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Modification of alternan by novel *Penicillium* spp.

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Four strains identified as *Penicillium* spp. were isolated from soil samples based on their capacity to modify the unique polysaccharide, alternan. Spores from these isolates germinated in medium containing alternan and reduced the apparent molecular weight of alternan as determined by high-performance size exclusion chromatography and viscometry. However, the fungi exhibited limited growth on alternan and did not consume the substrate. The rheological properties of the modified alternan resembled those of commercial gum arabic. Thus, treatment of native alternan with spores from these *Penicillium* spp. strains constitutes a simple bioconversion method to quantitatively produce novel and potentially useful modified alternan.
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Introduction

Alternan is a unique branched glucan produced by rare strains of *Leuconostoc mesenteroides* [4,6,12]. Alternan's backbone structure of alternating α-(1→6) and α-(1→3)-D-glucosidic linkages distinguishes this biopolymer from true dextrans [6,21–23]. Alternan's physical properties of high solubility and low viscosity have suggested a variety of potential commercial applications in foods and industrial processes [3,8]. Recent improvements in strains for alternan production have renewed interest in this biopolymer [14,17–20,25].

Native alternan has an apparent molecular weight average ($\bar{M}_{\bar{w}}$) of 10^6–10^7 [3,8]. Derivatives of alternan have been produced by ultrasonication, reducing the apparent molecular weight average to $<10^6$ and modifying the rheological properties of the polymer so that they more closely resemble gum arabic [3]. However, ultrasonication is a relatively expensive process that would be difficult to carry out on an industrial scale. Although alternan is resistant to hydrolysis by most known endoglucanases, a "limit alternan" has also been produced by treatment of native alternan with isomaltodextranase from *Arthrobacter globiformis* [3]. Limit alternan exhibits an apparent $\bar{M}_{\bar{w}}$ of 3.5×10^3 and is rheologically similar to oligosaccharides of maltodextrin [3]. Recently, *Bacillus* spp. isolates that produce an endoglucanase specific for alternan [1,7,29] were described. This enzyme produces a novel cyclic tetrasaccharide from alternan [5]. In the current study, we sought new organisms, particularly fungi, that could partially degrade or modify alternan for new applications. Because *L. mesenteroides* produces glucans only from sucrose and is frequently isolated from vegetable matter and sugar refineries, we screened soil samples from a sugar cane field.

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Materials and methods

Alternan enrichment cultures

Soil samples from a Louisiana sugar cane field, the kind gift of Dr. Donal F. Day, were serially diluted by a procedure similar to that of Will *et al* [28]. Specifically, 2 g of soil was diluted into 198 ml of sterile 0.2% agar in distilled water (water agar). This was shaken vigorously, and then 10 ml was transferred to 90 ml of water agar and mixed well. One milliliter of this suspension was then transferred into 9 ml of water containing 0.01% Triton X-100. Aliquots (0.1 ml) of these final dilutions were used to inoculate 10-ml enrichment cultures, composed of 1.0% alternan in a basal medium that favors fungal growth ("WW" of Koenig and Day [16], containing per liter: 2.5 g KH_2PO_4, 5.0 g $(NH_4)_2SO_4$, 0.1 g $CaCl_2$, 0.1 g $MgSO_4$ and 0.1 g NaCl, pH 4.5), amended with 100 μg/ml chloramphenicol to suppress bacterial growth. Enrichment cultures were incubated at 28°C and 200 rpm for 7 days. The initial screening of enrichment cultures for alternan modification was performed by measuring the optical density of culture supernatants at 225 nm. Similar to the findings of Kobayashi *et al* [15] in their studies of dextran, alternan solutions showed a maximal absorbance reading at 225 nm, and the optical density at this wavelength was proportional to the concentration of alternan. WW basal medium did not absorb strongly at 225 nm. Culture supernatants that showed a significant decrease in OD_{225} after 7 days were diluted for single colony isolations on potato dextrose agar (PDA; Difco, Detroit, MI). Isolates that retained their alternan modification phenotype were further purified by single spore isolation on Czapek's Agar (Difco).

Maintenance and growth of purified organisms

Penicillium spp. isolates were routinely maintained on PDA slants, and stock spore suspensions were stored at −80°C in 40% glycerol. Liquid cultures for alternan modification time course experiments contained the same medium used for enrichment

cultures, with the omission of chloramphenicol. For each experiment, fresh spore suspensions were harvested from PDA plates using sterile water containing 0.01% Triton X-100. Liquid cultures (10 ml in 50-ml flasks) were inoculated to 10^5 spores/ml and incubated at 28°C and 200 rpm for up to 17 days. For dry weight determinations, cultures were 100 ml in 500-ml flasks.

Analysis of modified alternan

One-milliliter samples of fungal cultures were filtered through Nanosep MF 0.45-μm spin tubes (Pall Gelman Laboratory, Ann Arbor, MI) and filtrates were dried under vacuum (Speed Vac; Savant Instruments, Holbrook, NY) and resuspended in 0.1 ml of distilled water. Samples (10 μl) were applied to a Shodex KB-806M high-performance size exclusion chromatography (HPSEC) column (Showa Denko, Tokyo, Japan) and eluted with water at 0.5 ml/min. Separations were monitored using a Shodex OR-1 optical rotation detector (Showa Denko). Pullulan standards were used to estimate the molecular weight averages of unknowns (Showa Denko). Methylation analysis of modified alternan was performed by the method of Slodki *et al* [24]. Total carbohydrate was estimated using the phenol–sulfuric acid method [10], utilizing maltose as a standard. Alternanase activity was measured using a reducing sugar assay similar to that previously described [1].

Results and discussion

In preliminary experiments, soil samples were screened directly for organisms that hydrolyzed alternan by plating them onto solid medium containing 1.0% Remazol brilliant blue-dyed alternan, similar to the procedure used by Wyckoff *et al* [29]. However, fungal colonies that appeared on these plates exhibited little or no clearing of the dyed substrate. Subsequently, alternan enrichment cultures were employed as described in Materials and Methods. Nearly half of approximately 40 enrichment culture supernatants exhibited a reduction in OD_{225} after 7 days of incubation, suggesting some degree of degradation or modification of alternan. The predominant organisms from these cultures were single-colony-purified and retested in liquid medium containing alternan.

Table 1 Growth of *Penicillium* spp. isolates on basal medium, glucose or alternan

Strain number	Dry weight (mg/ml)		
	Basal medium	1.0% Glucose	1.0% Alternan
NRRL 21966	0.02±0.03	0.98±0.23	0.22±0.03
NRRL 21967	0.04±0.02	1.20±0.30	0.29±0.03
NRRL 21968	0.22±0.12	1.05±0.20	0.27±0.02
NRRL 21969	0.08±0.01	1.06±0.23	0.25±0.06

Four isolates that consistently produced the greatest reductions in OD_{225} were single-spore-purified. These were identified as *Penicillium* spp. isolates and deposited in the ARS Patent Culture Collection as strains NRRL 21966, NRRL 21967, NRRL 21968 and NRRL 21969. These isolates were derived from separate enrichment cultures and differed slightly in colonial morphology, especially with respect to pigmentation. None of the purified isolates exhibited clearing on dyed alternan plates, suggesting that they were unable to degrade alternan to low-molecular-weight oligosaccharides that could easily diffuse in agar.

The four *Penicillium* spp. isolates were compared over a 17-day time course in parallel liquid medium cultures containing alternan (Figure 1). Strains NRRL 21966 and NRRL 21969 appeared to have a similar effect on alternan, reducing the OD_{225} of culture supernatants by approximately 3 OD units within 4 days. Strains NRRL 21967 and NRRL 21968 were somewhat slower in bringing about this change, although all four isolates appeared to produce a similar effect by the end of the time course. By microscopic examination, spores of all four strains germinated synchronously and rapidly in alternan medium. However, the strains exhibited only limited growth on alternan, even after 17 days. In contrast, the strains grew well in cultures containing glucose in place of alternan, as reflected by biomass dry weight measurements (Table 1). Phenol–sulfuric acid assays of alternan culture supernatants indicated no detectable change in total carbohydrate over 17 days. These results suggest that the *Penicillium* spp. isolates were largely unable to utilize alternan as a carbon source. However, microscopic examinations indicated that spores failed to germinate in basal medium containing no added carbon source. It is possible that spore

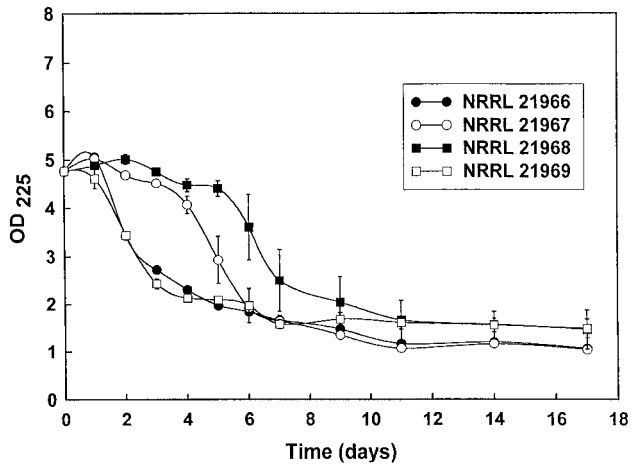

Figure 1 Effect of *Penicillium* spp. isolates on the OD_{225} of alternan culture supernatants. (●) NRRL 21966; (○) NRRL 21967; (■) NRRL 21968; (□) NRRL 21969.

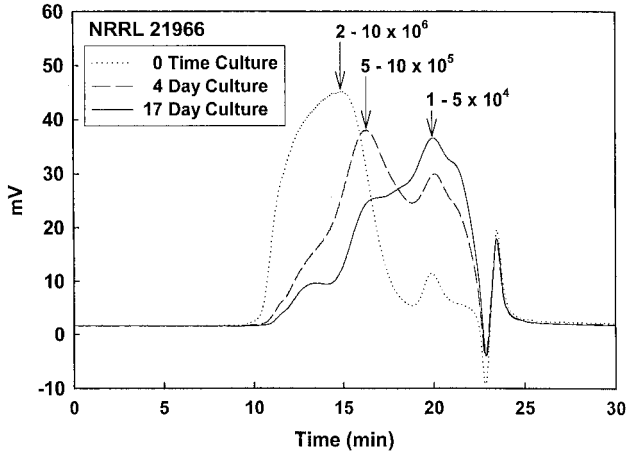

Figure 2 Characterization of strain NRRL 21966 alternan culture supernatants by HPSEC. Apparent averages are indicated for major molecular weight classes present in culture samples. Dotted line, 0 time culture medium; dashed line, 4-day cultures; solid line, 17-day cultures.

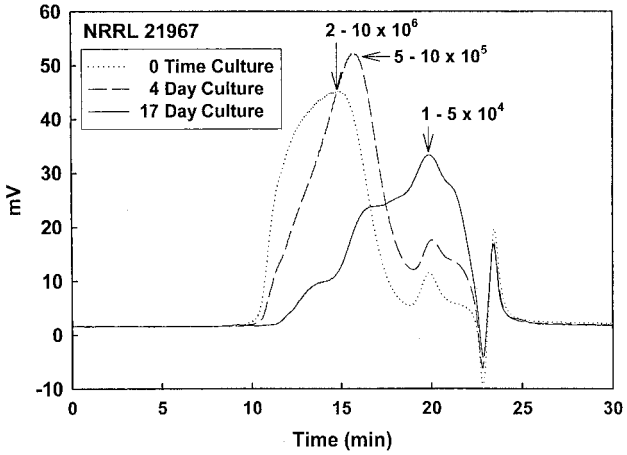

Figure 3 Characterization of strain NRRL 21967 alternan culture supernatants by HPSEC. Apparent averages are indicated for major molecular weight classes present in culture samples. Dotted line, 0 time culture medium; dashed line, 4-day cultures; solid line, 17-day cultures.

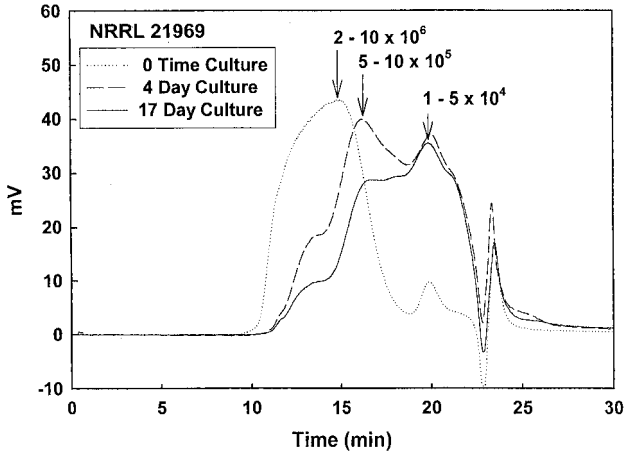

Figure 5 Characterization of strain NRRL 21969 alternan culture supernatants by HPSEC. Apparent averages are indicated for major molecular weight classes present in culture samples. Dotted line, 0 time culture medium; dashed line, 4-day cultures; solid line, 17-day cultures.

germination was supported by minor contaminants that may be present in alternan preparations, such as fructose.

HPSEC was used to characterize the molecular weight distribution of alternan from culture supernatants (Figures 2–5). Alternan present in the initial culture medium was predominantly represented by a high-molecular-weight peak of $2-10\times10^6$, consistent with previous characterizations of native alternan [3]. Interestingly, these zero time samples also contained a small amount of a lower-molecular-weight class of $1-5\times10^4$. Four-day cultures of all four isolates exhibited a reduction in high-molecular-weight native alternan and the appearance of an intermediate-molecular-weight class of $5-10\times10^5$. Strains NRRL 21966 and NRRL 21969, which caused the most rapid reduction in OD_{225}, also showed a dramatic increase in the $1-5\times10^4$ class at day 4. At 17 days, the $1-5\times10^4$ peak was predominant in cultures of all four isolates, with a peak shoulder corresponding to the intermediate-molecular-weight class. Methylation analyses confirmed that the modified material in culture supernatants was alternan as judged by its characteristic linkage pattern. Thus, the four fungal isolates appeared to differ principally in the rate at which they progressively modified alternan towards lower-molecular-weight classes.

The mechanism of alternan modification by *Penicillium* spp. isolates is not clear. Culture supernatants, cell suspensions and cell extracts were devoid of measurable alternanase enzyme activity. Although certain *Penicillium* species are known to produce dextranase and mutanase [2,11], alternan is considered to be impervious to these enzymes [3,21,26]. Furthermore, the pattern of progressive modification of alternan through seemingly discrete molecular weight classes was not expected. It is possible that the *Penicillium* spp. isolates produce novel, and possibly unstable, hydrolytic enzymes with unique specificities against alternan. Such enzymes would presumably differ from isomaltodextrase, which converts alternan to limit alternan and isomaltose [21]. Furthermore, methylation analyses indicated that modified alternan did not differ significantly from native alternan in its degree of branching. It is possible that enzymes from these *Penicillium* spp. isolates recognize some minor, as yet uncharacterized, structural feature of alternan.

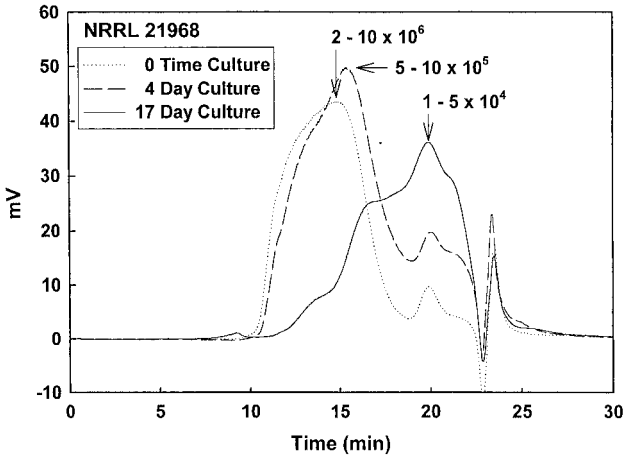

Figure 4 Characterization of strain NRRL 21968 alternan culture supernatants by HPSEC. Apparent averages are indicated for major molecular weight classes present in culture samples. Dotted line, 0 time culture medium; dashed line, 4-day cultures; solid line, 17-day cultures.

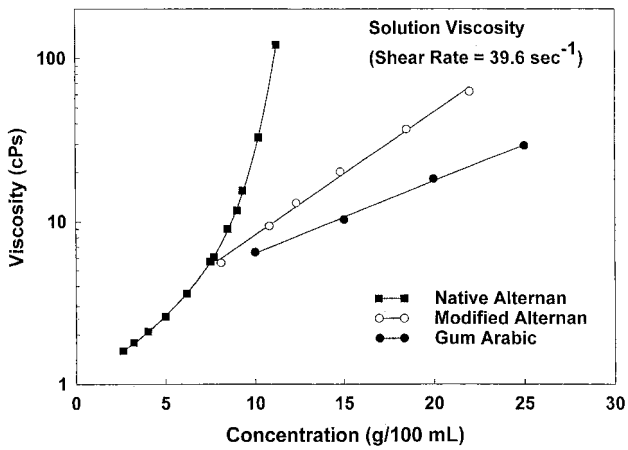

Figure 6 Rheological properties of native alternan and alternan modified by *Penicillium* spp. isolates compared with gum arabic. (■) Native alternan; (○) modified alternan; (●) gum arabic.

Alternatively, it is conceivable that *Penicillium* spp. spores secrete (or shed) materials during germination that promote disaggregation of native alternan. Alternan occasionally appears to aggregate during storage in solution (unpublished observations). The appearance of a minor $1-5\times10^4$ molecular weight class in native alternan preparations suggests that this is a naturally occurring form. Many fungi release hydrophobin-like proteins during spore germination, including *Penicillium chrysogenum* [9,13,27]. However, we found that *Schizophyllum commune*, a well-characterized source of hydrophobins, did not appear to affect alternan in culture medium.

The rheological properties of native alternan, modified alternan and commercial gum arabic were compared (Figure 6). As shown, modified alternan resembled gum arabic much more closely than did native alternan. This result suggests that modified alternan might be useful as a functional replacement for gum arabic in applications requiring a low-viscosity, highly soluble bulking agent. It should be noted, however, that certain uses of gum arabic depend on its emulsification capacity. Although the emulsification capacity of modified alternan was not tested in this study, native alternan exhibits low emulsification capacity.

In summary, novel *Penicillium* spp. isolates that modify alternan in a simple bioconversion process were identified. This modified alternan may find new industrial, food or research applications.

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