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Endoxanthanase, a Novel β-D-Glucanase Hydrolyzing Backbone Linkage of Intact Xanthan from Newly Isolated *Microbacterium* sp. XT11

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Abstract A novel endoxanthanase was produced and isolated from the culture of *Microbacterium* sp. XT11 growing on xanthan. Optimum pH and temperature for the enzyme activity were 6.0 and 35–40 °C, respectively. The endoxanthanase cleaves the backbone endo-β-1,4-glucosidic linkage of the xanthan molecule, which is specific to the intact xanthan. However, the lyase-treated xanthan or carboxymethyl cellulose could not be cleaved by endoxanthanase.

Keywords Xanthan · Endoxanthanase · Xanthan degradation · β-D-glucanase · *Microbacterium*

Introduction

Xanthan is an extracellular polysaccharide produced by phytopathogenic bacterium *Xanthomonas campestris* pv. *campestris* and is composed of a cellulosic backbone chain with trisaccharide side chains consisting of mannosyl–glucuronyl–mannose attached α-1,3 to alternating glucosyl residues [1]. The internal and terminal mannosyl residues of the side chain are frequently acetylated and pyruvylated respectively, depending on the bacterial strains and growth conditions [2]. Xanthan is considered to play a key role in the virulence of *Xanthomonas* bacterial cells against plants [3].

Xanthan has been widely used in food industry and microbial-enhanced oil recovery field because of its superior rheological properties [4, 5]. Xanthan degradation is very useful in altering the viscosity of suspensions injected into the underground oil bearing formations and in producing elicitor-active oligosaccharides from xanthan [4, 6]. However, xanthan is a highly stable polysaccharide not easily degraded by most microorganisms,

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although some microorganisms were reported to participate in depolymerization of xanthan [7–10]. Nankai et al. [11] elucidated the enzymatic route for xanthan degradation in *Bacillus* sp. strain GL1 by analyzing the structures of xanthan depolymerization product, in which xanthan is depolymerized after the pyruvylated mannosyl terminals are eliminated from side chains. Two types of xanthan-degrading enzymes, xanthan lyase and endoxanthanase (endo-β-1,4-D-glucanase), are included in the initial depolymerization [11]. Xanthan lyase, catalyzing the cleavage of the glycosidic bond between pyruvylated mannosyl and glucuronyl residues in xanthan side chains [12], has been well documented for xanthan modification. However, endoxanthanase catalyzing the hydrolysis of the main chain of xanthan has rarely been characterized. It was reported that such an endoxanthanase cleaved only backbone β-1,4-D-linkage of the lysed xanthan [7, 13]. Recently, we isolated a xanthan-degrading bacterium *Microbacterium* sp. strain XT11, which produced extracellular xanthan-degrading enzymes [14]. In this paper, we describe a novel xanthan depolymerase, endoxanthanase, which is specific to the intact xanthan with trisaccharide side chains but not to the lysed xanthan.

Materials and Methods

Microorganism and Culture Conditions

Microbacterium sp. strain XT11 was isolated from soil sample as described previously [14] and was maintained on the xanthan medium plates composed of 3 g xanthan, 0.5 g yeast extract, and 15 g agar in 1 L of the mineral salts solution. The mineral salts solution contained (per liter) 50 mg K_2HPO_4, 800 mg NaCl, 25 mg $MgSO_4 \cdot 7H_2O$, and 700 mg KNO_3, pH 7.0. For endoxanthanase production, 500 mL of xanthan medium in a 1.5-L Erlenmeyer flask was inoculated with 5 mL of an overnight culture grown in the same medium and aerobically incubated for 36 h at 30 °C and 150 rpm.

Enzyme Assay

Endoxanthanase activity was assayed viscometrically using xanthan as substrate. The reaction mixture consisted of 0.3% ($w\ v^{-1}$) of xanthan in 0.02 mol L^{-1} phosphate buffer (pH 6.0) and appropriately diluted enzyme was incubated at 35 °C for 15 min, and the activity was determined by measuring the reduction in viscosity [14]. Appropriate controls for the viscosity decrease with addition of the heated endoxanthanase (100 °C, 5 min) were included. One unit of the enzyme activity was defined as the amount of enzyme that produced a net decrease in viscosity of 1 centipoise (cp) per min under the above conditions.

Protein Determination

Protein content was determined by the dye-binding assay of Bradford [15], with bovine serum albumin as a reference protein, or was measured by light absorption at 280 nm. All experiments were done in triple.

Effect of Temperature and pH on Enzyme Activity

To assess the temperature optimum, the enzyme activity was measured at different temperatures according to the standard procedure described above. The thermostability of

endoxanthanase was assayed by measuring the residual enzyme activity after the endoxanthanase was incubated in 0.02 mol L^{-1} phosphate buffer for different time at 30 °C or 40 °C. To determine the optimal pH for endoxanthanase, the activity was measured as described above but xanthan was dissolved in 0.02 mol L^{-1} buffers of different pH values, in which citrate buffer is for pH range 3.4 to 6.0, phosphate buffer is for pH range 6.0 to 8.0, and phosphate-citrate buffer is for pH range 3.0 to 7.0. All experiments were done in triple.

Purification of Endoxanthanase

Unless otherwise specified, all operations were carried out at 4 °C; the buffer solution was 0.02 mol L^{-1} phosphate buffer.

Dual Precipitation of Culture Fluid with Ammonium Sulfate

The culture fluid of *Microbacterium* sp. strain XT11 was centrifuged at 10,000×*g* for 10 min, and the supernatant was precipitated by 40% saturation of ammonium sulfate and held for 4 h at 4 °C. After centrifugation at 15,000×*g* for 20 min, the additional ammonium sulfate was added into the supernatant to 70% of the final saturation with stirring and kept for 4 h at 4 °C. The precipitated protein was collected by centrifugation at 15,000×*g* for 20 min. The resultant pellet was dissolved in 0.02 mol L^{-1} phosphate buffer (pH 6.0) and dialyzed against the same buffer with three changes of the buffer.

CM-Cellulose (C22) Column Chromatography

The enzyme solution fractionated by ammonium sulfate above was applied to a carboxymethyl (CM)-Cellulose column (1.7×15 cm) previously equilibrated with 0.02 mol L^{-1} phosphate buffer. The column was washed with the same buffer, and then the enzyme was eluted with a stepwise gradient of NaCl (60 mL and 0.06 mol L^{-1} every stepwise) in the same buffer. The finial elution was applied to the column with 60 mL of 1 mol L^{-1} NaCl. Fractions were collected every 3 mL and protein was detected by UV light absorption at 280 nm.

DEAE-Sephadex Column Chromatography

The dual precipitated fraction of the culture fluid with ammonium sulfate was applied to a diethylaminoethyl (DEAE)-Sephadex column (1.7×15 cm) previously equilibrated with 0.02 mol L^{-1} phosphate buffer. The enzyme was washed with the same buffer and then was eluted, respectively, by 0.06, 0.12, 0.18, 0.24, 0.3, and 0.36 mol L^{-1} NaCl in the same buffer (60 mL each gradient). The final elution of column was carried out with the same buffer containing 1 mol L^{-1} NaCl. Fractions (3 mL) were collected and protein was detected by UV light absorption at 280 nm. Active enzyme fractions were combined, dialyzed against 0.02 mol L^{-1} phosphate buffer (pH 6.0), and stored at 4 °C.

SDS-PAGE

Endoxanthanases in the dual precipitated cell-free culture and in the fractions from different purification steps was determined using sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) by the method of Laemmli [16]. Proteins were stained with Coomassie Brilliant Blue G-250.

Results

Purification of Endoxanthanase

The dual precipitated enzyme with ammonium sulfate was applied to the CM-Cellulose column equilibrated with 0.02 mol L^{-1} phosphate buffer at pH 5.0, 7.0, or 9.0 separately and was eluted with a stepwise gradient of NaCl at the equilibrated pH. The elution profile was illustrated in Fig. 1. The enzyme activity was washed out when the phosphate buffer without NaCl was applied to the column at all the tested pH. Most of the protein was also washed out together with the endoxanthanase, resulted in a low purification fold (about 1). This suggested that the endoxanthanase could not be fractionated on the CM-Cellulose column.

The culture fluid precipitated by ammonium sulfate was applied to a DEAE-Sephadex column previously equilibrated with 0.02 mol L^{-1} phosphate buffer at pH 3.0, 5.8, or 7.0, respectively. As shown in Fig. 2 A, the endoxanthanase was eluted between fractions 17 and 36 with 0.06 mol L^{-1} NaCl, when the column was equilibrated with phosphate buffer at pH 3.0 and was eluted with a stepwise gradient of NaCl in the same buffer. The results in view of the elution profile indicated that the endoxanthanase was not fractionated effectively on the DEAE-Sephadex column when the pH value of the elution buffer is at pH 3.0. There were three active peaks occurred at fractions 72–82, 98–100, and 108–110, respectively, when the DEAE-Sephadex column was equilibrated and eluted at pH 7.0 (Fig. 2 C), which was eluted with 0.18, 0.24, and 0.30 mol L^{-1} NaCl separately. However, a big protein peak was eluted together with the last two active fractions. The endoxanthanase was also purified on DEAE-Sephadex column with a stepwise gradient of NaCl at pH 5.8 (Fig. 2 B). The enzyme was eluted at fractions 52–61, 87–97, and 117–122, respectively, the NaCl concentration of which used for the elution of the enzyme activity was 0.12, 0.24, and 0.30 mol L^{-1} NaCl separately. Most of the other protein was eluted out when the final

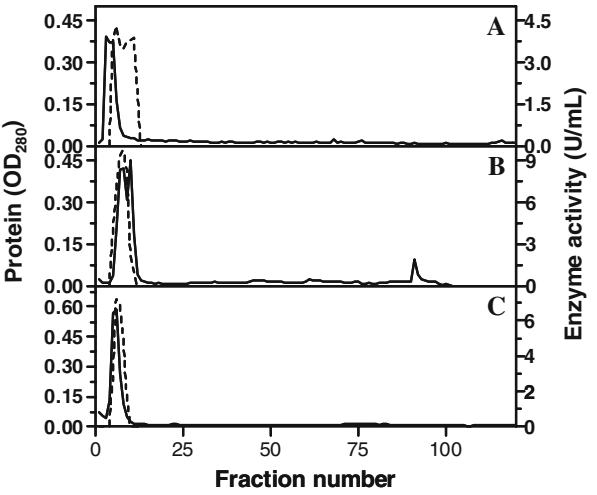

Fig. 1 CM-Cellulose column chromatography of endoxanthanase from culture broth of *Microbacterium* sp. XT11. Dual precipitated cell-free culture fluid of strain XT11 grown on xanthan was applied. *Column* was equilibrated and eluted with 0.02 mol L^{-1} phosphate buffer at *A* pH 5.0, *B* pH 7.0, or *C* pH 9.0 separately. Fractions (3 mL) were collected. *Symbols*: *dashed line*, protein; *broken line*, endoxanthanase activity

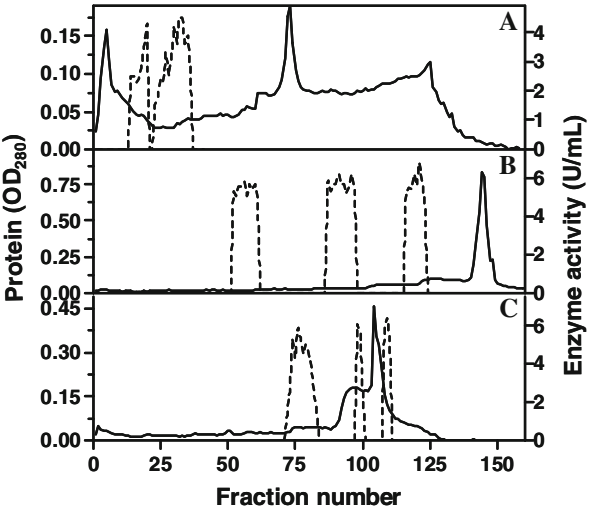

Fig. 2 Elution profile of the endoxanthanase on DEAE-Sephadex column. The column was previously equilibrated and eluted with 0.02 mol L^{-1} phosphate buffer at *A* pH 3.0, *B* pH 5.8, or *C* pH 7.0. Dual precipitated cell-free culture fluid of strain XT11 grown on xanthan was used. *Symbols*: *dashed line*, protein; *broken line*, endoxanthanase activity

elution (1 mol L^{-1} NaCl) was applied to column, which was separated completely from the active fractions. The analysis of the pooled fractions by SDS-PAGE was shown in Fig. 3. The active fractions 52–63 eluted with 0.12 mol L^{-1} NaCl at pH 5.8 were combined, dialyzed against 0.02 mol L^{-1} phosphate buffer (pH 6.0), and used for the characterization of the endoxanthanase.

Effect of Temperature and pH on Enzyme Activity

The enzyme activity was determined in 0.02 mol L^{-1} phosphate buffer (pH 6.0) at different temperatures. As shown in Fig. 4, the optimum temperature was found to be at 35–40 °C. The thermostability of the enzyme in 0.02 mol L^{-1} phosphate buffer (pH 6.0) was illustrated in Fig. 5. Only 18% of activity was lost after incubation for 120 min at 30 °C,

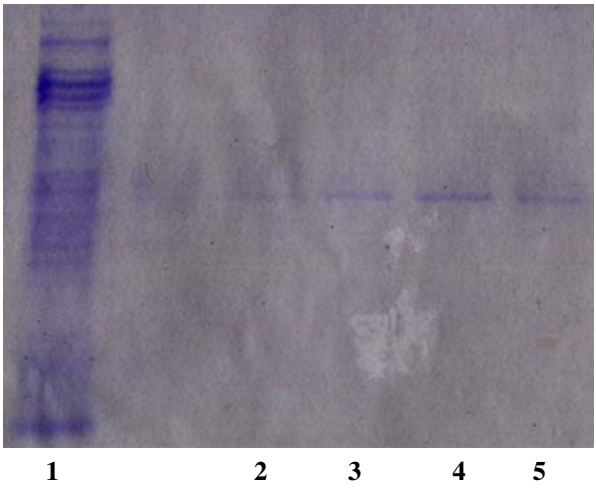

Fig. 3 SDS-PAGE analysis of the purification steps of endoxanthanase from *Microbacterium* sp. XT11. *Lane 1*, dual precipitated cell-free culture; *lane 2*, pooled fractions 72–82 on Fig. 2 C; *lane 3*, pooled fractions 52–61 on Fig. 2 B; *lane 4*, pooled fractions 87–97 on Fig. 2 B; *lane 5* pooled fractions 116–123 on Fig. 2 B

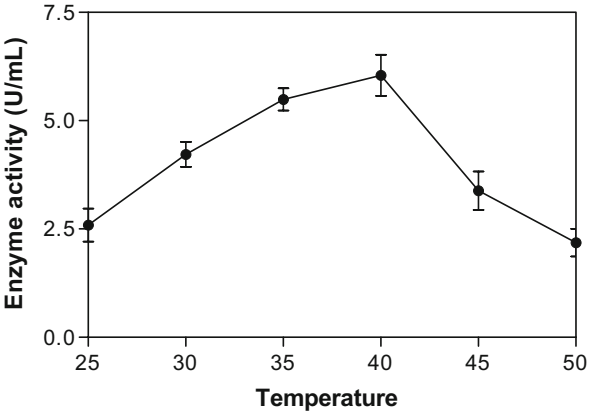

Fig. 4 Temperature profile of endoxanthanase from *Microbacterium* sp. XT11. Enzymatic reaction was performed for 15 min at different temperatures in 0.02 mol L^{-1} phosphate buffer (pH 6.0)

whereas the residual enzyme activity was 5.6% when the enzyme was incubated at 40 °C for 10 min, indicating that the endoxanthanase was unstable at 40 °C.

The effect of pH on enzyme activity was studied at 35 °C in the pH range 3.0 to 8.0 with different buffer. The enzyme was the most active at pH 5.5 for citrate buffer and pH 6.0 for both phosphate and phosphate-citrate buffer, respectively (Fig. 6). There was almost no difference in activity when the enzyme was assayed in different buffers of the optimum pH. To test the pH stability, the enzyme was incubated for 30 min at different pH and 30 °C, and the residual activity was determined at pH 6.0 and 35 °C. The results showed the narrow pH of stability at pH 6.0–7.0 (Fig. 6).

Substrate Specificity

To examine the substrate specificity of the endoxanthanase, the enzyme was incubated at 35 °C for 15 min in a mixture containing 0.02 mol L^{-1} phosphate buffer (pH 6.0) and various substrates (0.3%, *w* v^{-1}). Intact xanthan, especially pyruvylated xanthan, was degraded by endoxanthanase fast. However, the endoxanthanase could not depolymerize xanthan treated with xanthan lyase and carboxymethyl cellulose.

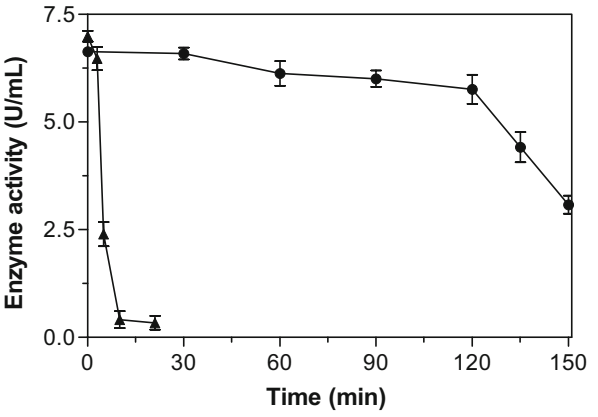

Fig. 5 Thermostability of endoxanthanase from *Microbacterium* sp. XT11. After the endoxanthanase was incubated in 0.02 mol L^{-1} phosphate buffer (pH 6.0) at 30 °C (*filled circle*) or 40 °C (*filled triangle*), respectively, for different time, the residual activity was measured at 35 °C

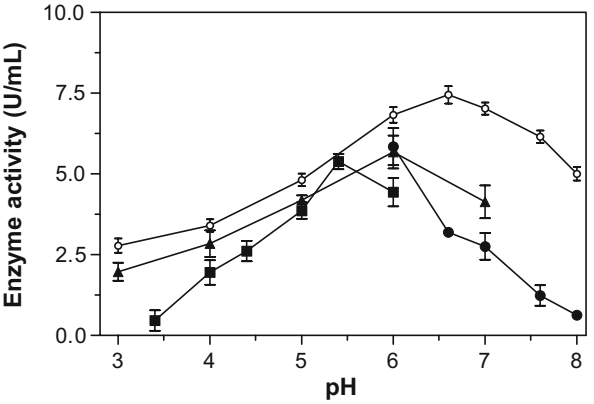

Fig. 6 pH optimum and stability of endoxanthanase from *Microbacterium* sp. XT11. Enzyme activity was assayed at 35 °C in 0.02 mol L^{-1} buffers of phosphate (*filled circle*), phosphate-citrate (*filled triangle*), and citrate (*filled square*), respectively. The pH stability (*unfilled circle*) was assayed by measuring the residual activity at pH 6.0 in phosphate buffer after incubation of endoxanthanase at 30 °C and different pH value

Michaelis Constant

The effect of xanthan concentrations on the enzyme activity in 0.02 mol L^{-1} phosphate buffer (pH 6.0) was studied at 35 °C. The K_m value calculated from Lineweaver–Burk plots of log velocity versus reciprocal of substrate concentration was 1.87.

Effect of Metal Ions on Enzyme Activity

The enzyme activity was determined in the presence of various metal ions. As shown in Table 1, the enzyme activity was inhibited in the presence of 10 or 100 mmol L^{-1} of $Cu2^{+}$, $Fe2^{+}$, Fe^{3+}, and Cr^{3+}. The endoxanthanase was almost completely inhibited by Fe^{3+} at 100 mmol L^{-1}, and Cu^{2+} inhibited endoxanthanase activity by 84% at 100 mmol L^{-1}. Ca^{2+} enhanced insignificantly enzyme activity at 10 mmol L^{-1} but inhibited enzyme activity at 100 mmol L^{-1}.

Discussion

As we know, xanthan lyases have been documented in modification of xanthan for food industry, while very little literatures exist on the purification and characterization of xanthan

Table 1 Influence of metal ions on endoxanthanase activity.

Ion concentration	0.01 mol L^{-1}	0.1 mol L^{-1}
Control	100±1.6	100±1.8
Cu^{2+}	58.4±1.3	16.1±2.1
K^{+}	93.6±2.1	91.2±1.7
Mg^{2+}	95.2±2.1	85.7±2.0
Mn^{2+}	96.1±1.5	59.5±2.4
Fe^{3+}	34.4±1.6	2.0±0.5
Cr^{3+}	37.1±2.5	69.6±1.6
Ca^{2+}	106.1±2.8	54.8±1.7
Fe^{2+}	63.3±2.5	50.0±1.3

The residual enzyme activity was expressed as the percentage against a control without metal ions addition. Results are presented as means ± standard deviation

Table 2 Purification of endoxanthanase from the xanthan-grown culture of *Microbacterium* sp. strain XT11.

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Cell-free culture broth	34	10,000	294.1	100	1
Ammonium sulfate precipitation (40–70%)	0.32	1,200	3,750	12	12.8
DEAE-Sephadex	0.022	120	5,454.5	1.2	18.5

The data for both total protein and total activity were means of three independent values and standard deviations were less than 5% of the means

depolymerase [7, 13]. Sutherland [17] reported that the partially purified β-glucanohydrolase from a *Bacillus* sp. cleaved both endo-β-1,4-linked and endo-β-1,3-linked polymers with side chains. Xanthan depolymerase was also purified from the culture of bacterial consortium, which hydrolyze the backbone linkages of the modified xanthan or carboxymethyl cellulose [7].

When *Microbacterium* sp. strain XT11 was cultured in the medium containing xanthan as the sole carbon source, the viscosity of the culture decreased with the increasing cell growth and reached the level of water when cell growth approached a plateau, indicating that the enzyme endolytically hydrolyzing backbone of xanthan occurred in the culture [6]. Both CM-Cellulose column and DEAE-Sephadex column were tested for endoxanthanase purification. However, the endoxanthanase could not be purified on the CM-Cellulose column, because the enzyme activity cannot be absorbed on CM-Cellulose column when all the tested pH values were examined (Fig. 1). The endoxanthanase was eluted out of the column together with the most other proteins by the balanced buffer. When the dual precipitated enzyme with ammonium sulfate was applied on the DEAE-Sephadex column, the endoxanthanase could be fractionated clearly depending on the pH values used for elution (Fig. 2). Extracellular endoxanthanase was purified 18.5-fold with a recovery of 1.2% from the culture fluid when the enzyme was fractionated on the DEAE-Sephadex column with 0.12 mol L^{-1} NaCl at pH 5.8 (Table 2). The enzyme, cleaving the β-1,4-linkage on the backbone of xanthan, was most active at pH 6.0 and 35–40 °C. It is different from β-D-glucanase of *Bacillus* specifically acting on xanthan after treatment with xanthan lyase [11]; the endoxanthanase of *Microbacterium* sp. XT11 acted on the intact xanthan with trisaccharide side chains but not on the lyase-treated xanthan. Carboxymethyl cellulose could not be cleaved by endoxanthanase either. It was presumed that the endoxanthanase of *Microbacterium* sp. XT11 was specific to the pyruvated mannose terminals in side chains rather than to the unsaturated terminals. The changes in OD235 was not detected during degradation of xanthan by endoxanthanase, indicated that there was no lyase activity occurred in endoxanthanase. Such an endoxanthanase could be used for the preparation of the bioactive oligosaccharides and the treatment of *Xanthomonas* infectious disease [6, 18] or has a potential application in biodegradation of xanthan injected into underground oil [4]. To our knowledge, this is the first report on the endoxanthanase cleaving the backbone linkage of the intact xanthan molecule with trisaccharide side chains.

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References

1. García-Ochoa, F., Santos, V. E., Casas, J. A., & Gómez, E. (2000). *Biotechnology Advances*, *18*, 549–579. doi:10.1016/S0734-9750(00)00050-1.
2. Sandford, P. A., Pittsley, J. E., Knutson, C. A., Watson, P. R., Cadmus, M. C., & Janes, A. (1977). In P. A. Sandford, & A. Laskin (Eds.), *Extracellular microbial polysaccharides* pp. 192–210. Washington, DC: American Chemical Society.
3. Yun, M. H., Torres, P. S., Oirdi, M. E., Rigano, L. A., Gonzalez-Lamothe, R., Marano, M. R., et al. (2006). *Plant Physiology*, *141*, 178–187. doi:10.1104/pp.105.074542.
4. Katzbauer, B. (1998). *Polymer Degradation & Stability*, *59*, 81–84. doi:10.1016/S0141-3910(97)00180-8.
5. Van Hamme, J. D., Singh, A., & Ward, O. P. (2003). *Microbiology and Molecular Biology Reviews*, *67*, 503–549. doi:10.1128/MMBR.67.4.503-549.2003.
6. Liu, H., Huang, C., Dong, W., Du, Y., Bai, X., & Li, X. (2005). *Process Biochemistry*, *40*, 3701–3706. doi:10.1016/j.procbio.2005.05.006.
7. Ahlgren, J. A. (1993). *Journal of Industrial Microbiology*, *12*, 87–92. doi:10.1007/BF01569906.
8. Cadmus, M. C., Jackson, L. K., Burton, K. A., Plattner, R. D., & Slodki, M. E. (1982). *Applied and Environmental Microbiology*, *44*, 5–11.
9. Hashimoto, W., Miki, H., Tsuchiya, N., Nankai, H., & Murata, K. (1998). *Applied and Environmental Microbiology*, *64*, 3765–3768.
10. Ruijssenaars, H. J., de Bont, J. A. M., & Hartmans, S. (1999). *Applied and Environmental Microbiology*, *65*, 2446–2452.
11. Nankai, H., Hashimoto, W., Miki, H., Kawai, S., & Murata, K. (1999). *Applied and Environmental Microbiology*, *65*, 2520–2526.
12. Hashimoto, W., Nankai, H., Mikami, B., & Murata, K. (2003). *Journal of Biochemistry*, *278*, 7663–7673.
13. Hou, C. T., Barnabe, N., & Greaney, K. (1986). *Applied and Environmental Microbiology*, *52*, 37–44.
14. Qian, F., An, L., Wang, M., Li, C., & Li, X. (2007). *Journal of Applied Microbiology*, *102*, 1362–1371. doi:10.1111/j.1365-2672.2006.03215.x.
15. Bradford, M. M. (1976). *Analytical Biochemistry*, *72*, 248–254. doi:10.1016/0003-2697(76)90527-3.
16. Laemmli, U. K. (1970). *Nature*, 227, 680–685. doi:10.1038/227680a0.
17. Sutherland, I. W. (1982). *The Journal of Applied Bacteriology*, *53*, 385–393.
18. Qian, F., An, L., He, X., Han, Q., & Li, X. (2006). *Process Biochemistry*, *411*, 582–1588.