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Elicitation of Lignin Peroxidase in *Streptomyces lividans*

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ABSTRACT

Using a novel starch-based medium (DJMM) which elicits high expression of lignin peroxidase (ALiP-P3) from *Streptomyces viridosporus* T7A, significant levels of ALiP-P3 (between 1135 and 1784 nmol/g cell·min) were excreted by *S. lividans* TK23, TK24, and TK64 with the supernatants capable of degrading dichlorophenol (these strains were previously reported to produce low levels of LiP). The *S. lividans* wild-type strains produced 1/9 to 1/6 the cell-specific LiP activity previously detected in *S. viridosporus* T7A cultures grown in the same starch-based medium; however, by using DJMM to increase the cell density, the volumetric activity of wild-type *S. lividans* TK23, TK24, and TK64 strains was increased 11- to 20-fold compared to cultivations in a yeast-extract-based medium. Consequently, this increase of LiP production allows the direct analysis of LiP activity in the supernatants of these strains without the need for enzyme concentration through ultrafiltration. Immunoblot analysis verified that a single 56.5 kDa band, secreted by all three strains, was extremely similar in size and immunologic reactivity to the 59.5 kDa ALiP-P3 isoform of *S. viridosporus* T7A. In addition, Western blot analysis was used to show that a previously cloned 4.1 kb chromosomal fragment of *S. viridosporus* T7A DNA did not contain the ALiP-P3 structural genes.

Index Entries: Lignin peroxidase; *Streptomyces;* dichlorophenol.

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INTRODUCTION

Chlorophenols, such as 2,4-dichlorophenol (DCP), are widely used in the synthesis of a range of herbicides (*1*). DCP is recognized as an EPA priority pollutant and, among all chlorophenols, has the highest annual rate of production in the world (*1*). The actinomycete, *Streptomyces viridosporus* T7A, produces a peroxidase (LiP) that utilizes DCP as a substrate and has been identified as an integral extracellular enzyme in the *S. viridosporus* T7A lignin-degrading system. This enzyme has been named ALiP-P3 and produces a major lignin degradation product, a modified water-soluble, acid-precipitable polymeric lignin (*2–6*). Esterases, endoglucanases, and xylanases that may be involved in the degradation of lignin have also been identified as extracellular enzymes produced by *S. viridosporus* T7A (*7,8*). In the presence of H_2O_2, LiP catalyzes the cleavage of the 1,2-diaryl propane (β-1) and arylglycerol-β-aryl ether (β-O-4) bonds of lignin (*6*). Although the role of ALiP-P3 in the degradation of lignin is not fully understood, the enzyme's use of DCP as a substrate suggests that ALiP-P3 may be used in the bioremediation of DCP and other chlorophenols.

Four isoforms of the extracellular *S. viridosporus* T7A peroxidase were detected by Ramachandra et al. (*6*) and the isoform with the greatest known substrate range, ALiP-P3, has been isolated using rabbit polyclonal antibodies (*9*). Whereas isoforms P1, P2, and P3 all had detectable peroxidase assay activity using an L-3,4-dihydroxypehnylalanine (L-Dopa) substrate, ALiP-P3 is the only isoform detected by a peroxidase assay using a 2,4-dichlorophenol substrate (*6*). Immunoblot analysis of affinity-purified ALiP-P3, purified by chromatography on a rabbit-anti-P3 IgG affinity column (*9*), resulted in one major band (*10,11*), which has been described as a 63 kDa band (*11*). However, in most cases, the analysis of ALiP-P3 required the use of several concentration steps to obtain sufficient amounts of protein. These steps often included ultrafiltration (*9*), gel filtration (*6*), and $(NH_4)_2SO_4$ precipitation (*9*), which require several hours and, due to protein degradation or inactivation, often resulted in protein recoveries of 30–50% (unpublished results of ultrafiltration followed by $(NH_4)_2SO_4$ precipitation).

To increase expression of ALiP-P3, a starch-based growth medium (DJMM) was developed for *S. viridosporus* T7A (*11*). This medium uses corn starch and casein to obtain high cell density cultures, and calcium carbonate to induce cellular production of ALiP-P3. Using DJMM, ALiP-P3 production by *S. viridosporus* T7A cultures was increased 60-fold volumetrically compared to cultivations in yeast-extract-based medium (*11*). Cultures grown in DJMM were also found to produce sixfold more ALiP-P3 per cell. This increase in ALiP-P3 production by *S. viridosporus* T7A precluded the need for enzyme concentration by ultrafiltration.

Because ALiP-P3 is capable of degrading DCP, *S. lividans* strains were screened in this work for enhanced ALiP-P3 when cultured in DJMM.

The results indicate that wild-type *S. lividans* strains are capable of producing significant amounts of lignin peroxidase using this medium and may be screened rapidly, without lengthy concentration steps.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

S. viridosporus T7A, *S. lividans* TK23, TK23.1, TK24, TK24.1, TK64, and TK64.1 (*12*) were obtained from Professor D. L. Crawford (University of Idaho, Moscow, ID). The *S. lividans* TK23.1, TK24.1, and TK64.1 recombinant strains harbor the pIJ702.LP plasmid, which was derived from the pIJ702 plasmid (thiostrepton^R), and contains the same 4.1 kb *S. viridosporus* T7A chromosomal DNA fragment in pBST4.1 (*13*). The presence of pIJ702.LP in the recombinant *S. lividans* TK23.1, TK24.1, and TK64.1 strains was verified by *Bgl*II digestion of *S. lividans* plasmid minipreps (*14*). Spore suspensions of each *Streptomyces* strain were made from stock cultures grown on R2YE slants (*14*) at 37°C for 1 to 3 wk, until sporulation. These 20% glycerol spore suspensions (~5 mL) were divided and stored at either −85°C (long-term spore suspensions) or −20°C (working-spore suspensions). All 50 mL *Streptomyces* cultures in this study were inoculated with 50 μL of working-spore suspensions and cultured at 37°C in a Series 25 rotary shaker at 250 rpm (New Brunswick Scientific Company Inc., Edison, NJ).

To determine the maximum LiP activity obtained from *S. lividans* TK23, TK24, TK64, and *S. viridosporus* T7A, each of the strains were grown in triplicate 250-mL Erlenmeyer flasks containing 50 mL DJMM (*11*) (all concentrations are w/v unless noted): 4% Kingsford corn starch (Albertson's Food and Drug Stores, Irvine, CA), 2% casein (Aldrich Chemical Co., Inc., Milwaukee, WI), 0.7% $CaCO_3$ (Fisher Scientific, Tustin, CA), 0.3% yeast extract (Difco Laboratories, Detroit, MI), 0.1% NH_4Cl (Fisher), and 0.1% v/v R2YE trace metals stock solution (*14*). Triplicate cultures of each *S. lividans* strain were also grown in 50 mL of yeast-extract-based medium (*15*) consisting of (w/v): 0.75% yeast extract, 0.53% Na_2HPO_4 (Fisher), 0.198% KH_2PO_4 (Fisher), 0.02% NaCl (Fisher), 0.02% $MgSO_4 \cdot 7H_2O$ (Fisher), 0.005% $CaCl_2$ (Fisher), 0.1% asparagine (Fisher), 0.1% glutamic acid (Fisher), 0.1% proline (Fisher), and 0.1% v/v R2YE trace metals stock solution.

Peroxidase Assay and Ultrafiltration

The initial rate of DCP oxidation by the peroxidase in supernatant samples was measured in the presence of H_2O_2 (*3,11*). The peroxidase oxidizes DCP which reacts with 4-aminoantipyrene to form a colored antipyrylquinonimine that strongly absorbs at 510 nm. The 1.0 mL aqueous

assay mixture contained final concentrations of 100 m*M* Tris-HCl buffer, pH 8.0 (Fisher), 5 m*M* 2,4-dichlorophenol (Eastman Kodak Co., Rochester, NY), 16.4 m*M* 4-aminoantipyrene (Sigma), 100 μL of LiP enzyme (1.0 mL culture supernatant centrifuged for 5 min at 14,000*g*), and 4.0 m*M* H_2O_2 (Fisher). The increase of absorbance at 510 nm was measured with a DU640 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA) at 25°C for 5 min. The activity was taken as the maximum rate of DCP oxidation obtained in a 1-min interval and calculated using a molar absorptivity of $18{,}500M^{-1}\ cm^{-1}$ for the antipyrylquinonimine product (*11*).

To concentrate extracellular LiP of *S. lividans* TK64.1 cultures grown in yeast-extract-based medium, the culture supernatant was first collected after the cells of a 1 L culture were allowed to settle at 4°C. The supernatant was then concentrated 20 times, at 4°C, using a YM10 ultrafiltration membrane (Amicon, Beverly, MA) and a Model 8200 stirred-cell ultrafiltration unit (Amicon) following the procedure of Magnuson et al. (*9*).

SDS-PAGE and Western Blot Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot samples of *S. lividans* culture supernatants were obtained from 1.0 mL centrifuged (5 min at 14,000*g*) samples of 6-d-old *S. lividans* cultures grown in 250 mL Erlenmeyer flasks containing 50 mL DJMM. The centrifuged supernatant was mixed with a 2X sample buffer (*16*), boiled 5 min, and analyzed on a 4% polyacrylamide stacking, 12% resolving gel (*17*). Gels were loaded with 20–70 μL of protein sample such that each *S. lividans* sample contained the volume of supernatant required to give a lignin peroxidase assay activity of 0.54 nmol/min (*S. viridosporus* T7A was loaded at 1.08 nmol/min). The SDS-PAGE was run on a P2X vertical gel electrophoresis unit (Owl Scientific Plastics, Cambridge, MA) at constant 30 mA through the stacking gel and then at constant 60 mA through the resolving gel for 3 h, followed by Coomassie blue staining (*18*). The size of the proteins on the SDS-PAGE were calculated using the calibration curve obtained as a plot of the log (size molecular weight standards) vs migration distance.

The same extracellular *S. lividans* and *S. viridosporus* T7A proteins were separated for Western blot analysis on a 4% polyacrylamide stacking, 12% resolving gel with a Mini-Protein II Dual Slab Cell at constant 150 V (Bio-Rad, Hercules, CA) with a target sample loading of 0.034 nmol/min LiP activity in each sample. The separated proteins were then transferred onto a 0.45 μm nitrocellulose membrane (Bio-Rad) at constant 102 V using a Mini Trans-Blot Cell (Bio-Rad) according to the manufacturer's specifications. Lignin peroxidase bands were detected by following the manufacturer's protocol for the ProtoBlot Western blot AP system (Promega Corp., Madison, WI) and using a 1:3000 dilution of rabbit-anti-ALiP-P3 antibody (*9*) (supplied by D. L. Crawford) and rabbit anti-IgG AP conjugate (Promega).

Dry Cell Mass Determination

The dry cell mass of each culture was obtained by centrifuging 5 mL of culture at 10,000*g* and 4°C (model J2-21, Beckman Instruments, Inc.), and washing twice with 10 mL distilled deionized water to remove residual medium components. The washed cell pellet was filtered through a preweighed, 1 μm nominal pore size, Type A/E glass-fiber filter (Gelman Scientific, Ann Arbor, MI) and dried overnight in an 80°C oven.

RESULTS

SDS-PAGE and Western Blot Analysis

The extracellular LiP produced by the *S. lividans* strains cultured in DJMM was visible on SDS-PAGE as shown in Fig. 1. For *S. viridosporus* T7A, a 59.5 kDa, ALiP-P3 band was clearly seen, along with a faint 56.5 kDa band on the SDS-PAGE, corresponding to the 63 kDa ALiP-P3 and 58 kDa bands, respectively, which were described previously (*11*). In each *S. lividans* sample, with and without the pIJ702.LP plasmid, only the 56.5 kDa band was visible (Fig. 1). An SDS-PAGE comparing equivalent amounts of culture supernatants (consistent cell density basis) from *S. lividans* strains TK23, TK24, and TK64 cultivated in either DJMM or yeast-extract-based medium showed that no protein bands were visible from the supernatants of yeast-extract-based cultures; however, the 56.5 kDa band was the only band clearly visible in samples obtained from the DJMM cultures containing almost 20 times more volumetric LiP activity (gel not shown). In addition, when *S. lividans* TK64.1 cultures in yeast-extract-based medium were concentrated 20 times by ultrafiltration, the 56.5 kDa band then became clearly visible on SDS-PAGE (gel not shown). Hence, ALiP-P3 activity and the 56.5 kDa protein are closely related.

To corroborate that the 56.5 kDa band of *S. lividans* was immunologically similar to that of ALiP-P3, a Western blot analysis was performed. The Western blot (Fig. 2) shows that the 56.5 kDa band in all the *S. lividans* strains grown in DJMM (with and without pIJ702.LP) was the only band that reacted to the *S. viridosporus* T7A-derived ALiP-P3 antibody. The most prominent bands for *S. viridosporus* T7A were 59.5 kDa and 56.5 kDa bands. Since each sample for the Western blot contained the same amount of ALiP-P3 activity, the results indicate that the ALiP-P3 band, from *S. viridosporus* T7A, is very similar in size and intensity to the sole band appearing in the wild-type *S. lividans* TK23, TK24, and TK64 samples. Furthermore, a Western blot analysis comparing *S. lividans* TK64 and TK64.1 cultures in DJMM and yeast-extract-based medium also showed that in both media, the 56.5 kDa protein was the only extracellular protein produced by the *S. lividans* strains which reacted with the ALiP-P3 antibodies (Western blot not shown). Interestingly, although the

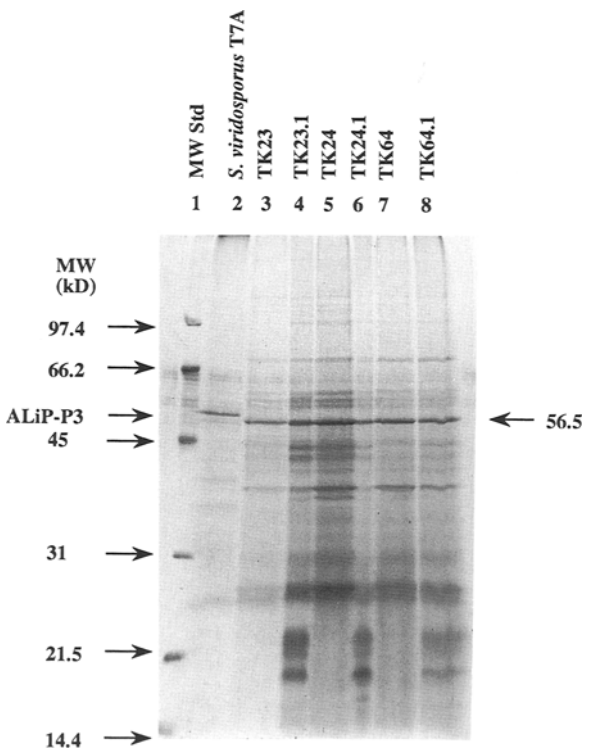

Fig. 1. SDS-PAGE of supernatants from *S. lividans* DJMM cultures. Each well contains equivalent amounts of LiP activity (0.54 nmol/min), except *S. viridosporus* T7A (1.08 nmol/min). Lane 1: Molecular weight standards, Lane 2: *S. viridosporus* T7A, Lane 3: *S. lividans* TK23, Lane 4: *S. lividans* TK23.1, Lane 5: *S. lividans* TK24, Lane 6: *S. lividans* TK24.1, Lane 7: *S. lividans* TK64, Lane 8: *S. lividans* TK64.1. Molecular weight standards (kilodaltons): phosphorylase *b* (97.4), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), soybean trypsin inhibitor (21.5), and lysozyme (14.4).

56.5 kDa band was faintly visible in the *S. viridosporus* T7A SDS-PAGE sample (Fig 1), the corresponding 56.5 kDa band in the Western blot of the same *S. viridosporus* T7A sample preparation (Fig. 2) was distinctly visible and appeared even more intense than the 59.5 kDa ALiP-P3 band.

Although the recombinant *S. lividans* TK23.1, TK24.1, and TK64.1 strains contain the *S. viridosporus* T7A-derived pIJ702.LP, only two new, lower-molecular-weight proteins (18 and 21 kDa) were distinctly visible on SDS-PAGE compared to the wild-type *S. lividans* and *S. viridosporus* T7A samples (Fig. 1). The 59.5 kDa ALiP-P3 band of *S. viridosporus* T7A was not seen in the recombinant strains; although, like the *S. lividans* wild-type strains, a distinct, immunologically-related 56.5 kDa band was clearly visible. The two new protein bands did not bind to the ALiP-P3 antibodies, indicating that the new proteins were not immunologically related to ALiP-P3, and they are not related to the single 30 kDa protein encoded by the thiostrepton-resistance gene in pIJ702.LP (*14*).

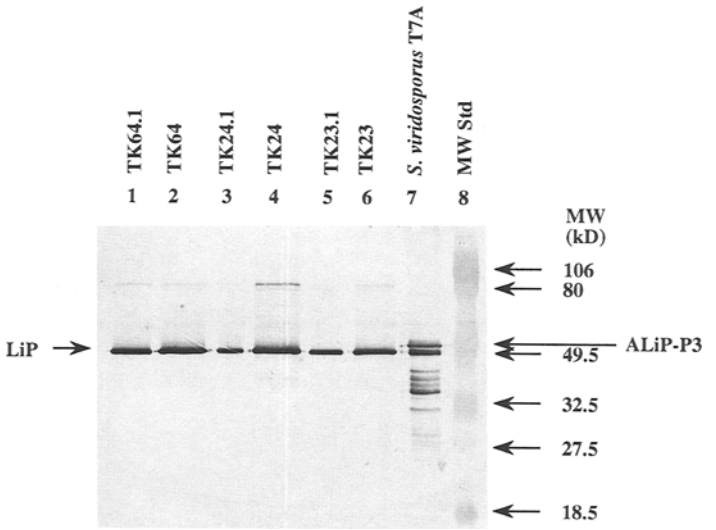

Fig. 2. Western blot of supernatants from *S. lividans* DJMM cultures. Each well contains equivalent amounts of LiP activity (0.034 nmol/min). Lane 1: *S. lividans* TK64.1, Lane 2: *S. lividans* TK64, Lane 3: *S. lividans* TK24.1, Lane 4: *S. lividans* TK24, Lane 5: *S. lividans* TK23.1, Lane 6: *S. lividans* TK23, Lane 7: *S. viridosporus* T7A, Lane 8: Molecular weight standards (prestained, kilodaltons): phosphorylase *b* (106), bovine serum albumin (80), ovalbumin (49.5), carbonic anhydrase (32.5), soybean trypsin inhibitor (27.5), and lysozyme (18.5).

Maximum LiP Activities

When wild-type *S. lividans* TK23, TK24, and TK64 were cultured in the starch-based DJMM, the *volumetric* LiP activities (30.3 to 36.8 nmol/mL·min, Table 1) averaged 17-fold higher than similar cultures grown in yeast-extract medium (*15*) (2.0 to 2.2 nmol/mL·min, Table 1). This increase resulted from the higher cell densities of the DJMM cultures. Consequently, the *cell-specific* LiP activities of the wild-type *S. lividans* were only marginally higher in DJMM compared to yeast-extract-based medium (17–65% higher). However, the recombinant *S. lividans* TK23.1, TK24.1, and TK64.1 cultured in yeast-extract-based medium had 2.6–4.0-fold higher cell-specific activities (3514, 5405, and 5514 nmol/g cell·min, respectively, Table 1) compared to the corresponding strains cultivated in DJMM (1351, 1838, and 1405 nmol/g cell·min, respectively, Table 1).

The maximum ALiP-P3 activity obtained for each *S. lividans* strain (Table 1) indicates that the presence of pIJ702.LP did not improve LiP production in DJMM; the maximum cell-specific ALiP-P3 activity for the six strains analyzed were very similar (1135 to 1838 nmol/g cell·min), with the highest wild-type activity obtained from *S. lividans* TK23 (1783.8 nmol/g cell·min).

Table 1
Maximum LiP Activity of Supernatants of *S. lividans* Strains Cultured in DJMM and Yeast-Extract-Based Medium (YM)

	Maximum volumetric LiP activity, nmol/(mL·min)		Maximum specific LiP activity, nmol/(g·min)		Cell density, mg/mL	
Strains	DJMM	YM	DJMM	YM	DJMM	YM
S. lividans						
TK23	36.8 ± 16.2	2.2 ± 0.2	1,784 ± 757	1,081 ± 22	20.3 ± 0.3	2.1 ± 0.1
TK23.1	30.8 ± 13.0	1.5 ± 0.2	1,351 ± 432	3,514 ± 595	22.5 ± 0.3	0.4 ± 0.1
TK24	34.6 ± 5.4	2.0 ± 0.4	1,622 ± 108	1,189 ± 378	21.2 ± 2.0	1.8 ± 0.3
TK24.1	38.4 ± 11.4	3.7 ± 0.3	1,838 ± 432	5,405 ± 378	20.8 ± 1.7	0.7 ± 0.1
TK64	30.3 ± 2.20	1.9 ± 0.3	1,135 ± 270	973 ± 108	26.8 ± 5.2	1.9 ± 0.3
TK64.1	37.8 ± 5.9	2.1 ± 0.3	1,405 ± 216	5,514 ± 1,297	26.5 ± 0.1	0.4 ± 0.1
S. viridosporus T7A[a]	183.8 ± 21.6	3.0 ± 0.3	10,865 ± 1,568	1,805 ± 162	17.4 ± 1.3	1.7 ± 0.1

[a] Yee, Jahng, and Wood, 1996 (*11*).

DISCUSSION

A starch-based medium, optimized for maximum ALiP-P3 production by *S. viridosporus* T7A, was used to analyze *S. lividans* strains for production of a lignin peroxidase similar to ALiP-P3. It was found that wild-type *S. lividans* are capable of producing significant extracellular concentrations of LiP when cultured in DJMM. The reaction of the *S. lividans* LiP with 2,4-dichlorophenol (ALiP-P3 is the only *S. viridosporus* T7A isoform capable of oxidizing 2,4-DCP) and the very strong binding of the *S. viridosporus* T7A-derived ALiP-P3 antibody to the 56.5 kDa *S. lividans* samples indicate that the wild-type *S. lividans* peroxidase is very similar immunologically to the ALiP-P3 peroxidase produced by *S. viridosporus* T7A. *S. lividans* is known to glycosylate proteins (*19–21*) and differences in glycosylation between *S. viridosporus* T7A and *S. lividans* may explain the small size difference between the LiP proteins from the two strains.

Previous researchers have indicated that pIJ702.LP either contains a regulatory element for ALiP-P3 production or the ALiP-P3 structural genes by showing that *S. lividans* strains transformed with pIJ702.LP decolorized Poly B-411 dye-containing agar plates (*12*), produced an active band in L-Dopa-activity-stained PAGE gels (*10*), and restored ALiP-P3 activity in formerly peroxidase-deficient *S. viridosporus* T7A mutants which were transformed with pIJ702.LP (*22*). However, the decolorization of Poly B-411 may not be a specific peroxidase screening method. For example, Remozal Brilliant Blue (RBB), a dye very similar to Poly B-411 (*23*), is often used in an RBB-based plate assay for the selection of xylanase-producing *S. lividans* (*24–26*). Our SDS-PAGE results, showing additional 18 kDa and 21 kDa bands for the recombinant *S. lividans* samples (which were not seen in either the *S. viridosporus* T7A or wild-type *S. lividans* samples grown in DJMM), indicate that any increased activity in pIJ702.LP-containing strains may be due to a regulatory protein. Considering that wild-type *S. lividans* produce LiP at the same high level as pIJ702.LP-containing *S. lividans* in DJMM, that no proteins reacting to ALiP-P3 antibodies are detected in induced, recombinant *E. coli* strains containing the 4.1 kb *S. viridosporus* T7A chromosomal DNA (unpublished results using *E. coli* JM109/pBST4.1), and that the recombinant *S. lividans* do not produce an additional 59.5 kDa ALiP-P3 band on the Western blot, our results support the suggestion of Wang et al. (*12*) that a regulatory element was cloned into pIJ702.LP.

Wild-type *S. lividans* TK23, TK24, and TK64 produced between 1135 and 1784 nmol/(g cell·min) units of LiP when cultivated in starch-based DJMM, but produced much lower maximum LiP activities compared to *S. viridosporus* T7A cultured in DJMM: 10,865 nmol/(g cell·min) (*11*). The enhanced volumetric activities of *S. lividans* cultures in DJMM compared to yeast-extract-medium cultures were primarily due to the increased cell density of the starch-based cultures.

Other researchers have found that *S. viridosporus* T7A does not produce maximum amounts of LiP with the same growth medium component concentrations as *S. lividans* TK64.1 (*15*). Because DJMM was formulated specifically for high LiP production in *S. viridosporus* T7A, the concentrations of corn starch, casein, and yeast extract may not be optimal for high LiP production in other *Streptomyces* strains, such as *S. lividans*. Based on our preliminary data using *S. lividans* TK24, in which additional 3-fold improvements in specific LiP activity were seen, further improvements in cell-specific LiP expression may also be obtained by optimizing culture conditions for *S. lividans* grown in DJMM (e.g., reactor size and configuration). The use of DJMM in *S. lividans* cultivations allows for the rapid screening of peroxidase-producing strains which may be used to degrade DCP.

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REFERENCES

1. Desmurs, J. R. and Ratton, S. (1992), in *Encyclopedia of Chemicals and Technology*, vol. 6, Howe-Grant, M., ed., Academic, New York, NY, pp. 157–168.
2. Pasti, M. B., Hagen, S. R., Korus, R. A., and Crawford, D. L. (1991), *Appl. Microbiol. Biotechnol.* **34,** 661–667.
3. Spiker, J. K., Crawford, D. L., and Thiel, E. C. (1992), *Appl. Microbiol. Biotechnol.* **37,** 518–523.
4. Adhi, T. P., Korus, R. A., and Crawford, D. L. (1989), *Appl. Environ. Microbiol.* **55,** 1165–1168.
5. Crawford, D. L., Pometto, A. L., and Crawford, R. L. (1983), *Appl. Environ. Microbiol.* **45,** 898–904.
6. Ramachandra, M., Crawford, D. L., and Hertel, G. (1988), *Appl. Environ. Microbiol.* **54,** 3057–3063.
7. Deobald, L. A. and Crawford, D. L. (1987), *Appl. Microbiol. Biotechnol.* **26,** 158–163.
8. Ramachandra, M., Crawford, D. L., and Pometto, A. L. (1987), *Appl. Environ. Microbiol.* **53,** 2754–2760.
9. Magnuson, T. S., Roberts, M. A., Crawford, D. L., and Hertel. G. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 433–443.
10. Magnuson, T. S. and Crawford, D. L. (1992), *Appl. Environ. Microbiol.* **58,** 1070–1072.
11. Yee, D. C., Jahng, D., and Wood, T. K. (1996), *Biotechnol. Prog.* **12,** 40–46.
12. Wang, Z., Bleakley, B. H., Crawford, D. L., Hertel, G., and Rafii, F. (1990), *J. Biotechnol.* **13,** 131–144.
13. Crawford, D. L. (1993), Personal communication.
14. Hopwood, D. A., Bibb, M. G., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M., and Shrempf, H. (1985), *Genetic manipulations of* Streptomyces: *a laboratory manual,* John Innes Foundation, Norwich, England.

15. Korus, R. A., Lodha, S. J., Adhi, T. P., and Crawford, D. L. (1991), *Biotechnol. Prog.* **7,** 510–515.
16. Jahng, D. and Wood, T. K. (1994), *Appl. Envirion. Microbiol.* **60,** 2473–2482.
17. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), *Molecular cloning: a laboratory manual,* 2nd ed., Cold Spring Harbor Laboratory, New York.
19. MacLeod, A. M., Gilkes, N. R., Escote-Carlson, L., Warren, R. A. J., Kilburn, D. G., and Miller Jr., R. C. (1992), *Gene* **121,** 143–147.
20. Ong, E. D., Kilburn, G., Miller Jr., R. C., and Warren, R. A. J. (1994), *J. Bacteriol.* **176,** 999–1008.
21. Vilches, C., Hernandez, C., Mendez, C., and Salas, J. A. (1992), *J. Bacteriol.* **174,** 161–165.
22. Wang, Z., Crawford, D. L., Magnuson, T. S., Bleakley, B. H., and Hertel, G. (1991), *Can. J. Microbiol.* **37,** 287–294.
23. Pasti, M. B. and Crawford, D. L. (1991), *Can. J. Microbiol.* **37,** 902–907.
24. Kleupfel, D. (1988), in *Methods in Enzymology,* vol. 160, Academic, New York: NY, pp. 180–186.
25. Kleupfel, D., Shareck, F., Mondou, F., and Morosoli, R. (1986), *Appl. Microbiol. Biotechnol.* **24,** 230–234.
26. Mondou, F., Shareck, F., Morosoli, R., and Kleupfel, D. (1986), *Gene* **49,** 323–329.