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Enhancement of Lignin Degradation in *Streptomyces* spp. by Protoplast Fusion†

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Protoplast fusion was investigated as a technique for genetically manipulating two lignin-degrading *Streptomyces* strains, *Streptomyces viridosporus* T7A and *Streptomyces setonii* 75Vi2. Four of 19 recombinants tested showed enhanced production of acid-precipitable polymeric lignin (APPL), producing 155 to 264% more APPL from corn stover lignocellulose than was produced by the wild-type *S. viridosporus* T7A. APPLs are lignin degradation intermediates known to be potentially valuable chemical products produced by bioconversion of lignin with *Streptomyces* spp. The prospects of utilizing protoplast fusion to construct APPL-overproducing *Streptomyces* strains was considered especially promising.

Currently, there is interest in the potential for microbial conversion of lignin to useful phenolic compounds and modified lignin polymers (4, 6). *Streptomyces* spp. are known to degrade lignin (3, 10) by an oxidative mechanism which involves aromatic ring demethylations, ring cleavage reactions, and oxidative attack on phenylpropanoid side chains (5). Several single-ring aromatic phenols released during the degradation of hardwood, softwood, and grass lignins by *Streptomyces viridosporus* have been identified (4). In addition, a polymeric intermediate, acid-precipitable polymeric lignin (APPL), has been identified as a quantitatively major water-soluble catabolite of lignin degradation by *Streptomyces* spp. (7). Both low-molecular-weight phenols and APPLs have potential uses as industrial chemicals (7). However, to obtain economical yields of lignin-derived products, increased rates of lignin degradation by *Streptomyces* spp. will be required. Protoplast fusion has been demonstrated as a means of genetic recombination in a number of actinomycete strains (11). Here, we report on the use of protoplast fusion as a technique for genetically manipulating lignin-degrading *Streptomyces* strains.

The procedures for protoplast formation and regeneration were modified from those of Okanishi et al. (9), Baltz (1), and Baltz and Matsushima (2). *Streptomyces* strains *S. viridosporus* T7A and *Streptomyces setonii* 75Vi2 were grown in shaken culture in tryptone yeast extract (TYE) broth at 37°C, aseptically homogenized, and transferred into TYE broth containing 0.4% (wt/vol) glycine. The cells were harvested in the late exponential phase and were subsequently fragmented by homogenization and sonication. Mycelia to be subjected to protoplast fusion were washed twice in medium P of Okanishi et al. (9), homogenized, sonicated, and treated with 3 mg of lysozyme per ml for 1 to 2 h. Then, the protoplast suspension was filtered through sterile cotton and a 5-μm Nuclepore filter (Nuclepore Corp., Pleasanton, Calif.) to exclude mycelial forms. After two washings with medium P, the filtered suspension was serially diluted and plated on regeneration medium (MR2) of Baltz (1) and TYE agar to determine regeneration efficiency. Incubation occurred at 31°C for 4 to 7 days. Protoplast fusion procedures were modified from those of Okanishi et al. (9) and Baltz (1). Polyethylene glycol 8000-treated protoplasts of the mixed *Streptomyces* strains were serially diluted and plated on MR2 and TYE agar after fusion proceeded for 1 to 2 min. Colonies arising on MR2 were compared with those of the parental types on regeneration plates, and colonies which differed in form were selected for further examination. These morphologically distinct colonies were designated as tentative protoplast fusion recombinants.

The ability of the tentative recombinants to produce the APPL intermediate of lignin degradation (7) was investigated by growing cultures on dampened (7) 50-mg samples of lignocellulose prepared from *Zea mays* as previously described (4, 5). Sterilized lignocellulose was inoculated with 1.0 ml of a cell suspension previously grown for 24 to 48 h in a mineral salts broth containing 0.6% yeast extract. Inoculated lignocellulose cultures were then incubated at 37°C for 3 weeks in a humid incubator. Afterwards, 9.0 ml of distilled water was added to each test tube, which was then steamed at 100°C for 1 h. The mixture was then filtered, and the APPL was recovered in the aqueous phase. APPL present was measured by a turbidometric assay (7). Recombinants which produced substantial APPL by the turbidometric assay were studied further. Two-gram samples of sterilized lignocellulose were inoculated with 20 ml of a cell suspension previously grown for 24 to 48 h in the mineral salts-yeast extract medium. Inoculated cultures were incubated at 37°C for 4 weeks in a humid incubator. Flasks were harvested by the addition of 250 ml of distilled water followed by steaming at 100°C for 1 h. This mixture was filtered through preweighed filter paper disks. Residues on the filters were air dried and weighed to determine lignocellulose weight loss (3). APPLs were precipitated from culture filtrates by acidification to pH 1.5. Precipitates were collected by centrifugation, washed with acidified water, and then air dried and weighed.

Regeneration efficiencies of 53.3 and 24.3% were obtained for *S. viridosporus* T7A and *S. setonii* 75Vi2, respectively. Recombination frequencies varying between 1 and 10% were obtained by using 40% (wt/vol) polyethylene glycol 8000 and direct selection of recombinants on the basis of colonial morphology on MR2. The turbidometric assay for APPL production in test-tube cultures by the parental strains *S. viridosporus* T7A and *S. setonii* 75Vi2 showed absorbances at 600 nm of 0.21 and 0.07, respectively (Table 1). The 19 tentative recombinants varied in APPL-producing abilities, with observed absorbances ranging between 0.06 and 0.36. Four recombinants (SR-3, SR-4, SR-10, and SR-14) appar-

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TABLE 1. APPL production of the *Streptomyces* parental strains and protoplast fusion recombinants as measured by turbidometric assay[a]

Strain	Optical density (600 nm)[b]
Uninoculated control	0.04 ± 0.01
S. viridosporus T7A	0.21 ± 0.03
S. setonii 75Vi2	0.07 ± 0.01
SR-1	0.21 ± 0.01
SR-2	0.23 ± 0.01
SR-3	0.35 ± 0.01
SR-4	0.35 ± 0.01
SR-5	0.06 ± 0.01
SR-6	0.25 ± 0.01
SR-7	0.29 ± 0.01
SR-8	0.16 ± 0.01
SR-9	0.26 ± 0.01
SR-10	0.36 ± 0.01
SR-11	0.15 ± 0.01
SR-12	0.22 ± 0.02
SR-13	0.19 ± 0.02
SR-14	0.35 ± 0.02
SR-15	0.22 ± 0.02
SR-16	0.23 ± 0.01
SR-17	0.26 ± 0.02
SR-18	0.15 ± 0.01
SR-19	0.19 ± 0.00

[a] Incubation occurred at 37°C for 3 weeks in the dampened lignocellulose culture system. Aqueous extracts were acidified to precipitate APPLs present, and the optical density at 600 nm of the resulting suspension was measured.

[b] Values are means plus or minus standard deviations of three replicates.

ently produced more APPL under these cultural conditions than *S. viridosporus* T7A did. These four were selected for more detailed study. As shown in Table 2, *S. viridosporus* T7A degraded 2.0 g of corn lignocellulose to a weight loss of 36.0% after 4 weeks, and 34.6 mg of APPL was recovered from the acidified aqueous extracts of the degraded lignocellulose. *S. setonii* 75Vi2 degraded the lignocellulose to a weight loss of 4.9%, and 3.9 mg of APPL was recovered. The uninoculated control showed a weight loss of 3.5% and a recovery of 11.0 mg of APPL. The four selected recombinants produced higher levels of APPL than *S. viridosporus* did, although they degraded the lignocellulose to weight losses which were similar to those with *S. viridosporus* T7A.

The present study shows that protoplast fusion can be utilized to construct APPL-overproducing *Streptomyces* strains which may in turn be useful in bioconversions of lignin to chemicals. As reported here, 21% of the recombinants examined produced between 155 and 264% more APPL than *S. viridosporus* T7A did when grown on corn stover lignocellulose. Enhanced APPL production may have been the result of fusion between two or more protoplasts of *S. viridosporus* T7A since a self-fusion experiment also yielded recombinants which showed enhanced APPL production as measured by the turbidometric assay (7). According to Hopwood (8), fusion between three or four protoplasts are about as frequent as those involving only two protoplasts. Recombinants derived from *S. setonii* 75Vi2 and more than one *S. viridosporus* T7A protoplast may explain the variations in APPL-producing abilities. Also, fragmentation of the *S. viridosporus* T7A genome after self-fusion may

TABLE 2. Corn stover lignocellulose degradation and APPL production by *Streptomyces* parental strains and selected protoplast fusion recombinants after 4 weeks of incubation at 37°C in the dampened lignocellulose system

Strain	Wt loss of lignocellulose (%)[a]	APPL recovered (mg)[a]
Uninoculated control	3.5 ± 0.0	11.0 ± 1.9
S. viridosporus T7A	36.0 ± 1.5	34.6 ± 1.6
S. setonii 75Vi2	4.9 ± 0.0	3.9 ± 0.2
SR-3	36.4 ± 0.0	125.9 ± 3.3
SR-4	35.3 ± 0.0	108.0 ± 6.7
SR-10	32.9 ± 0.0	102.3 ± 12.9
SR-14	35.5 ± 0.0	88.1 ± 3.3

[a] Values are means plus or minus standard deviations of two replicates.

have resulted in an incomplete enzyme system necessary for APPL production. Transformation may also have occurred at a very low frequency since DNase was not added to the protoplast fusion medium. Stability of recombinants is an important consideration if certain strains are to be useful as bioconversion organisms. The four recombinants which showed enhanced APPL production retained that ability after 10 transfers on TYE agar and have remained stable since then through multiple transfers on routine laboratory stock culture media.

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