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Natamycin as a Fungicide in Agar Media

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Fungal inhibition in four commonly used agar media was improved by substituting natamycin (pimaricin) for cycloheximide. The recovery of bacteria was not affected by natamycin, whereas fungal contamination from a variety of samples was significantly suppressed. Furthermore, natamycin lacks the occupational health hazards of cycloheximide. Medium-dependent natamycin degradation occurred during the preparation and refrigerated storage of agar plates, but the addition of natamycin at 21.6 μg/ml resulted in effective residual activity.

Enumeration of bacteria from environmental samples by the spread plate technique is usually performed by using agar media containing the fungicide cycloheximide (actidione) (1, 24). However, the use of cycloheximide has drawbacks; it is genotoxic (clastogenic but not mutagenic) in several test systems (2), has reproductive toxicity effects, and can cause contact dermatitis (5, 9, 13, 17, 20).

Among possible alternatives to cycloheximide is natamycin (pimaricin), a polyene antifungal antibiotic produced by *Streptomyces natalensis*. The antifungal activities of natamycin and other polyenes are dependent on their binding to cell membrane sterols, primarily ergosterol, the principal sterol in fungal membranes, thereby making them leaky (3, 11, 21). Bacteria are not susceptible to natamycin since their membranes are devoid of sterols (14, 21), and accordingly, reported MICs of natamycin against bacteria are high (>10,000 μg/ml) (6). Naturally occurring fungal resistance to natamycin is rare (3); the level of resistance is low compared with that to cycloheximide (23) and does not increase during exposure to natamycin (7, 8). The use of natamycin in the prevention of the growth of molds on certain cheeses and sausages is permitted in many countries (4, 6, 25).

The objective of the present study was to compare fungal inhibitions and recoveries of specific bacteria from soil on natamycin- and cycloheximide-containing agar media. Also, we examined the stability of natamycin during the preparation and refrigerated storage of four commonly used agar media.

Inhibition of fungi. Four commonly used agar media, Luria-Bertani (LB) (12), nutrient agar (NA; Nutrient Broth [Difco Laboratories, Detroit, Mich.] plus 15 g of agar per liter), yeast-peptone-dextrose (pH 4.5) (YPD) (22), and Micro Assay Culture Agar (MACA; Difco) were prepared devoid of fungicide and with the addition of either cycloheximide (Sigma Chemical Co., St. Louis, Mo.) or natamycin (Gist-Brocades, Delft, The Netherlands) in settings protected from UV light. Cycloheximide was added at 25 μg/ml (1) from filter-sterilized, frozen (−20°C) stocks containing 6.25 mg of cycloheximide per ml of distilled water. Natamycin was added at 21.6 μg/ml on the basis of published fungal MICs (8, 14, 15) from refrigerated stocks containing 0.864 mg of natamycin per ml of methanol. When the melted agar medium had cooled to about 50°C, the fungicides were added and the plates were poured immediately. Spore suspensions containing 10^5 conidia per ml were prepared from seven fungal strains propagated on YPD as described by Rusul and Marth (19). The plates were inoculated with 10 μl of spore suspension (three spots on each of two plates) and incubated at 30°C. Colony diameters were measured on the plates after 3 and 7 days. Each experiment was repeated two or three times. Data were analyzed by analysis of variance and mean separation procedures by using the program SAS/STAT version 6.04 (Statistical Analysis Systems, SAS Institute, Cary, N.C.).

Fungal colony growth (Table 1) was significantly influenced by fungal strain, fungicide treatment, and medium ($P < 0.0001$); interactions between these parameters were also significant ($P < 0.0001$). Growth of all fungi on the four natamycin-containing media was inhibited relative to that on untreated or cycloheximide-containing plates on day 7, while only five of seven fungi (*Aspergillus niger*, *Geotrichum candidum*, *Geotrichum* sp., *Penicillium commune*, and *Penicillium roqueforti*) were significantly inhibited by cycloheximide. Although more effective inhibition of growth by natamycin was noted when the plates were read earlier, the present experiments were extended for 7 days to test natamycin in a worst-case situation. Only two of the seven fungi, i.e., *Penicillium chrysogenum* and *Aspergillus flavus*, could grow on natamycin-containing plates. Until day 2, inhibition of these two strains with natamycin was complete; from day 3, inhibition was highly variable but significantly influenced by strain and medium (Table 2). A high tolerance to natamycin has previously been reported for *A. flavus* (6, 16).

Test for degradation of natamycin. The four agar media were prepared with the addition of 21.6 μg of natamycin per ml, and the plates were stored at 6°C in the dark. The natamycin concentration was determined immediately after preparation and after 2, 4, and 8 weeks in duplicate samples by use of a procedure developed for cheese (10); two discs of agar were cut from each of three plates and pooled, extracted with 80% methanol, cleaned by solid-phase extraction (Sep-Pak cartridge, C_{18}), and analyzed by high-performance liquid chromatography. Natamycin was determined by UV absorption at a λ of 303 nm.

The natamycin decomposition rate was medium dependent and occurred both during medium preparation and during storage (Fig. 1). Analysis of variance showed that natamycin stability in all four media (absolute or relative to day 0) was different at the 1% level (Duncan's multiple-range test), the order of decreasing stability being NA, MACA, YPD, and LB. The rate of natamycin degradation in LB was subsequently confirmed by a repeat experiment.

Natamycin losses during plate preparation (average of two experiments) were 11% for NA, 28% for MACA, 34% for YPD, and 40% for LB. Degradation during storage (6°C)

TABLE 1. Colony diameter of fungi grown for 7 days on media devoid of fungicide (untreated) or containing natamycin or cycloheximide

Fungus	Colony diam (mm)[a]			n^b
	Untreated	Cycloheximide treated	Natamycin treated	
A. flavus SNACC 7A	44 ± 6	46 ± 5	19 ± 10	24/24/48[c]
A. niger IBT 3256	43 ± 8	8 ± 13	0	60/66/72[c]
G. candidum IL	25 ± 3	23 ± 5	0	48
Geotrichum sp. strain AS1	25 ± 3	22 ± 3	0	48
P. chrysogenum 3	33 ± 9	33 ± 8	18 ± 16	72
P. commune IBT 3429	14 ± 2	12 ± 2	0	48
P. roqueforti IBT PV	12 ± 3	4 ± 6	0	72

[a] Values are means ± standard errors of the means and are averages of six determinations on four media, repeated two or three times. Values connected by horizontal bars are not significantly different ($P > 0.05$, Duncan's multiple-range test).

[b] Number of observations in each treatment.

[c] Different numbers of observations in treatments as indicated (untreated/cycloheximide/natamycin) due to missing values from one replicate experiment.

could be described by first-order kinetics (r^2, ~0.9) but was better described by the equation $y = a\sqrt{x} + b$, where y is the natamycin concentration and x is the time (r^2 ~0.97). This equation predicts that the half-lives of natamycin were 17 days for LB, 31 days for YPD, 41 days for MACA, and 134 days for NA. This pattern of medium-dependent degradation was in agreement with the pattern of fungal inhibition on the four media (Table 2).

Surprisingly, natamycin was degraded during medium preparation. Natamycin is considered to be stable even when heated to 100°C for short periods (17). It is known that chemical oxidants and UV light cause rapid degradation of natamycin in aqueous solution (15). The observed degradation was probably due to chemical degradation that is accelerated at 50°C. In food applications, natamycin degradation is of little importance because crystalline natamycin is used; this provides longer protection when the fungicide is slowly released (solubility in water is about 50 μg/ml [3]).

Enumeration of bacteria from soil. Strains of *Enterobacter cloacae* and *Pseudomonas fluorescens* resistant to nalidixic acid were inoculated into triplicate microcosms containing 60 g of a soil/sand mixture (1:1) at 2×10^5 cells per g and incubated at 20°C for 7 days. Bacteria were enumerated in duplicate by using LB plates containing nalidixic acid (500 μg/ml) and either cycloheximide or natamycin (prepared as described above). Plates were incubated at 30°C overnight (*E. cloacae*) or for 2 days (*P. fluorescens*). Colony counts

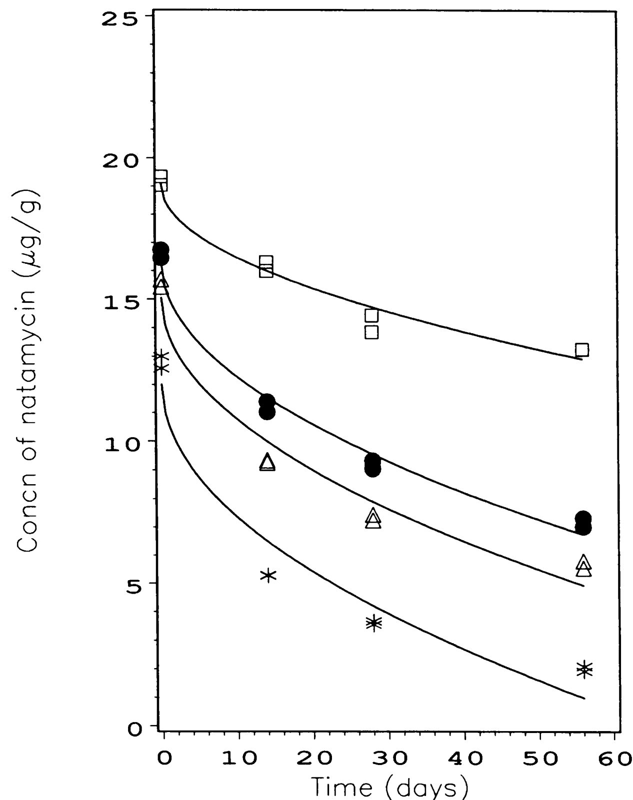

FIG. 1. Natamycin concentration in four different agar media to which 21.6 μg of natamycin per ml had been added to the molten agar. Samples were taken immediately after the plates had hardened (day 0) and at intervals during refrigerated storage. Each value represents pooled samples from three agar plates. The concentration-time relationship is described by the regression curve $y = a\sqrt{x} + b$ (y is the natamycin concentration and x is the time) (average r^2, ~0.97). Symbols: □, NA; ●, MACA; △, YPD; *, LB.

were converted to CFU per gram of dry matter. No significant differences were observed between bacterial counts on the two media ($P > 0.05$, Duncan's multiple-range test; Table 3), and there was no fungal growth on the plates. This agrees with the general finding that natamycin does not inhibit bacteria (6, 14, 21).

Since the completion of the above experiments, natamycin-containing agar media have been used successfully in our laboratory for the enumeration of bacteria in soil, cattle dung, and earthworm casts and on bean and barley leaves

TABLE 2. Mycelial growth inhibition of *P. chrysogenum* 3 and *A. flavus* SNACC 7A on natamycin-containing agar plates relative to untreated controls (18)

Medium	% Inhibition (mean ± SEM)[a]	
	P. chrysogenum	*A. flavus*
NA	74 ± 35a	100 ± 0a
YPD	62 ± 39b	77 ± 25b
MACA	61 ± 34b	62 ± 22c
LB	57 ± 39b	59 ± 32c

[a] Means with the same letter are not significantly different ($P > 0.05$, Duncan's multiple-range test). The numbers of observations in each treatment were 30 for *P. chrysogenum* (days 3 and 7) and 12 for *A. flavus* (day 3).

TABLE 3. Enumeration of bacteria from soil microcosms on LB medium containing nalidixic acid and either cycloheximide or natamycin

Addition to medium	Log CFU/g of dry soil[a]			
	E. cloacae A107		*P. fluorescens*	
	Expt 1	Expt 2	Expt 1	Expt 2
Natamycin	5.92a	5.61a	6.05a	6.04a
Cycloheximide	5.85a	5.65a	6.03a	6.01a

[a] Values are means $n = 6$ (two plates from each of three microcosms). Values within a column followed by the same letter are not significantly different ($P > 0.05$) by Duncan's multiple-range test.

from fields and microcosms (data not shown). These experiments suggest that natamycin is an efficient suppressor of fungal contamination on agar plates, enabling enumeration of the bacteria of interest.

In conclusion, the substitution of natamycin for cycloheximide in agar media is advantageous because its antifungal activity has been shown to be significantly higher than that of cycloheximide and natamycin lacks the occupational health hazards of cycloheximide. In addition, natamycin does not affect recovery of bacteria from soil.

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REFERENCES

1. **Armstrong, J. L., G. R. Knudsen, and R. J. Seidler.** 1987. Microcosm method to assess survival of recombinant bacteria associated with plants and herbivorous insects. Curr. Microbiol. **15:**229–232.
2. **Bašić-Zaninović, T., D. Papeš, and J. Franekić.** 1991. Cycloheximide genotoxicity in in vitro and in vivo test systems. Mutat. Res. **263:**203–210.
3. **Classen, H.-G., P. S. Elias, and W. P. Hammes.** 1987. Toxicologic/hygienic evaluation of food constituents, additives and contaminants (in German). Paul Parey, Berlin.
4. **Commission of the European Communities.** 1979. Reports of the Scientific Committee for Food, 9th series. Commission of the European Communities, Brussels.
5. **Cronin, E.** 1980. Contact dermatitis. Churchill Livingstone, Ltd., London.
6. **De Boer, E.** 1988. Food preservatives, p. 268–273. *In* R. A. Samson and E. S. van Reenen-Hoekstra (ed.), Introduction to food-borne fungi, 3rd ed. Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.
7. **De Boer, E., H. Labots, M. Stolk-Horsthuis, and J. N. Visser.** 1979. Sensitivity to natamycin of fungi in factories producing dry sausage. Fleischwirtschaft **59:**1868–69.
8. **De Boer, E., and M. Stolk-Horsthuis.** 1977. Sensitivity to natamycin (pimaricin) of fungi isolated in cheese warehouses. J. Food Prot. **40:**533–536.
9. **Gousselin, R. E., R. P. Smith, H. C. Hodge, and J. E. Braddock.** 1984. Clinical toxicology of commercial products, 5th ed. The Williams & Wilkins Co., Baltimore.
10. **Guldborg, M.** 1983. Development and testing of a method for determination of natamycin in cheese rind. Report A83005. National Food Agency, Søborg, Denmark. (In Danish.)
11. **Kobayashi, G. S., and G. Medoff.** 1977. Antifungal agents: recent developments. Annu. Rev. Microbiol. **3:**291–308.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **National Institute of Occupational Health and Safety.** 1990. RTECS, registry of toxic effects of chemical substances. National Institute of Occupational Health and Safety, Washington, D.C.
14. **Noordervliet, P. F.** 1978. Sorbic acid and pimaricine as preservatives on cheese and sausages surfaces. A comparative literature study. Nordeuropæisk Mejeri Tidsskrift **4:**121–127+XVIII.
15. **Raab, W. P.** 1972. Natamycin (pimaricin). Its properties and possibilities in medicine. Georg Thieme Publishers, Stuttgart, Germany.
16. **Ray, L. L., and L. B. Bullerman.** 1982. Preventing growth of potentially toxic molds using antifungal agents. J. Food Prot. **45:**953–963.
17. **Reynolds, J. E. F. (ed.).** 1982. Martindale. The extra pharmacopoeia, 28th ed. The Pharmaceutical Press, London.
18. **Roberts, M., and C. B. C. Boyce.** 1972. Principles of bioassay, p. 155–190. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 7A. Academic Press, Ltd., London.
19. **Rusul, G., and E. H. Marth.** 1988. Growth and aflatoxin production by *Aspergillus parasiticus* NRRL 2999 in the presence of potassium benzoate or potassium sorbate and at different initial pH values. J. Food Prot. **50:**820–825.
20. **Shepard, T. H.** 1983. Catalogue of teratogenic agents, 4th ed. The Johns Hopkins University Press, Baltimore.
21. **Stock, R.** 1981. How effective are antimycotic drugs? Pharmacy Int. **2:**232–236.
22. **Van Uden, N., and L. Do Carmo Sousa.** 1957. Yeasts from the bovine caecum. J. Gen. Microbiol. **16:**385–395.
23. **Van Vliet, T.** 1977. Interactions between adsorbed macromolecules. Measurements on emulsions and liquid films. Mededelingen Landbouwhogeschool Wageningen 77–1. H. Veenman & Zonen B.V., Wageningen, The Netherlands.
24. **Wollum, A. G., II.** 1982. Cultural methods for soil microorganisms, p. 781–802. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Methods of soil analysis, part 2, 2nd ed. American Society of Agronomy, Inc./Soil Science Society of America, Inc., Madison, Wis.
25. **World Health Organization.** 1969. Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics. Twelfth Report of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organization, Geneva.