L drum of raw material.[6]

21.7 TAXONOMY OF IMPORTANT HEAT-RESISTANT MOLDS

The basis for high heat resistance in these molds is the production of a teleomorph; that is, they form ascospores. Ascospores are produced, generally, in groups of eight, within a closed sac, the ascus (plural, asci); ascospores are the prime characteristic of the class of fungi called ascomycetes. In nearly all ascomycete genera, asci are in turn enclosed, in large numbers, within larger bodies. In genera of interest here, these bodies may have a solid, totally enclosed wall (a cleistothecium) or be composed of fine, interwoven hyphae (a gymnothecium). Only in *Byssochlamys* are asci borne singly and unenclosed on a layer of fine contorted hyphae.

As well as ascospores, ascomycetes generally produce an anamorph with asexual spores called conidia (singular, conidium). Conidia are not very heat resistant and are usually readily destroyed by pasteurizing heat processes or the screening techniques outlined above. The molds of interest here produce conidial states characteristic of the genera *Aspergillus, Paecilomyces,* and *Penicillium*.

21.71 Identification of Isolates

To identify heat-resistant mold isolates, proceed as follows. Inoculate each isolate onto media in four 90-mm diameter petri dishes, two each of CYA and MEA. Inoculate each plate at three equally spaced points. Incubate one plate each of MEA and CYA at 25°C, and the others at 30°C.

After incubation for 7 days, examine plates by eye, measuring colony diameters with a ruler, and make wet mounts to examine small pieces of mold under a compound microscope. The following key will assist in identification of common heat-resistant molds. For less common species, see Hocking and Pitt.[10, 28]

21.72 Key to Common Heat-resistant Fungi

1. Asci produced in discrete bodies with totally enclosed walls (cleistothecia)—*Neosartorya fischeri*
   Asci produced in bodies with walls of woven hyphae (gymnothecia) or openly—2
2. Asci enclosed in gymnothecia—*Talaromyces macrosporus*
   Asci produced openly; fine hyphae may be present, but asci not enclosed—3
3. Colonies on CYA and MEA predominately buff or brown—*Byssochlamys fulva*
   Colonies of CYA and MEA persistently white or cream—*Byssochlamys nivea*

21.721 Genus Byssochlamys *Westling*

Byssochlamys has the distinction of being almost uniquely associated with food spoilage and in particular with the spoilage of heat-processed acid foods. Its natural habitat appears to be soils, but the genus is mentioned very seldom in lists of molds from soils other than those used for the cultivation of fruits.

Byssochlamys is an ascomycete genus characterized by the absence of cleistothecia, gymnothecia, or other bodies that in most ascomycetes envelop asci during development. Asci in *Byssochlamys* are borne in open clusters, in association with, but not surrounded by, unstructured wefts of fine white hyphae.

In our experience, the temperature range for observation of *Byssochlamys* asci and ascospores in the laboratory is sometimes very narrow. Cultures need to be incubated at 30°C as some isolates do not produce asci at 25°C or 37°C. However, presumptive evidence of the presence of *Byssochlamys* or other heat-resistant molds can be made from plates incubated at 25°C or 37°C if the isolate has come from heat-processed food or raw materials.

Byssochlamys fulva Olliver and G. Smith (Figure 2)
Anamorph: *Paecilomyces fulvus* Stolk and Samson

At 25°C, colonies on CYA and MEA are at least 60 mm diameter, often covering the whole petri dish, relatively sparse, low, or somewhat floccose, with conidial production heavy, uniformly colored olive brown, and reverse in similar colors or pale. At 30°C, colonies on CYA and MEA usually cover the entire surface, and are low to moderately deep, sparse, with moderate conidial production, colored brown, overlaid by white hyphae from which asci are produced; reverse is olive brown to deep brown.

Teleomorph, observed as single asci, which are borne from, but not enveloped by, wefts of contorted white hypae, best developed at 30°C, maturing in 7 to 12 days, occasionally formed at 25°C in fresh isolates but maturing slowly if at all, with asci spherical to subspheroidal, 9 to 12 µm diameter, and ascospores ellipsoidal, hyaline, or straw-colored, 5 to 7 µm long, and smooth-walled.

Anamorph is best observed at 25°C, consisting of penicilli borne from surface hyphae or long, trailing, aerial hyphae, stipes 10 to 30 µm long, with phialides of variable appearance, flask-shaped or narrowing gradually to the apices, 12 to 20 µm long, and conidia mostly cylindrical or barrel-shaped, narrow and 7 to 10 µm long, but sometimes longer, wider, or ellipsoidal from particular phialides, smooth-walled.

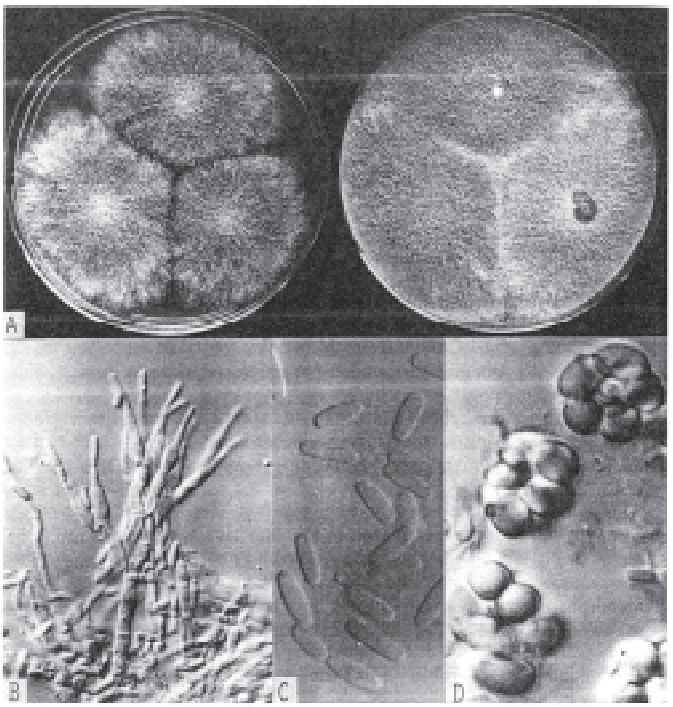

Figure 2. *Byssochlamys fulva: (A) colonies on CYA and MEA, 7 days, 25°C; (B) penicillus, x 750; (C) conidia, x 1875; (D) asci and ascospores, x 1875.*

Byssochlamys nivea Westling
Anamorph: *Paecilomyces nivea* Stolk and Samson

At 25°C, colonies on CYA are 40 to 50 mm diameter, low and quite sparse, white to slightly grey, with reverse pale to mid-brown. Colonies on MEA cover the whole petri dish, low and sparse, white to creamish, with small knots of dense hyphae, and reverse pale to brownish. At 30°C on CYA, colonies cover the whole petri dish, similar to those on MEA at 25°C, but often more dense, enveloping distinct knots of dense hyphae.

Teleomorph is similar to that of *B. fulva* except for slightly smaller asci (8 to 11 µm diam) and ascospores (4 to 6 µm diam), maturing in 10 to 14 days at 25°C and in 7 to 10 days at 30°C.

Anamorphs of two kinds are produced, aleurioconidia and penicilli; aleurioconidia borne singly, common at 30°C and 37°C, spherical to pear-shaped, 7 to 10 µm diam, with irregular penicilli sparsely produced and phialides sometimes borne solitarily from hyphae as well, phialides 12 to 20 µm long, cylindrical then gradually tapering, and conidia ellipsoidal to pear-shaped, 3 to 6 µm long, smooth-walled.

21.722 Genus Neosartorya *C. R. Benjamin*

A genus of soil fungi, *Neosartorya* is of interest in food microbiology only because its highly heat-resistant ascospores occur from time to time in heat-processed foods and it has occasionally been reported as a cause of spoilage. Although there are several species, only *N. fischeri* is commonly isolated from foods.

Neosartorya fischeri (Wehmer) Malloch & Cain (Figure 3)
Anamorph: *Aspergillus fischerianus* Samson & W. Gams

On CYA and MEA at 25°C, colonies 50 to 65 mm or more diameter, low and sparse to moderately deep, of cottony white to cream mycelium, surrounding abundant white developing cleistothecia and overlaid by scattered, usually inconspicuous blue to green conidial heads, with reverse pale to yellow. At 30°C, colonies cover the whole petri dish, similar to those at 25°C, but often are deeper and more luxuriant.

Cleistothecia white, 300 to 400 µm diameter, mature in 1 to 2 weeks at 25°C, with ascospores ellipsoidal, overall 6 to 7 × 4 to 5 µm, ornamented with two prominent, sinuous, longitudinal ridges and usually with other irregular ridges as well. Anamorph *Aspergillus*, with sparse conidiophores, 300 to 1000 µm long, terminating in small swellings, 12 to 18 µm diameter, with phialides crowded, 5 to 7 µm long, and conidia spheroidal, 2.0 to 2.5 µm diameter, with finely roughened walls.

21.723 Genus Talaromyces *C. R. Benjamin*

Talaromyces is characterized by the production of yellow or white gymnothecia in association with anamorph characteristics of *Penicillium*, *Paecilomyces,* or *Geosmithia*. It is a genus of about 25 species, mostly soil inhabiting. The commonly encountered species is *T. flavus*, and until recently this was believed to be the correct name for the species which sometimes occurs in heat processed foods. However, the heat resistant species is now considered to be distinct and is referred to as *T. macrosporus*.

Talaromyces macrosporus (Stolk and Samson) Frisvad et al. (Figure 4)
Anamorph: *Penicillium macrosporus* Frisvad et al.

At 25°C, colonies on CYA are usually 20 to 40 mm diameter, plane, low, and quite sparse to moderately deep and cottony, with mycelium pale to bright yellow, in most isolates concealing developing gymnothecia, also clear to reddish exudate present occasionally, and reverse sometimes yellow, more usually orange, reddish, or brown. Colonies on MEA are 30 to 50 mm diameter, generally similar to those on CYA, but gymnothecia are more abundant, with reverse usually dull orange or brown, but sometimes deep brown or deep red. At 30°C on CYA, colonies are 30 to 45 mm diameter, and generally similar to those at 25°C, but sometimes with white or brown mycelium or overlaid with grey conidia or with conspicuous red soluble pigment and reverse color. At 30°C on MEA, colonies are similar to those at 25°C, usually produce abundant gymnothecia, and in reverse sometimes produce red or olive colors also.

Gymnothecia of tightly interwoven mycelium, bright yellow, about 200 to 500 µm diameter, closely packed, mature within 2 weeks, with ascospores yellow, ellipsoidal, commonly 5.0 to 6.5 µm long, with spinose walls. Anamorph *Penicillium*, with conidiophores borne from aerial hyphae and stipes 20 to 80 µm long, bear terminal biverticillate or, less commonly, monoverticillate penicilli, and have phialides needle-shaped, 10 to 16 µm long, and conidia ellipsoidal, 2.5 to 4.0 µm long, with smooth to spinulose walls.

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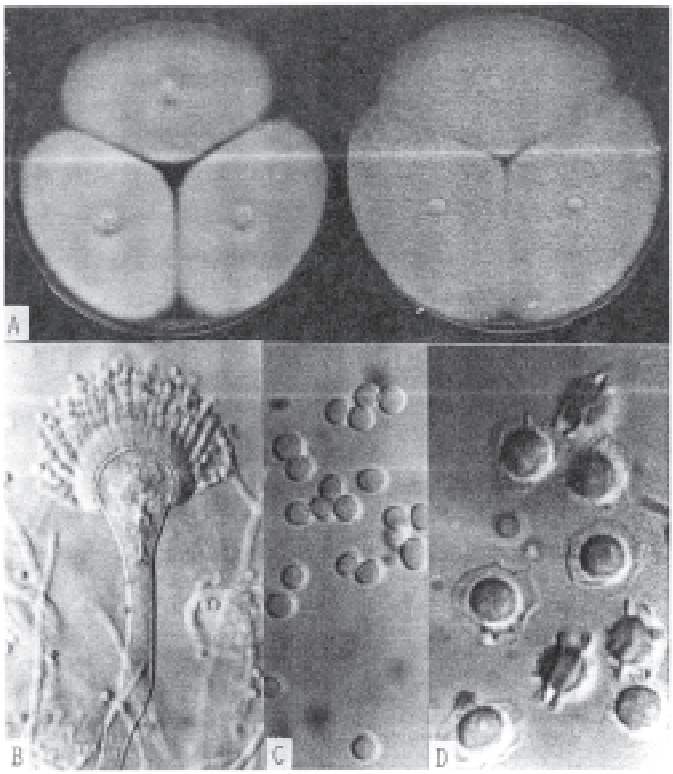

Figure 3. *Neosartorya fischeri: (A) colonies on CYA and MEA, 7 days 25°C; (B) conidiophore, x 750; (C) conidia, x 1875; (D) ascospores, x 1875.*

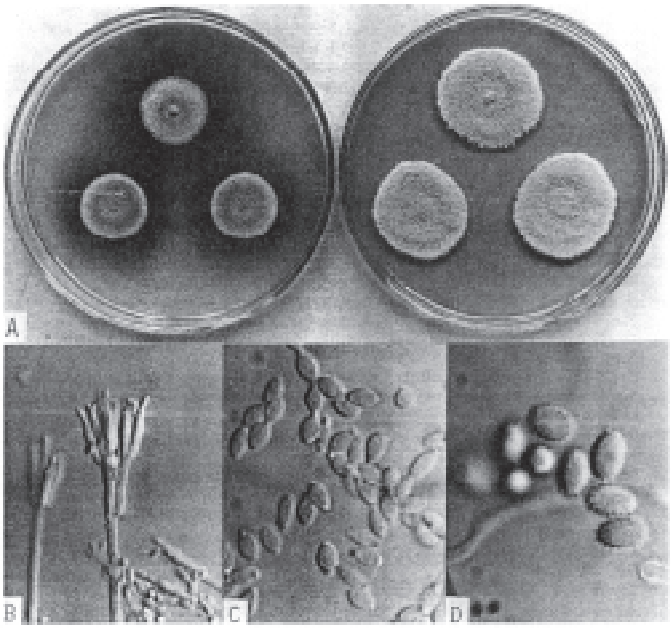

Figure 4. *Talaromyces marcosporus: (A) colonies on CYA and MEA, 7 days, 25°C; (B) penicillus, x 750; (C) conidia, x 1875; (D) ascospores, x 1875.*

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