BYSSOCHLAMYS: SIGNIFICANCE OF HEAT RESISTANCE AND MYCOTOXIN PRODUCTION

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1. INTRODUCTION

Byssochlamys species produce ascospores that are very heat-resistant and survive heating above 85°C for considerable periods (Beuchat and Rice, 1981; Splittstoesser, 1987). Besides their heat resistance, *Byssochlamys* species are also able to grow under very low oxygen tensions (Tanawaki, 1995) and are capable of producing pectinolytic enzymes. The combination of these three physiological characteristics make *Byssochlamys* species very important spoilage fungi in pasteurized and canned fruit in which they can cause great economical losses. The natural habitat of *Byssochlamys* is soil. Fruit that grow near the soil, or are harvested from the ground may thus become contaminated with *Byssochlamys* (Olliver and Rendle, 1934; Hull, 1939).

Besides causing spoilage of pasteurized products, some *Byssochlamys* species are also capable of producing mycotoxins, including patulin, byssotoxin A and byssochlamic acid (Kramer et al., 1976; Rice, 1977). An antitumor metabolite, byssochlamysol, a steroid against IGF-1 dependent cancer cells, is also produced by *Byssochlamys nivea* (Mori et al. 2003).

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In the taxonomic revision of Samson (1974), three *Byssochlamys* species were accepted: *B. fulva*, *B. nivea* and *B. zollerniae*. *B. verrucosa* was subsequently described (Samson and Tansey, 1975). Only *B. nivea* and *B. fulva* are currently considered important food spoilage fungi or mycotoxin producers.

Recently we have encountered numerous food spoilage problems in which species of *Byssochlamys* and their *Paecilomyces* anamorphs were involved. To elucidate the taxonomic and ecological characteristics of these isolates in relation to heat resistance and mycotoxin production we have investigated *Byssochlamys* and *Paecilomyces* isolates from various origins, including pasteurized fruit, ingredients based on fruit, and soil. Using a polyphasic approach, a classification of food-related heat resistant *Byssochlamys* species is presented.

2. MATERIALS AND METHODS

2.1. Isolates

The 39 isolates of *Byssochlamys* and *Paecilomyces* studied are listed in Table 1. All isolates are maintained in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

2.2. Media

The following media were used in this study: Czapek Yeast Autolysate agar (CYA), Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Yeast Extract Sucrose agar (YES), Hay infusion agar (HAY), Cornmeal agar (CMA), Creatine sucrose agar (CREA) (Samson et al., 2004) and Alkaloid agar (ALK) (Vinokurova et al.).

2.3. Macromorphological characterisation

The macroscopical features used were acid production on CREA, colony diameter on MEA after 72 h. and colony diameter and degree of growth on CYA after 7 days at 30°C.

2.4. Micromorphological characterisation

Isolates were grown on several media for 5-70 d. Micromorphology of the anamorphic *Paecilomyces* states was characterised on MEA,

Table 1. Byssochlamys and *Paecilomyces* isolates used in this study

CBS[a] No.	Species	Source, remarks about culture	Asco-spores
132.33	*Byssochlamys fulva*	Bottled fruit, Type of *Paecilomyces fulvus*	+
146.48	*Byssochlamys fulva*	Bottled fruit, Type of *Byssochlamys fulva*	–
135.62	*Byssochlamys fulva*	Fruit juice, Type of *Paecilomyces todicus*	+
604.71	*Byssochlamys fulva*	Unknown source	–
113245	*Byssochlamys fulva*	Pasteurized fruit juice	+
113225	*Byssochlamys fulva*	Multifruit juice	+
100.11	*Byssochlamys nivea*	Unknown source, Type of *Byssochlamys nivea*	+
133.37	*Byssochlamys nivea*	Milk of cow, Type of *Arachniotus trisporus*	–
606.71	*Byssochlamys nivea*	Oat grain, received as *Byssochlamys musticola*	–
546.75	*Byssochlamys nivea*	Unknown source	+
271.95	*Byssochlamys nivea*	Mushroom bed	+
102192	*Byssochlamys nivea*	Pasteurized drink yoghurt	+
113246	*Byssochlamys nivea*	Apple compote	+
373.70	*Byssochlamys laguncullariae*	Wood of *Laguncularia racemosa* (Mangue), Type of *B. nivea* var. *laguncullariae*	+
696.95	*Byssochlamys laguncullariae*	Pasteurized strawberries	+
338.51	*Byssochlamys spectabilis*	Fruit juice	–
102.74	*Byssochlamys spectabilis*	Unknown source, Type of *Paecilomyces variotii*	–
298.93	*Byssochlamys spectabilis*	Man, breast milk of patient	–
101075	*Byssochlamys spectabilis*	Heat processed fruit beverage, Type of *Talaromyces spectabilis*	+
109072	*Byssochlamys spectabilis*	Pectin, teleomorph present	+
109073	*Byssochlamys spectabilis*	Pectin, teleomorph present	+
110431	*Byssochlamys spectabilis*	Rye bread	–
284.48	*Byssochlamys divaricatum*	Mucilage bottle with library paste, Type of *Penicillium divaricatum*	– initials
110428	*Byssochlamys divaricatum*	Pectin	–, initials
110429	*Byssochlamys divaricatum*	Pectin	–, initials

Table 1. Byssochlamys and *Paecilomyces* isolates used in this study—*cont'd*

CBS[a] No.	Species	Source, remarks about culture	Asco-spores
110430	*Byssochlamys divaricatum*	Pectin	+
604.74	*Byssochlamys verrucosa*	Nesting material of *Leipoa ocellata*	+
605.74	*Byssochlamys verrucosa*	Nesting material of *Leipoa ocellata,* Type	+
374.70	*Byssochlamys zollerniae*	Wood of *Zollernia ilicifolia* and *Protium heptaphyllum*, Type	+
628.66	*Paecilomyces maximus*	Quebracho-tanned sheep leather, France	–
371.70	*Paecilomyces maximus*	Annona squamosa, Brazil, Type of *Paecilomyces maximus*	–
990.73B	*Paecilomyces maximus*	Unknown source, Type of *Monilia Formosa*	–
296.93	*Paecilomyces maximus*	Man, bone marrow of patient	–
297.93	*Paecilomyces maximus*	Man, blood of patient	–
323.34	*Paecilomyces dactylethromorphus*	Unknown source, Type of *Paecilomyces mandshuricus* var. *saturatus*	–
223.52	*Paecilomyces dactylethromorphus*	Leather	–
251.55	*Paecilomyces dactylethromorphus*	Acetic acid, Type of *Paecilomyces dactylethromorphus*	–
990.73A	*Paecilomyces dactylethromorphus*	Unknown source, Type of *Penicillium viniferum*	–
492.84	*Paecilomyces dactylethromorphus*	*Lepidium sativum*	–

[a]CBS is the culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands

HAY and YES agars (Samson et al., 2004). The latter was used for the determination of the presence of chlamydospores. For the analyses of the features of the teleomorphic *Byssochlamys* state, OA and PDA (Samson et al., 2004) were used. The microscopical features recorded included shape and size of conidia, size and ornamentation of ascospores and presence and ornamentation of chlamydospores. Ornamentation of the surface of the conidia, chlamydospores and ascospores was determined by light microscopy after prolonged incubation up to 70 days.

2.5. Multivariate analyses

A matrix consisting of 39 objects (fungal isolates) and 10 variables (macro- and microscopical features) was constructed. Cluster analysis by unweighted pair-group method, arithmetic average (UMPGA) was performed on the data matrix using BIOLOMICS™ software (Bioaware S.A., Hannut, Belgium).

2.6. Secondary metabolite analysis

Isolates studied (see Table 1) were three point inoculated on MEA, YES, PDA, OA, CYA and ALK agars. All isolates were analysed for secondary metabolites after two weeks growth at 30°C. The cultures were extracted according to the method of Smedsgaard (1997) and analysed by HPLC with diode array detection (Frisvad and Thrane, 1993). The metabolites found were compared with a spectral UV library made from authentic standards run under the same conditions, and retention indices were compared with those of standards. The maximal similarity was a match of 1000.

2.7. Heat resistance

The strain *Talaromyces spectabilis* CBS 109073 (now considered to be a *Byssochlamys* species, see below) was inoculated at three points on CMA and incubated for 45 days at 30°C. After incubation, the parts of the colony where ascospores were produced were combined and transferred to 10 mM ACES buffer (pH 6.8, N-[2-acetamido]-2-aminoethane-sulfonic acid; Sigma) supplemented with 0.05% Tween 80. The intact asci were ruptured by suction through a 0.9 mm hypodermic needle with a syringe and by agitation with glass beads. The suspension was sonicated briefly (3 times for 30 s) and filtered through sterile glass wool. The ascospores together with conidia and other fungal fragments were centrifuged at 1,100×g (5 min) and washed three times in buffer.

D values in ACES buffer were determined at 85°C in duplicate. The spore suspension was pre-treated by heating at 65°C for 10 min to eliminate conidia, chlamydospores and hyphae. The suspension was then heated at 85°C at various times (see Figure 2). After heating, the suspension was cooled, serially diluted in sterile water, then spread plated onto CYA and incubated for 5-10 days at 30°C. Colonies were counted and D_{85} values calculated.

3. RESULTS

3.1. Morphological analyses

Paecilomyces variotii sensu lato and anamorphs of *Byssochlamys* species share several micromorphological characteristics, including phialides with cylindrical bases tapering abruptly into long cylindrical necks. The conidia are produced in long divergent chains (Samson, 1974). There are characters that are constant at the species level but distinct between species. Microscopical features such as the shape of the conidia, the sizes of the conidia and ascospores and the presence of chlamydospores can be used to group species belonging to *Byssochlamys* and *Paecilomyces*. Acid production on CREA, colony diameters and degree of growth on CYA are useful characters too. Table 2 summarises the results of the micro- and macroscopical analyses.

The classification of the *Byssochlamys* and *Paecilomyces* taxa based on phenotypical characters is also supported by a molecular taxonomic study of this complex (partial β-tubulin gene sequencing) (Samson et al., submitted).

Figure 1 shows that nine clades could be distinguished among the isolates studied (Table 1). Clades 1, 2 4 and 5 separate the four known species of *Byssochlamys*: *B. verrucosa, B. zollerniae, B. fulva* and *B. nivea*. Clade 6 includes the ex-type culture of *B. nivea* var. *lagunculariae* (CBS 373.70) and CBS 696.95, isolated from strawberries. Clades 3, 7, 8 and 9 are isolates have been classified in *Paecilomyces variotii* complex, now seen to include several taxa. Clade 8 includes the ex-type cultures of *Paec. variotii* and *Talaromyces spectabilis*. Re-examination of the ex-type culture of *T. spectabilis* shows that it logically belongs in *Byssochlamys* and the formal combination *Byssochlamys spectabilis* (Udagawa & Suzuki) Samson et al. is proposed (Samson et al., submitted). Strains which produce ascospores are rare and if ascospores are formed, they often develop only after prolonged incubation. In this clade many strains isolated from drinking yoghurt and pectin are accommodated.

Clade 3 contains the ex-type culture of *Penicillium divaricatum. Pen. divaricatum* Thom 1910 was considered to be a synonym of *Paec. variotii* by Thom (1930). In one isolate, CBS 110430, we have observed ascospore production of the *Byssochlamys* type after prolonged incubation (70 d). We have therefore erected the new name *Byssochlamys divaricata* Samson et al. for *Pen. divaricatum* (Samson et al., submitted). The strains examined were isolated from pectin and

Table 2. Macro- and microscopical features of *Byssochlamys* and *Paecilomyces* isolates

Species	Conidial size (µm) and shape	Chlamydo-spores[a]	Ascospore size (µm) and ornamentation	Colony diameter (mm)[b]	Degree of growth[c]	Acid[d]
B. divaricatum	3.2-4.6 × 1.6-2.5; ellipsoidal to cylindrical with truncate ends	– (+)	5.3-7.0 × 3.8-4.9, smooth	10-17	Moderate	–
B. fulva	3.7-7.5 × 1.4-2.5; cylindrical with truncate ends	– (+)	5.3-7.1 × 3.3-4.3, smooth	(50), >80	Good	+
B. laguncullariae	2.7-4.5 × 2.2-3.3; globose with flattened base	+, smooth	3.8-5.0 × 3.0-3.9, smooth	45-55	Good	–
B. nivea	3.0-4.7 × 2.3-4.0; globose to ellipsoidal with flattened base	+, smooth to finely roughened	4.1-5.5 × 2.9-3.9, smooth	(8) 28-50	Weak	– (+)
B. spectabilis	3.3-6.1 × 1.5-4.4; mostly ellipsoidal and ellipsoidal with truncated ends	+, smooth to finely roughened	5.2-6.8 × 3.5-4.5, almost smooth, sl. roughened	25-40 (56)	Good	–
B. verrucosa	6.3-13.1 × 1.6-4.7; cylindrical with truncate ends	–	6.6-8.4 × 4.0-6.1, rough	25-40	Good	–
B. zollerniae	2.5-4.0 × 1.5-3.0; globose to ellipsoidal, apiculate	+, warted	3.0-4.5 × 2.5-3.0, smooth	30-35	Weak	–
P. dactylethro-morphus	2.3-7.0 × 1.7-3.4; mostly cylindrical and ellipsoidal **without** truncated ends	+, smooth	No ascospores detected	22-55	Good	–
P. maximus	3.0-10 × 1.8-3.5; ellipsoidal to cylindrical with truncate ends	+, smooth and often pigmented	No ascospores detected	18 – >80	Good	+

[a] +, chlamydospores present; –, chlamydospores absent, (+) chlamydospores produced by some isolates after prolonged incubation (40 days); [b] Colony diameter on CYA, 72 h, 30°C; [c] Degree of growth on CYA, 7 d, 30°C; [d] Acid production in CREA, 7 d, 30°C

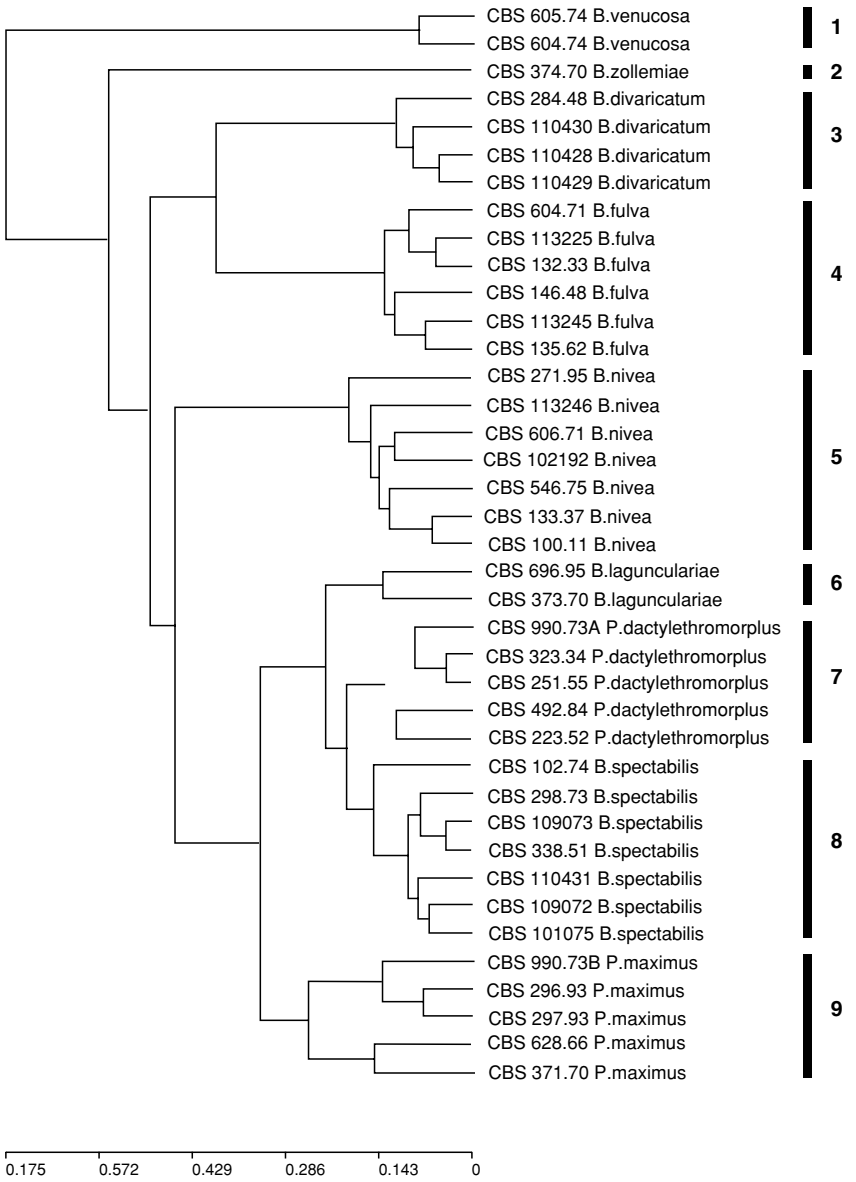

Figure 1. An UPGMA dendrogram based upon micro- and macromorphological characteristics of *Paecilomyces variotii* and *Byssochlamys* isolates.

fruit concentrates, and the ex-type culture came from a mucilage bottle with library paste. Clade 7 includes the ex-type cultures of *Paec. dactylethromorphus* Batista & H. Maia and *Paec. mandshuricus* var. *saturatus* Nakazawa et al. while the ex-type culture *Paec. maximus* C.

Ram is accommodated in Clade 9. These three taxa are now considered to be synonyms of *Paec. variotii*. Both clades contain strictly conidial isolates and no ascospores have been observed.

3.2. Mycotoxin analysis

Species in *Byssochlamys* and related *Paecilomyces* species can also be distinguished by differences in secondary metabolites. The results of the analyses are summarized in Table 3. *B. spectabilis* produced the mycotoxin viriditoxin. Some isolates of *B. nivea* and *P. dactylethromorphus* produced patulin, however, many did not. Mycophenolic acid was produced by *B. nivea* and *B. laguncullariae*, whereas emodin was produced by *B. divaricatum*. Byssotoxin A has been reported to be produced by isolates of *B. fulva* (Kramer, 1976) but because the structure of byssotoxin A was not elucidated, isolates were not screened for the presence of this mycotoxin.

3.3. Heat resistance

Paecilomyces and *Byssochlamys* isolates in the CBS collection were re-identified as described above. The results of the identification were related to the origin of the isolates. In Table 4 the number of strains isolated from heat-treated products or samples is correlated with the total number of isolates. The Table shows that species with a teleomorph are more often found in heat treated products, with the exception of *B. verrucosa* and *B. zollerniae* which do not occur in foods. The presence of *P. variotii sensu stricto* (the anamorph of *B. spectabilis*) in heat treated products could be explained by the production of heat resistant ascospores.

Table 3. Mycotoxin production by *Byssochlamys* and *Paecilomyces* species[a]

Species	Known mycotoxins
Byssochlamys fulva	Byssochlamic acid
Byssochlamys nivea	Patulin, mycophenolic acid, byssochlamic acid
Byssochlamys laguncullariae	Mycophenolic acid, byssochlamic acid
Byssochlamys spectabilis	Viriditoxin
Byssochlamys divaricatum	Emodin
Byssochlamys verrucosa	Byssochlamic acid
Byssochlamys zollerniae	No known mycotoxins detected
Paecilomyces maximus	No known mycotoxins detected
Paecilomyces dactylethromorphus	Patulin

[a] Many other metabolites are produced but not listed here

Table 4. Overview of isolates in CBS collection correlated with origin[a]

Species	No. isolates investigated	No. isolates from heat-treated products	Percentage from heat-treated products
Byssochlamys fulva	5	5	100
Byssochlamys nivea	5	3	60
Byssochlamys langucullariae	2	1	50
Byssochlamys spectabilis	17	6	35
Byssochlamys divaricatum	4	3	75
Byssochlamys verrucosa	2	0	0
Byssochlamys zollerniae	1	0	0
Paecilomyces maximus	6	0	0
Paecilomyces dactylethromorphus	5	0	0

[a] CBS is the culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands. Isolates of unknown origin are excluded

Two laboratory experiments were conducted on 45 day-old ascospores of *B. spectabilis* in ACES buffer. Conidia, chlamydospores and other fungal fragments were inactivated as described above (Section 2.7.). Figure 2 shows one of the two thermal death rate curves. Between 0 and 8 minutes of the heat-treatment, activation of the ascospores occurs, then a linear correlation exists between time and the logarithm of surviving ascospores. Regression analyses on the best fit resulted in two equations:

$$\log CFU = -0.0170^{*}T\ (min) + 5.9779\ (r\ 0.972,\ p < 0.05\ );$$

and

$$\log CFU = -0.0179^{*}T\ (min) + 6.379\ (r = 0.976,\ p < 0.05),$$

for experiments 1 and 2, respectively. The D_{85} value was calculated from these equations. Experiment 1 resulted in a D_{85} of 59 min, and the D_{85} value in experiment 2 appeared to be 56 min. Taking the 95% confidence level into account, the D_{85} value may vary between 49 and 75 min in experiment 1 and between 47 and 70 min in experiment 2.

4. DISCUSSION

This study has demonstrated that *Byssochlamys* and its associated anamorph species can be separated into at least nine taxa. *B. nivea* var.

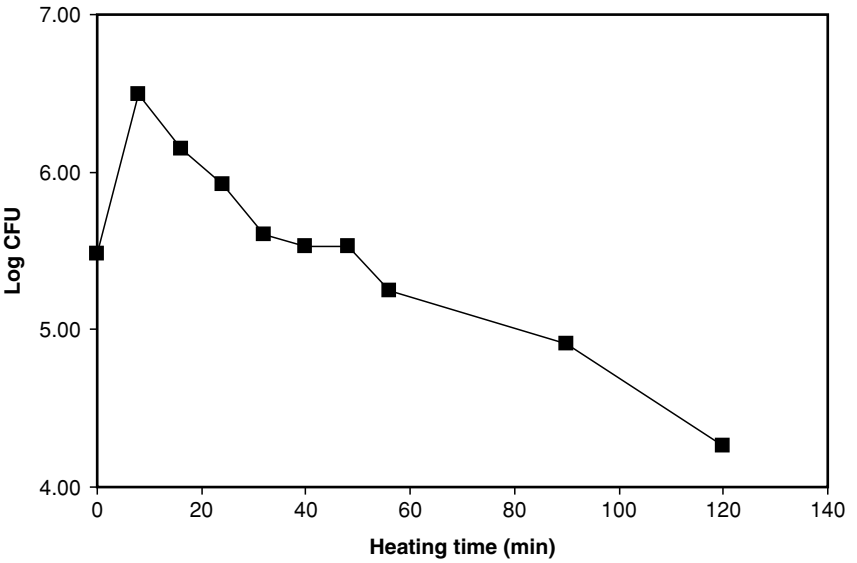

Figure 2. Thermal death curve of *Byssochlamys spectabilis* CBS 109073 at 85°C.

laguncullariae can be considered as a separate taxon, in addition to the known taxa, *B. fulva, B. nivea, B. verrucosa* and *B. zollerniae*. Within the species complex, *Byssochlamys* teleomorphs are observed in two other taxa, *B. spectabilis* and *B. divaricata*, which are clearly different from the other members of the genus *Byssochlamys*.

According to the literature, *Paec. variotii sensu lato* plays a role in mycotoxicoses described in several types of animals. In 1916, Turresson reported that rabbits died after ingestion of conidia and mycelium of *Pen. divaricatum* (= *B. divaricata*). *B. divaricata* produces the mycotoxin emodin, a genotoxic and diarrhoeagenic anthraquinone (Müller et al., 1996), which could be the cause of this described mycotoxicosis. Our investigation showed that byssochlamic acid is formed by *B. nivea*, *B. fulva*, *B. laguncullariae* and *B. verrucosa*. Byssochlamic acid was shown by Raistrick and Smith (1933) to be toxic to mice, and it is weakly hepatotoxic to guinea pigs (Gedek, 1971).

Patulin was reported to be produced by *B. nivea* (Karrow and Foster, 1944; Kis et al., 1969) and *B. fulva* (Rice et al., 1977). However, we could not detect patulin production by any of the investigated *B. fulva* isolates. Patulin production by strains of *B. nivea* was confirmed and its production by *Paec. dactylethromorphus* is described. Patulin

was not produced by any of the strains of *B. laguncullariae* examined in this study. Mycophenolic acid was produced by strains of *B. nivea* and *B. laguncullariae*. This metabolite is an antibiotic, with anti-tumour, anti-psoriasis and immunosuppressive features (Bentley, 2000) and may be of relevance for secondary mycotoxicosis (bacterial infections caused by intake of an immunosuppressive mycotoxin).

Paec. variotii sensu lato is a rather common fungus in the air, in soil (subtropical and tropical climates), in compost (Knösel and Rész, 1973) and on wood (Ram, 1968). It is also common in foods such as rye bread, margarine, peanuts and peanut cake (Joffe, 1969; King et al., 1981), cereals (Pelhate, 1968) and heat treated fruit juices (S. Udagawa, A. D. Hocking , unpublished data).

From our study it can be concluded that the D_{85} value of *B. spectabilis* in ACES buffer was between 47 and 75 minutes. Comparing these results with other data, it seems that the ascospores of this species are one of the most heat resistant fungal ascospores. As this species is also capable of producing viriditoxin, it is an important spoilage fungus in pasteurized food and feed.

Paec. maximus commonly occurs in subtropical and tropical soils and *Paec. dactylethromorphus* is isolated from products such as acetic acid, leather and wood. Both species form chlamydospores, but we have never detected them from heat-treated samples. This indicates that ascospores, not thick walled chlamydospores, are the survival structures.

B. divaricata has also been isolated from heat-treated samples. This fungus does not form chlamydospores and therefore the mode of heat-survival is probably due to ascospores. *B. divaricata* and *B. spectabilis* make ascomata in culture only sparsely (and only after prolonged incubation at 30°C), nevertheless these structures should be present in nature. Soil (Udagawa et al., 1994) could be its natural habitat but also wood (Cartwright, 1937; Ram, 1968) should not be excluded.

5. ACKNOWLEDGEMENTS

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