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Spheroplast formation and partial purification of microbodies from hydrocarbon-grown cells of *Cladosporium resinae*

David B. Carson and Joseph J. Cooney

Environmental Sciences Program, University of Massachusetts at Boston, Boston, MA, U.S.A.

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SUMMARY

Cells of *Cladosporium resinae* form greater numbers of microbodies when grown on *n*-alkanes than when grown on glucose. To facilitate isolation of microbodies, hydrocarbon-grown cells were spheroplasted. Of four spheroplasting agents and five osmotic supports examined, best results were obtained after a 4-h incubation with Novozym 234 plus chitinase and with 0.8 M sorbitol as osmotic support. Equal numbers of spheroplasts were obtained at pH 5.8 and at pH 7.0. Catalase was used as a marker for microbodies and cytochrome-*c* oxidase as a marker for mitochondria. Urate oxidase, a second marker for microbodies, was not detected in cell extracts. Microbodies were extremely fragile; of eight spheroplast disruption techniques attempted, the best yield of microbodies was obtained using a Teflon homogenizer for 5 min. Microbodies were partially purified by differential and density gradient centrifugation. Best results were obtained with discontinuous Percoll gradients which yielded a fraction enriched in microbodies and one enriched in mitochondria.

INTRODUCTION

The hydrocarbon-degrading filamentous fungus *Cladosporium (Amorphotheca) resinae* has been isolated from soil [25], from air [26], from fresh, estuarine, and marine waters [1], and from hydrocarbon-rich environments [26]. This ubiquitous microorganism degrades a variety of hydrocarbons, including some which persist in the environment [3,21,22,33,39]. However, growth is best on normal alkanes of intermediate chain length [3,33]. Shorter *n*-alkanes (C_6–C_8) support little or no growth and hexane is toxic [35]. The organism is a potential source of malfunctions in the fuel systems of jet aircraft [27] and in diesel fuel for ships powered by gas turbines [23].

A working model has been developed for use of *n*-alkanes by *C. resinae*. The fungus takes up substrate *n*-alkane without altering it [4,37]. Cells grown on hydrocarbon contain large vacuoles [32]

Correspondence: J. J. Cooney, Environmental Sciences Program, University of Massachusetts at Boston, Boston, MA 02125, U.S.A.

and lysis of spheroplasts yields the substrate hydrocarbon [4], suggesting that the hydrocarbon may be accumulated in vacuoles. The hydrocarbon can inhibit enzymes involved in glucose metabolism [31]. *n*-Alkanes are oxidized to the homologous fatty acid [36] by enzymes that are constitutive [35].

Examination of thin sections or of freeze-fracture replicas showed that cells of *C. resinae* grown on kerosene contain significant numbers of microbodies, while cells grown on glucose contain few microbodies [4,32]. Osumi et al. [24] proposed that catalase is a marker enzyme for microbodies. In hydrocarbon-grown cells of *C. resinae,* the specific activity of catalase is twice that found in cells grown on glucose [32]. In addition, use of the cytochemical stain 3,3′-diaminobenzidine (DAB) showed that microbodies contain catalase [5]. However, microbodies are not the likely site of initial oxidation of *n*-alkanes since cells grown on glucose contain hydrocarbon-oxidizing enzymes [35] even though they rarely contain microbodies [32].

Therefore, the role of microbodies in hydrocarbon metabolism remains to be elucidated. The goal of the present work was to isolate and purify microbodies as a prelude to determining their role in the ability of *C. resinae* to grow on hydrocarbons.

MATERIALS AND METHODS

Organism and culture conditions. Cultures of *Cladosporium resinae* ATCC 22711 were maintained on Sabouraud Dextrose Agar slants which were incubated at 30°C and then stored at 4°C. The basal medium consisted of the salts solution of Bushnell and Haas (BHS) [2] supplemented with 0.1% (w/v) yeast extract. The pH was adjusted to 5.8 with HCl, and 500-ml volumes were dispensed into 2800-ml Fernbach flasks. The salts solution was sterilized by autoclaving. It was then overlaid with filter-sterilized *n*-dodecane to a final concentration of 2% (v/v). A loopful of cells from a slant was used as inoculum for static cultures incubated at 28°C. Cells were harvested after 6 days, at which time the culture was in early trophophase.

Spheroplast formation and disruption. Cells were collected on 20-μm-aperture nylon mesh by vacuum filtration and washed three times on the filter with BHS. Cells were then washed twice with hexane, a process which removes extracellular hydrocarbon without removing intracellular hydrocarbon [37], followed by three additional washes with BHS.

After the wet weight was recorded, cells were suspended in 20 ml of 0.14 M mercaptoethylamine and 0.04 M ethylenediaminetetraacetic acid and held for 30 min at 30°C as described by Duell et al. [8]. Cells were then collected by filtration on a 20-μm nylon mesh, washed twice with BHS, and suspended in 15 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM $MgCl_2$, lytic enzyme preparation, and osmotic stabilizer. Commercially available lytic enzymes evaluated were Driselase (20 mg/ml), a cellulase preparation from the snail *Irpex lacteus* (Plenum, Hackensack, NJ); Glusulase (6.6 mg/ml), a preparation of digestive enzymes from the snail *Helix pomatia* (Dupont Pharmaceuticals, Wilmington, DE); chitinase (1 mg/ml), from *Streptomyces antibioticus* (Calbiochem, La Jolla, CA); and Novozym 234 (10 mg/ml), a multienzyme preparation from *Trichoderma harzianum* (Novo Lab Inc., Wilton, CT). The osmotic supports tested included: $MgSO_4$ (0.5 M), $(NH_4)_2SO_4$ (0.6 M), KCl (0.7 M), sucrose (0.5 M and 0.6 M), and sorbitol (0.72 M and 0.8 M). Cells, lytic enzymes, and buffered osmotic support were incubated at 30°C for 4 h. Production of spheroplasts was monitored by phase contrast microscopy; numbers of spheroplasts were counted using a hemacytometer. Trypan blue was used to assess spheroplast viability: spheroplasts which excluded the dye were considered viable [13].

All centrifugation procedures for spheroplasts and cell-free extracts were carried out at 4°C and cells and extracts were kept on ice. Undigested hyphae were removed by passing the suspension of treated cells through a 1-cm layer of glass wool. Spheroplasts and spores were sedimented by centrifuging the resulting filtrate for 10 min at 2000 × *g*. The supernatant fluid was removed and spheroplasts (plus spores) were washed once in a solution which contained 0.8 M sorbitol, 2 mM $MgCl_2$, and 50 mM potassium phosphate (pH 7.0).

In most experiments, spheroplasts were disrupted in 15 ml of 20 mM potassium phosphate buffer (pH 7.0) containing 0.35 M sucrose, 2.5% (w/v) Ficoll, 0.15 mM spermine, 0.5 mM spermidine, 0.1% (w/v) bovine serum albumin (BSA), 2 mM $MgCl_2$, 2 mM KCl, 1 mM ethylene glycol tetraacetic acid (EGTA), and 1 mM dithiothreitol using a Teflon homogenizer for 5 min. Other techniques for the disruption of spheroplasts which were evaluated included mechanical breakage using a Dounce homogenizer, dilution, ultrasound, metabolic lysis [14], and polybase-induced lysis [9]. The suspension of treated spheroplasts was centrifuged for 10 min at 2000 × *g*. Spheroplast disruption was then monitored by following the release of catalase activity, a marker for microbodies [24], from treated spheroplasts into the supernatant fluid.

Fractionation by differential and density gradient centrifugation. Differential centrifugation was carried out in a Sorvall Superspeed RC2-B refrigerated centrifuge with an SS-34 rotor. The suspension of disrupted spheroplasts was centrifuged for 10 min at 2000 × *g* to sediment spores, non-broken spheroplasts, and nuclei. The pellet was resuspended in a known volume of the buffer used in disruption and saved; the pellet and supernatant fluid are referred to as pellet 1 and supernatant 1, respectively. Supernatant 1 was then centrifuged for 20 min at 20000 × *g*. The second supernatant solution (supernatant 2) was retained, and for density gradient separation the pellet (pellet 2) was suspended in 3.0 ml of the buffer used in disruption. In most experiments, density gradients were made with Percoll. However, sucrose density gradients, both discontinuous and linear, were also utilized. The suspension containing pellet 2 (1.5 ml) was layered over a discontinuous gradient with concentrations (v/v) of 5%, 15%, 30%, 45%, and 60% Percoll, each containing 0.4 M sucrose and 20 mM potassium phosphate (pH 7.0). Gradients were comprised of 0.45 ml of 60%, and 2.7 ml each of 45%, 30%, 15%, and 5% Percoll mixtures. Centrifugation conditions were 45 min at 28400 × *g* using an SW 40 rotor in a Beckman L8-M ultracentrifuge.

Fractions, collected using an Auto-Densi Flow IIC fractionator (Buchler Instruments Inc., Fort Lee, NJ), were assayed for catalase and the mitochondrial marker cytochrome-*c* oxidase. Catalase (EC 1.11.1.6) was measured by the decrease in absorbance at 240 nm as H_2O_2 was decomposed [40]. Cytochrome-*c* oxidase (EC 1.9.3.1) was assayed by the decrease in absorbance of reduced cytochrome *c* at 550 nm [29]. In some experiments, urate oxidase (EC 1.7.3.3) was measured as a second marker for microbodies. It was assayed by the decrease in absorbance at 290 nm resulting from the oxidation of uric acid to allantoin [40]. Protein was measured by the Lowry method [19], using BSA as standard.

RESULTS AND DISCUSSION

Of the five osmotic supports evaluated for their effect on spheroplast formation and stability, the largest number of spheroplasts was obtained by using 0.5 M $MgSO_4$ or 0.8 M sorbitol. However, a majority of the spheroplasts formed in the presence of 0.5 M $MgSO_4$ were highly vacuolated. DeVries and Wessels [7] reported a similar phenomenon in protoplasts produced from the basidiomycete *Schizophyllum commune*. Also, Picataggio et al. [28] noted that spheroplasts prepared with $MgSO_4$ (0.2–0.7 M) from the deuteromycete *Trichoderma*

Table I

Effect of lytic agents on spheroplast formation

Washed cells were incubated for 4 h at 30°C in 5 ml of 50 mM potassium phosphate buffer (pH 7.0) containing lytic agent(s), 0.8 M sorbitol, and 2 mM $MgCl_2$. Final concentrations of lytic agents were: Novozym 234, 10 mg/ml; chitinase, 1 mg/ml; Driselase, 20 mg/ml; and Glusulase, 6.6 mg/ml. Each value represents a mean of four microscopic counts ± S.E.

Agent	Spheroplasts ($\times 10^4$)/mg dry wt. mycelium
Novozym 234 + chitinase	14.7 ± 0.30*
Novozym 234	13.0 ± 0.50*
Driselase	1.7 ± 0.27
Glusulase	0.4 ± 0.15
Chitinase	0.1 ± 0.06

* Values were significantly different at the 95% confidence level when the Student's *t*-test was applied.

reesei tended to float and were difficult to collect by centrifugation. In contrast, spheroplasts formed from *C. resinae* with 0.8 M sorbitol as osmotic support exhibited a granular cytoplasm and sedimented during centrifugation. Therefore, 0.8 M sorbitol was chosen as the osmotic support for further experiments.

Of lytic enzyme preparations tested, a combination of Novozym 234 and chitinase produced the greatest yield of spheroplasts (Table 1). Spheroplast formation was complete after a 4-h incubation (Fig. 1). Further incubation, as long as 24 h, did not increase the yield of spheroplasts. Equal numbers of spheroplasts were obtained from preparations incubated at pH 5.8 or at pH 7.0. Spheroplasts were sensitive to osmotic shock and greater than 80% were viable as determined by vital staining using Trypan blue.

Eight methods were tested to achieve satisfactory recovery of organelles from disrupted spheroplasts. For each method, spheroplast disruption was monitored by following the release of catalase activity from treated spheroplasts into the 2000 × *g* supernatant fraction (supernatant 1). Supernatant 1 was then centrifuged at 20000 × *g* to yield particulate (pellet 2) and soluble (supernatant 2) fractions. The amount of catalase activity recovered in pellet 2 was used as an index of the release of undamaged microbodies from broken spheroplasts. Of four mechanical methods evaluated for spheroplast disruption, best results were obtained using a Teflon homogenizer for 5 min (Table 2). Homogenization treatments of 1 cycle, 2 cycles, 1 min, or 3 min did not improve the yield of organelles from broken spheroplasts. Prolonged homogenization of spheroplasts resulted in an increase of catalase activity in supernatant 1. However, the corresponding increase in activity was detected in supernatant 2, rather than in pellet 2, suggesting loss of enzyme from damaged microbodies.

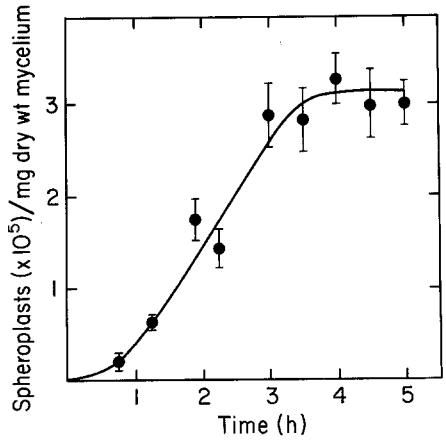

Fig. 1. Spheroplast formation from *C. resinae* during treatment with Novozym 234 (10 mg/ml) plus chitinase (1 mg/ml). Cells were incubated at 30°C in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing the lytic agents and 0.8 M sorbitol as osmotic support. Error bars indicate ± 1 S.E.M.

Table 2

Methods used for the disruption of spheroplasts

A suspension of washed spores and spheroplasts was treated to disrupt spheroplasts. Spores, intact spheroplasts, and nuclei were removed by centrifuging the treated suspension at 2000 × *g* for 10 min. The supernatant fraction was then centrifuged at 20000 × *g* for 20 min. The catalase activity present in the resulting pellet (pellet 2) was considered particulate activity.

Method	Catalase (% total activity in pellet 2)[a]
No treatment	27.8
Teflon homogenizer, 5 min	31.6
Teflon homogenizer, 10 min	25.8
Dounce homogenizer, 5 min	21.2
Ultrasound, 3 × 5 s	10.9

[a] Percent total catalase activity in pellet 2 was determined by: (activity in pellet 2/total activity) × 100.

Four other methods used to disrupt spheroplasts (data not shown) did not increase the yield of intact microbodies. Protoplasts of the yeast *Saccharomyces carlsbergensis* NCYC 74 underwent metabolic lysis in the presence of glucose and a chelating agent [14]. However, spheroplasts of *C. resinae* in osmotic equilibrium with a 0.65 M sorbitol solution increased in size but did not lyse upon addition of glucose and EDTA. Polybase-induced lysis of spheroplasts from *C. utilis* NCYC 737 released intact vacuoles [9]. Although spheroplasts of *C. resinae* lysed in the presence of DEAE-Dextran or polylysine, catalase activity was detected primarily

in supernatant 2, suggesting that microbodies were also lysed by the polybasic macromolecules. In spheroplasts disrupted by osmotic shock or a combination of osmotic shock and homogenization, the majority of catalase activity was also observed in supernatant 2. Consequently, spheroplasts were disrupted using a Teflon homogenizer for 5 min to yield preparations for density gradient centrifugation.

For each disruption technique, the majority of catalase activity was recovered in the soluble fractions, indicating the fragile nature of microbodies. Kawamoto et al. [17] observed significant levels of catalase in soluble fractions prepared from the *n*-alkane-grown yeast *Candida tropicalis* pK 233. In addition, Jenkins et al. [16] reported low sedimentability of catalase in cell free extracts from *C. stellatoidea* grown on *n*-hexadecane.

Fig. 2 shows the distribution of marker enzymes detected in fractions collected after centrifuging pellet 2 through a discontinuous Percoll gradient. Catalase activity was highest in fraction 2 whereas cytochrome-*c* oxidase was greatest in fraction 3.

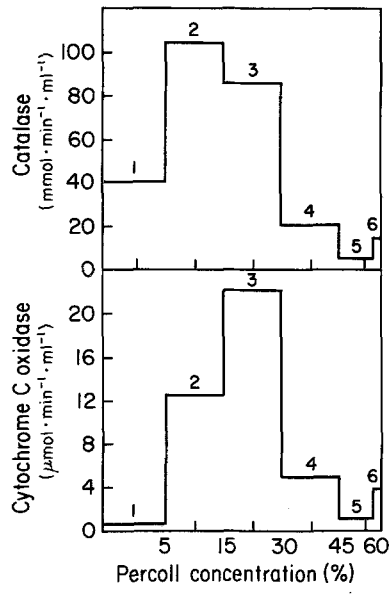

Fig. 2. Distribution of catalase and cytochrome-*c* oxidase in fractions resulting from centrifugation of pellet 2 through a discontinuous density gradient. The volumes of fractions were as follows: (1) 2.85 ml; (2–4) 2.7 ml each; (5) 1.575 ml; and (6) 0.225 ml.

These results suggest that fraction 2 was enriched in microbodies, although mitochondrial contamination was present. Vital staining of gradient fractions using Janus green B showed that fraction 3 was enriched in mitochondria. We were not able to obtain preparations of microbodies which were free of the mitochondrial marker cytochrome-*c* oxidase. Separation of microbodies from mitochondria was not improved when pellet 2 was centrifuged through a continuous gradient of percoll or sucrose.

Catalase activity present at the top of the gradient, in fraction 1, was assumed to have resulted from disruption of or leakage from microbodies. Hayashi et al. [12] noted that catalase was released most easily while urate oxidase was not solubilized when peroxisomes isolated from rat liver were treated with detergent or were repeatedly frozen and thawed. Therefore, in some experiments we attempted to use urate oxidase as a second marker for microbodies. However, this enzyme was not detected in cell extracts or gradient fractions.

Three additional approaches were taken to increase the separation of microbodies from mitochondria in Percoll gradients. The growth medium can affect the density of fungal mitochondria [18,30], and in the present work when *C. resinae* was grown on glucose, mitochondria isolated in Percoll gradients were more dense than those isolated from cells grown on *n*-alkanes. An attempt was made to use this difference in separating organelles. Cells were grown on glucose for 2 days, washed, and then transferred to hydrocarbon medium in the hope that microbody synthesis would occur more rapidly than synthesis of new, lighter mitochondria. In Percoll density gradients, however, significant mitochondrial contamination remained in the microbody-enriched fraction.

Changing the tonicity or hydrophobicity of the gradient medium did not enhance removal of mitochondria from the fraction enriched in microbodies. Kobr and Vanderhaeghe [18], working with *Neurospora crassa*, observed that the tonicity of the medium in which organelles were suspended affected the density of 'glyoxysome-like' particles while the density of mitochondria was unaffected. Nevertheless, we did not improve upon the purification

of microbodies by using density gradient centrifugation with Percoll gradients containing 0.4, 0.5, or 0.6 M sucrose. Failure to separate microbodies and mitochondria might also be due to hydrophobic interactions among microbodies and mitochondria. In addition, intracellular *n*-alkane or a metabolite of the *n*-alkane may be responsible for chemical interactions which may influence the mobility of the organelles within the density gradient. Jackson et al. [15] were able to separate mitochondria from chloroplast fragments in cell extracts prepared from green leaves of spinach or cos lettuce only when the hydrophobicity of the gradient medium was altered. However, when we replaced part of the sucrose in the 5% Percoll layer with 50 mM propane-1,2-diol, the combined effects of density gradient separation and partitioning in an aqueous polymer phase system did not remove mitochondrial contamination from the microbody-enriched fraction.

Cells of the yeast *C. tropicalis* pK 233 grown on a mixture of *n*-alkanes contain microbodies which can be separated from mitochondria by centrifugation through discontinuous gradients of sucrose [17]. They contain catalase, D-amino-acid oxidase, urate oxidase, isocitrate lyase, malate synthase, NADP-linked isocitrate dehydrogenase, acyl-CoA synthetase, and a fatty acid β-oxidation system. Microbodies from *C. resinae* appear to be more fragile than those from *C. tropicalis* pK 233 based upon sedimentability of catalase on sucrose density gradients. While only 15–20% of the catalase activity from cell extracts of *C. tropicalis* pK 233 was recovered at the top of density gradients [17], in our work 43–53% of the same marker from microbodies of *C. resinae* was detected in the top fraction of similar gradients.

Microbodies from filamentous fungi have rarely been isolated and characterized. Microbodies isolated from *Aspergillus tamarii* grown on ethanol contained catalase, isocitrate lyase, and malate synthase [11]. These same enzymes are compartmentalized in the water mold *Blastocladiella emersonii* in a 'symphyomicrobody', a larger organelle formed by the union of small microbodies during sporogenesis [20]. Two types of microbodies were found in a plasmodioid wall-less slime mutant of *N. crassa* [38]. Marker enzymes for microbodies were distributed between two particulate fractions which sedimented at different densities in sucrose gradients.

While microbodies of alkane- and methanol-grown yeasts have been well characterized [10], little information exists on microbodies in hydrocarbon-using filamentous fungi. Microbodies were not observed in thin sections from a *Penicillium* sp. grown on *n*-hexadecane [6]. In contrast to results obtained in our laboratory [32], Turner et al. [34] observed that microbodies were present in a different strain of *C. resinae* grown on either *n*-hexadecane or glucose, suggesting that the organelle is not specifically induced by growth on *n*-alkanes. At present, we attribute this difference to strain variation.

It is unlikely that the initial enzymes of alkane oxidation are located in microbodies because those enzymes, being present in glucose-grown cells, are constitutive [35], while *C. resinae* does not form significant numbers of microbodies when it is grown on glucose [32]. We will use these preparations from Percoll gradients to help characterize microbodies from *C. resinae* and to elucidate their relation to the ability of the organism to grow on *n*-alkanes.

REFERENCES

1 Ahearn, D.G. and S.P. Meyers. 1972. The role of fungi in the decomposition of hydrocarbons in the marine environment. In: Biodeterioration of Materials (Walters, H.A. and E.H. Huck-Van Der Plas, eds.), pp. 12–18, Applied Science Publishers Ltd., London.

2 Bushnell, L.D. and F.F. Haas. 1941. The utilization of certain hydrocarbons by microorganisms. J. Bacteriol. 41: 653–673.

3 Cofone, L., J.D. Walker and J.J. Cooney. 1973. Utilization of hydrocarbons by *Cladosporium resinae*. J. Gen. Microbiol. 76: 243–246.

4 Cooney, J.J., C. Siporin and R.A. Smucker. 1980. Physiological and cytological responses to hydrocarbons by the hydrocarbon-using fungus *Cladosporium resinae*. Bot. Mar. 23: 227–232.

5 Cooney, J.J. and R.A. Smucker. 1983. Cytological and physiological responses of *Cladosporium resinae* exposed to hydrocarbon. In: Biodeterioration 5 (Oxley, T.A. and S. Barry, eds.), pp. 361–372, John Wiley and Sons Ltd., New York.

6 Cundell, A.M., W.C. Mueller and R.W. Traxler. 1976. Mor-

phology and ultrastructure of a *Penicillium* sp. grown on *n*-hexadecane or peptone. Appl. Environ. Microbiol. 31: 408–414.

7 DeVries, O.M.H. and J.G.H. Wessels. 1972. Release of protoplasts from *Schizophyllum commune* by a lytic enzyme preparation from *Trichoderma viride*. J. Gen. Microbiol. 73: 13–22.

8 Duell, E.A., S. Inoue and M.F. Utter. 1964. Isolation and properties of intact mitochondria from spheroplasts of yeasts. J. Bacteriol. 88: 1762–1773.

9 Dürr, M., T. Boller and A. Wiemken. 1975. Polybase induced lysis of yeast spheroplasts. Arch. Microbiol. 105: 319–327.

10 Fukui, S. and A. Tanaka. 1979. Peroxisomes of alkane- and methanol-grown yeasts: metabolic functions and practical applications. J. Appl. Biochem. 1: 171–201.

11 Graves, L.B., V.N. Armentrout and D.P. Maxwell. 1976. Distribution of glyoxylate-cycle enzymes between microbodies and mitochondria in *Aspergillus tamarii*. Planta 132: 143–148.

12 Hayashi, H., T. Suga and S. Niinobe. 1971. Studies on peroxisomes. I. Intraparticulate localization of peroxisomal enzymes in rat liver. Biochim. Biophys. Acta 252: 58–68.

13 Hoskins, J.M., G.G. Meynell and F.K. Sanders. 1956. A comparison of methods for estimating the viable count of a suspension of tumour cells. Exp. Cell Res. 11: 297–305.

14 Indge, K.J. 1968. Metabolic lysis of yeast protoplasts. J. Gen. Microbiol. 51: 433–440.

15 Jackson, C., J.E. Dench, D.O. Hall and A.L. Moore. 1979. Separation of mitochondria from contaminating subcellular structures utilizing silica sol gradient centrifugation. Plant Physiol. 64: 150–153.

16 Jenkins, R.O., T.G. Cartledge and D. Lloyd. 1983. Subcellular fractionation of *Candida stellatoidea* after growth with glucose or *n*-hexadecane. J. Gen. Microbiol. 129: 1171–1185.

17 Kawamoto, S., A. Tanaka, M. Yamamura, Y. Teranishi, S. Fukui and M. Osumi. 1977. Microbody of *n*-alkane-grown yeast: enzyme localization in the isolated microbody. Arch. Microbiol. 112: 1–8.

18 Kobr, M.J. and F. Vanderhaeghe. 1973. Changes in density of organelles from *Neurospora*. Experientia 29: 1221–1223.

19 Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.

20 Mills, G.L. and E.C. Cantino. 1975. Isolation and characterization of microbodies and symphyomicrobodies with different buoyant densities from the fungus *Blastocladiella emersonii*. Biochem. Biophys. Res. Commun. 67: 1256–1263.

21 Mukherjee, B.B., G. Kraidman and I.D. Hill. 1973. Synthesis of glycols by microbial transformation of some monocyclic terpenes. Appl. Microbiol. 25: 447–453.

22 Mukherjee, B.B., G. Kraidman and I.D. Hill. 1974. Transformation of 1-menthene by a *Cladosporium:* accumulation of β-isopropyl glutaric acid in the growth medium. Appl. Microbiol. 27: 1070–1074.

23 Neihof, R.A. and ME. May. 1982. Microbial contamination of fuels. Dev. Ind. Microbiol. 22: 781–787.

24 Osumi, M., M. Naoto, Y. Teranishi, A. Tanaka and S. Fukui. 1974. Ultrastructure of *Candida* yeast grown on *n*-alkanes. Appearance of microbodies and its relationship to high catalase activity. Arch. Microbiol. 99: 181–201.

25 Parbery, D.G. 1968. The soil as a natural source of *Cladosporium resinae*. In: Biodeterioration of Materials (Walters, H.A. and J.A. Elphick, eds.), pp. 371–380, Elsevier, London.

26 Parbery, D.G. 1969. The natural occurrence of *Cladosporium resinae*. Trans. Br. Mycol. Soc. 53: 15–23.

27 Parbery, D.G. 1971. Biological problems in jet aviation fuel and the biology of *Amorphotheca resinae*. Mater. Org. 6: 161–208.

28 Picataggio, S.K., D.H.J. Schamhart, B.S. Montenecourt and D.E. Eveleigh. 1983. Sphaeroplast formation and regeneration in *Trichoderma reesei*. Eur. J. Appl. Microbiol. Biotechnol. 17: 121–128.

29 Polakis, E.S., W. Bartley and G.A. Meek. 1964. Changes in the structure and enzyme activity of *Saccharomyces cerevisiae* in response to changes in the environment. Biochem. J. 90: 369–374.

30 Schwitzguebel, J.P., I.M. Møller and J.M. Palmer. 1981. Changes in density of mitochondria and glyoxysomes from *Neurospora crassa:* a reevaluation utilizing silica sol gradient centrifugation. J. Gen. Microbiol. 126: 289–295.

31 Siporin, C. and J.J. Cooney. 1976. Inhibition of glucose metabolism by *n*-hexadecane in *Cladosporium (Amorphotheca) resinae*. J. Bacteriol. 128: 235–241.

32 Smucker, R.A. and J.J. Cooney. 1981. Cytological responses of *Cladosporium resinae* when shifted from glucose to hydrocarbon medium. Can. J. Microbiol. 27: 1209–1218.

33 Teh, J.S. and K.H. Lee. 1973. Utilization of *n*-alkanes by *Cladosporium resinae*. Appl. Microbiol. 25: 454–457.

34 Turner, A.P.F., I.J. Higgins and K. Gull. 1980. Microbodies in *Cladosporium (Amorphotheca) resinae* grown on glucose and *n*-alkanes. FEMS Microbiol. Lett. 9: 115–119.

35 Walker, J.D. and J.J. Cooney. 1973. Oxidation of *n*-alkanes by *Cladosporium resinae*. Can. J. Microbiol. 19: 1325–1330.

36 Walker, J.D. and J.J. Cooney. 1973. Pathway of *n*-alkane oxidation in *Cladosporium resinae*. J. Bacteriol. 115: 635–639.

37 Walker, J.D. and J.J. Cooney. 1973. Aliphatic hydrocarbons of *Cladosporium resinae* cultured on glucose, glutamic acid, and hydrocarbons. Appl. Microbiol. 26: 705–708.

38 Wanner, G. and R.R. Theimer. 1982. Two types of microbodies in *Neurospora crassa*. Ann. N.Y. Acad. Sci. 386: 269–284.

39 Willets, A.J. 1973. Microbial metabolism in alkylbenzene sulphonates. Fungal metabolism of 1-phenylundecane-*p*-sulphone and 1-phenyldodecane-*p*-sulphonate. Antonie van Leeuwenhoek J. Microbiol. Serol. 39: 585–597.

40 Worthington Biochemical Corp. 1978. Enzymes and Related Biochemicals, 210 pp., Freehold, NJ.