Improved Production of Mannanase by *Streptomyces lividans*

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Replacement of the natural promoter of the β -mannanase gene of *Streptomyces lividans* by *lacp* resulted in a 15-fold increase in enzyme production over that of the previously reported clone *S. lividans* IAF36, a clone carrying multiple copies of *manA*, and a 350-fold increase over that of the wild-type strain *S. lividans* 1326. In addition, the use of *lacp* in the shuttle vector pIAF199 allowed synthesis of the enzymes on carbon sources that did not contain mannan, such as xylan and whey, which offers interesting possibilities for industrial production of the enzyme.

β -Mannanase (1,4- β -D-mannan mannanohydrolase; EC 3.2.1.78) catalyzes the hydrolysis of β -1,4 mannose linkages of the backbone of β -mannans. These polysaccharides are found in various seeds and beans, where they play an important role in the mechanical resistance and the swelling that occur during germination. Mannans are also a major component of the hemicellulose fraction in soft woods. Mannanases are useful in several industrial processes, such as the extraction of vegetable oils from leguminous seeds and the reduction of the viscosity of coffee extracts during manufacture of instant coffee (5, 6). In the pulp and paper industry, mannanases can act synergistically with xylanases as biological prebleaching agents for soft-wood pulp, allowing a significant reduction of environmental pollution compared with the use of chlorine-based chemical agents (4, 12). Despite these potential applications, the use of mannanases in industry is limited, essentially because of low yields and high production costs.

Previously, we reported the cloning and characterization of mannanase A (ManA) from *Streptomyces lividans* (1). In this study, we investigated the possibility of increasing enzyme production by *S. lividans* by replacement of the promoter in the homologous expression vector.

A 1.7-kb *Sph*I fragment which contains the coding region of ManA was subcloned from pIAF36 (1) into pTZ19 downstream of *lacp*, as shown in Fig. 1. *Escherichia coli* transformants were screened on locust bean gum (LBG) plates, and clones showing activity were selected. Then, the plasmid pIAF199, containing the origin of replication and the thiostrepton resistance gene from plasmid pIJ702 (8), was inserted into the unique *Hind*III site located at the 3' end of the structural *manA* gene. The final construct, designated pIAF37, was used to transform *S. lividans* 1326. The wild-type strain 1326 was preferred over the cellulase- and xylanase-negative mutant *S. lividans* IAF10-164 that had been used previously as the *manA* cloning host (1). The mutation affects the transport of cellobiose and xylobiose (7) but does not influence the expression of the chromosomal *manA*; both strains produce 0.3 IU of ManA activity per ml. How-

ever, the wild-type strain showed considerably better growth patterns.

Spore suspensions of *S. lividans* IAF37, obtained from 7-day-old Bennett agar plates, served to inoculate Erlenmeyer flasks containing Trypticase soy broth (Difco) to which 5 μ l of thio-strepton had been added per ml as a selection marker. Abundant growth was achieved after 24 h of incubation at 34°C on a rotary shaker. A 10-ml aliquot of this preculture served to inoculate a 500-ml Erlenmeyer flask containing 100 ml of minimal medium M14 (11) to which 1% LBG, oat spelt xylan, or whey had been added as the sole carbon source. Incubations were carried out at 34°C on a rotary shaker at 240 rpm. The growth of the cultures on LBG or on xylan was followed by determination of the total protein content in the fermentation broth. For this, 1 ml of culture was homogenized in an Ultrasonic Processor XL (Heat Systems, Farmingdale, N.Y.) and the protein content was determined according to the method of Lowry et al. (10). In the case of whey, because of its initially high protein content, growth was determined by centrifugation as a percentage of packed mycelium per volume.

S. lividans IAF37 grew equally well with either xylan or LBG

FIG. 1. Plasmid IAF37. The ManA gene is under the control of *lacp* (*plac* in the figure) contained in pTZ19. Amp^r and Tsr^r indicate resistance to ampicillin and thiostrepton, respectively.

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FIG. 2. Relationship between mannanase production (A), mycelial growth (B), and protease synthesis (C) on different carbon sources. ○, LBG; ●, xylan; □, whey.

as the carbon source but showed a distinct lag period on whey, with which maximum growth occurred after 96 h of incubation (Fig. 2B).

Enzyme activity was determined on culture filtrates obtained by centrifugation of 5-ml samples withdrawn at intervals. The ManA activity was assayed by adding an appropriately diluted sample of the supernatant to a 0.5% (wt/vol) solution of LBG in 50 mM sodium citrate buffer, pH 6.7. The substrate had been purified as described previously (1). The reducing sugars released by enzyme activity after a 10-min incubation at 56°C were determined by performing the *p*-hydroxybenzoic acid hydrazide method of Lever (9) in a miniaturized form adapted to enzyme-linked immunosorbent assay plates and reading the A_{405} . A standard curve was established with mannose (0 to 1 mM). The enzyme activities are expressed in international units, where 1 IU is the amount of enzyme that releases 1 μ mol of reducing sugar (expressed as mannose) in 1 min.

The best mannanase production was achieved on LBG after 48 h of incubation, with levels reaching 115 IU/ml (Fig. 2A). On the noninducing carbon substrates, the enzyme production was lower, reaching 80 IU/ml on oat spelt xylan and 63 IU/ml on whey. On the same substrates, the clone *S. lividans* IAF36, in which the multicopy *manA* gene is under the control of its natural promoter (1), yielded 2 to 3 IU/ml (results not shown). Enzyme production was followed over the 96-h incubation period by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis with specific antimannanase antibodies, which confirmed that *S. lividans*

FIG. 3. Comparison of mannanase production by *S. lividans* IAF36 and IAF37 on LBG by Western blot analysis of culture filtrates (15 μ l) after 24, 48, 72, and 96 h of incubation. Lane 1, purified mannanase (1 μ g); lanes 2 to 5, *S. lividans* IAF36; lanes 6 to 9, *S. lividans* IAF37. Molecular mass standard positions are shown on the left.

FIG. 4. Slot blot analysis of total RNA extracted from clones, after 30 h of growth on LBG, carrying pIAF36 (A) or pIAF37 (B). Ten micrograms of total RNA was spotted onto a nitrocellulose membrane. The mannanase probe consisted of *manA* which was 32 P labelled by nick translation. The spots were quantified by densitometry (optical density $\times$ square millimeters [OD $\times$ mm 2]).

IAF37 secreted the same 37.6-kDa protein as *S. lividans* IAF36 (Fig. 3).

S. lividans IAF36 and IAF37 differed significantly in their enzyme yields. This could be attributed to differences in the *manA* transcription levels. Slot blot analysis of total RNA, as described previously (11), showed that the level of transcription for *S. lividans* IAF37 was indeed 10 times higher than that for IAF36, which explains the higher degree of expression obtained when *manA* is under the control of *lacp* (Fig. 4). This also confirms the observation by Bibb and Cohen that *E. coli* regulatory signals are well recognized by *S. lividans* (3).

The mannanase activity decreased once the stationary growth phase was reached. To determine if this decrease was due to proteolysis, protease activity in the supernatants was measured by the azocasein method (2). The results are shown in Fig. 2C. Protein hydrolysis was more pronounced with the noninducing substrates than with the mannan-containing LBG, indicative of the latter's role in protecting the enzyme. This stabilizing effect was confirmed in vitro by the addition of LBG to 64-h-old enzyme preparations (Fig. 5).

The remarkable 350-fold increase in mannanase production obtained on a mannan-rich substrate, such as LBG, with this new construct could be useful for industrial processes. Other such substrates might be available in waste products from extraction of agar from red algae like *Porphyra umbilicalis*. Alternatively, this study has shown that even on substrates that do not contain mannan, *S. lividans* IAF37 yielded significantly higher enzyme levels than the previously reported strains (1). This opens the possibility of investigating other waste products as potential carbon sources for large-scale production.

FIG. 5. Stability of mannanase in supernatants of 64-h xylan-grown cultures of *S. lividans* IAF37, incubated over a period of 24 h at 37°C in the presence (open symbols) or absence (closed symbols) of 1% LBG. The mannanase activities are expressed as percentages of the activity at time zero.

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