Journal of Industrial Microbiology, 7 (1991) 243–250
Elsevier

SIM 00325

Original Papers

Polygalacturonate lyase production by *Thermomonospora curvata* on protein-extracted lucerne fiber

Fred Stutzenberger

Department of Microbiology, Clemson University, Clemson, SC, U.S.A.

(Received 10 August 1990; revision received 31 October 1990; accepted 2 November 1990)

Key words: Actinomycete; Exoenzyme; Pectin; Pectinase; Protease; Thermophile

SUMMARY

Protein-extracted lucerne fiber was used as carbon and energy source for production of extracellular polygalacturonate lyase by *Thermomonospora curvata*. The optimal fiber concentration was 1.5% (w/v); peak lyase activity in culture fluid occurred after 3 days growth at 53 °C. During that time, lyase biosynthesis was controlled through induction; production was accelerated by adding small amounts of pectin or by grinding the fiber to 40-mesh particle size to release more inducer. After 3 days growth, lyase activity decreased; inactivation of the enzyme was delayed by the presence of 1 mM Ca or by inhibition of serine proteases with 0.05 mM phenylmethylsulfonyl fluoride. The molecular weight of the lyase produced during growth on the fiber was 35 kDa compared to 56 kDa for the enzyme produced on pure pectin. The K_m of the 35-kDa form was 0.54% pectin compared to 0.06% for the 56-kDa form. The smaller form was rapidly inactivated at 60 °C, the optimal temperature for activity of the larger form.

INTRODUCTION

Lucerne (alfalfa) protein has an excellent amino acid composition for human consumption [12]. High protein productivity (which is about eight times that of rice, corn or soy plants [21]), perennial growth and nitrogen fixation make lucerne an attractive protein source for developing countries [9,22]. After protein extraction, the cellulosic residue (protein-extracted lucerne fiber, PELF) is suitable for industrial enzymatic conversion to soluble sugars since its reducing sugar yield averages about 60% of the theoretical maximum compared with 20–30% for other widely studied biomasses [29]. *Thermomonospora curvata* is well adapted as a source of enzymes for this bioconversion since its natural habitat is heating masses of hay and other complex cellulosic materials [8]. During growth on PELF, *T. curvata* produces multiple cellulases which solubilize about two-thirds of the total fiber polysaccharide [26]. The action of pectinolytic enzymes renders these polysaccharide components in plant biomass more susceptible to hydrolysis [2]. Since PELF has a relatively high pectin content (13–17% of dry weight), the action of pectinases may play a significant role in the bioconversion process. This study describes the production of the pectinolytic enzyme, polygalacturonate lyase (PL), from *T. curvata* growing on the fiber, and shows that its characteristics differ from those of the enzyme produced on purified pectin.

Correspondence: F. Stutzenberger, Department of Microbiology, Clemson University, Clemson, SC 29634-1909, U.S.A.

MATERIALS AND METHODS

Organism and growth conditions

The actinomycete employed in this study formed the dominant population during the heating stage in municipal solid waste compost [23] and was identified as *Thermomonospora curvata* by Dr. T. Cross (University Bradford, England). However, the Thermomonosporaceae is a family of convenience which includes organisms exhibiting a continuum of characteristics and also many with relatively few similarities [8]. This strain shares some characteristics in common with *Thermomonospora fusca*, but differs as to sugar utilization patterns, growth requirements and morphology. At present, the species designation as *curvata* has been retained.

Inocula were prepared from late-exponential phase cells grown on 0.5% citrus pectin (Sigma Chemical Co. no. 9135) in mineral salts minimal medium [24]. Cells were concentrated about 10-fold by centrifugation and stored in 25% glycerol at −70 °C. Flasks routinely received 0.5% v/v inoculum.

PELF was prepared by the method of Vaughn et al.

[29]. The fiber was exhaustively washed in a Buchner funnel until the filtrate contained no detectable trace of either protein or reducing sugar, then dried under vacuum at 55 °C. The polysaccharide content of the fiber averaged 40% cellulose, 14% hemicellulose, 18% lignin and 14% pectin. To make the fiber more susceptible to hydrolysis in some experiments, it was ground in a Wiley mill to 40-mesh particle size or treated with 0.75 N NaOH for 30 min at 80 °C. PELF (final concentrations of 0.2–4.0% w/v) were autoclaved separately and mixed aseptically with other medium components after cooling. 100-ml cultures were shaken at 150 rpm, 53 °C, in 250-ml baffled Bellco Erlenmeyer flasks inclined at 30° from vertical.

The serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), was dissolved at 10^{-1} M concentration in absolute ethanol, filter-sterilized, and added aseptically to cultures at a final concentration of 5×10^{-5} M. This concentration was the highest which did not cause cessation of growth in preliminary experiments, yet inhibited serine protease activity in culture fluid by about 80%.

Analyses

Culture fluid samples for estimation of extracellular protein and PL activity were clarified by centrifugation for 15 min at 12200 × *g* in conical tubes at room temperature. PL activity was estimated spectrophotometrically at room temperature by the rate of increase in absorbance at 235 nm [1] in reaction mixtures as previously described [25]. Standard deviation (SD) based on five replicate determinations was 2.1%. PL units were calculated as μmol of product formed per min, with a molar extinction coefficient of 4600 [11]. Protein concentrations (SD of 3.7%) were determined by the Coomassie Blue dye-binding method [7] using the protocol outlined in Bio-Rad technical bulletin 89-0931. Total reducing sugar concentrations (SD of 1.3%) were estimated by the method of Bernfeld [3].

Analysis of sample variation

The average SD values for PL activity and soluble protein concentration between duplicate flasks within the same experiment were 7.3% and 5.1% respectively. The average SD values for PL and protein for flasks of duplicate experiments were 13.8% and 9.2% respectively. The data in this study, unless otherwise noted, are averages of flasks from at least two duplicate experiments.

Thermal inactivation studies

Tests of PL thermal stability were carried out in stoppered glass tubes. Enzyme samples were added to the pre-heated tubes, and aliquots were transferred at timed intervals from the heated samples directly to the PL reaction mixture for assay. Rates of inactivation were calculated using the formula, $\log_{10}(N/N_o) = -Kt$, where N_o is the initial PL activity, N is the residual activity at any time, t, and K is the inactivation constant.

Chromatography

Extracellular proteins were concentrated four- to 10-fold by ultrafiltration (Amicon YM10 membrane under 15 PSI nitrogen, 5 °C). High performance liquid chromatography (HPLC) of concentrated samples was done as previously described [25] using a Toyo Soda TSK2000 analytical size exclusion column.

RESULTS

The accumulation of soluble protein and PL in *T. curvata* cultures was influenced by initial PELF concentration in the medium (Fig. 1). Peak protein during 6 days growth increased with initial fiber concentration up to about 1%, but declined slightly at the highest concentration. It was unlikely that a major portion of this extracellular protein was derived from the fiber itself; shaking 1.5% (w/v) PELF at culture pH and temperature under sterile conditions overnight released only 17 μg protein/ml. Furthermore, treatment of a 2% fiber concentration under those conditions with concentrated exoenzymes produced during growth on PELF resulted in extensive binding of protein to the fiber and a 77% reduction in soluble protein concentration. The PL production was very sensitive to PELF concentration. A sharp production peak was obtained at 1.5% PELF; doubling the initial fiber concentration in the medium resulted in a three-fold decrease in peak PL production.

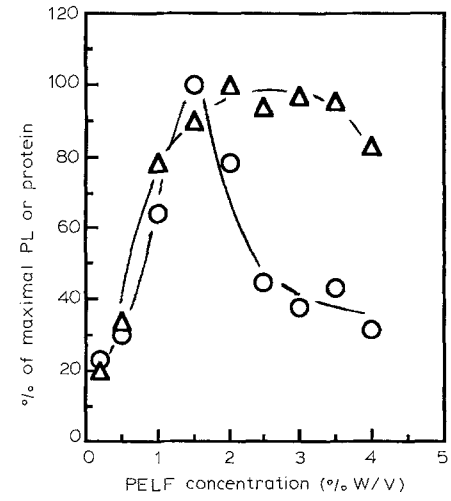

Fig. 1. Influence of initial PELF concentration on PL (○) and protein (△) levels in culture fluid. The 100% values were 2.9 U/ml and 625 μg/ml for PL and protein respectively.

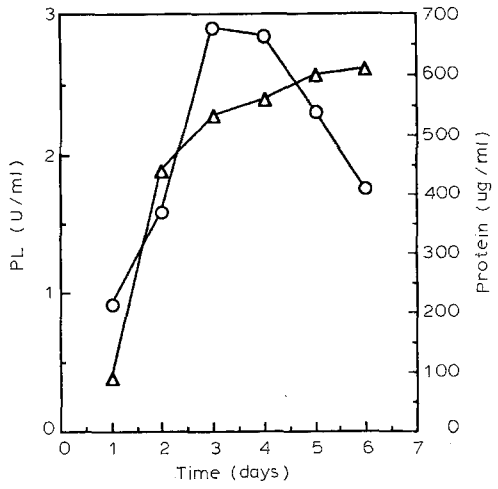

Fig. 2. A time course of PL (○) and protein (△) accumulation in culture fluid during growth on 1.5% PELF.

A time course of extracellular protein and PL accumulation at the optimal PELF concentration of 1.5% is shown in Fig. 2. Extracellular protein continued to accumulate throughout the incubation period, but PL activity declined rapidly beyond 4 days. It was of interest to determine whether this decline in PL biosynthesis was due to the repressive effect of soluble sugars in the medium or to the depletion of pectic substances able to maintain induction. Within the first 2 days of growth on 1.5% PELF, the total reducing sugar concentration of culture fluids reached the range of 370–420 μg glucose equivalents per ml. To test whether induction or repression was the major factor controlling PL biosynthesis, small amounts of pectin were added to 2-day PELF-grown cultures. The effect on the rate of PL biosynthesis is shown in Fig. 3. During

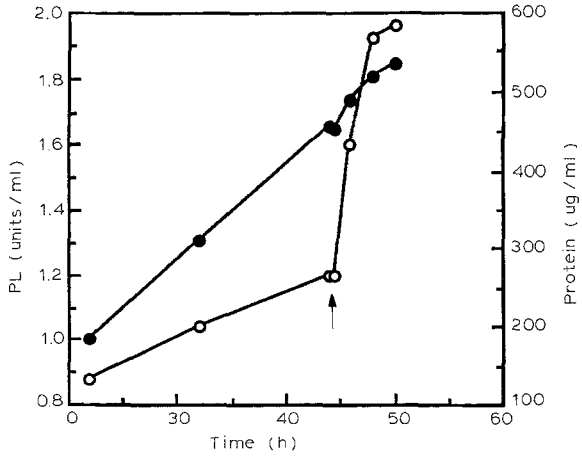

Fig. 3. Increased rate of PL biosynthesis by 200 μg pectin/ml of PELF-grown culture (time of addition indicated by arrow). Symbols: PL activity (○), soluble protein (△).

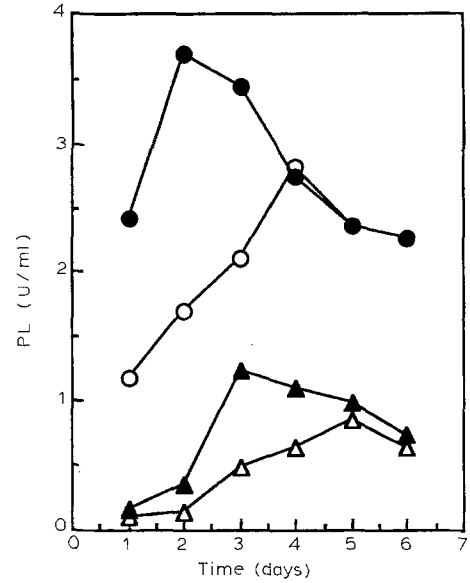

Fig. 4. Acceleration of PL production by the use of ground PELF (solid symbols) compared to the unground fibers (open symbols) at concentrations of 0.5% (triangles) and 1.5% (circles).

the following pectin addition, the rate of PL accumulation in culture fluid increased about 13-fold while the rate of extracellular protein accumulation continued essentially unchanged. Therefore, it appeared that PL biosynthesis was limited by the pectin available for induction. Some of the fiber (which consisted largely of strands one to five cm long) was ground to 40-mesh particle size in an effort to increase the surface available to enzymatic attack and therefore make more pectin available for induction. A comparison of PL productions on ground versus unground PELF is shown in Fig. 4; peak PL levels occurred earlier and at somewhat higher maxima when the fiber was ground. Replacing ground fiber by that treated with NaOH sharply reduced PL production. The stimulation of PL production through the use of ground fiber was only achieved at PELF concentrations up to 1.5%; the decline in PL activity still occurred during the latter stages of growth.

The rapid decline in PL activity after 4 days was suggestive that it was more thermolabile than other previously-studied *T. curvata* exoenzymes. The temperature range optimal for the growth of *T. curvata* and production of extracellular enzymes is 53–60 °C [4,13]. Therefore, the stability of the crude PL was tested over that temperature range. In preliminary experiments, the crude PL from pectin-grown cultures was stable when shaken overnight at temperatures to 60 °C (detailed data not shown). However, the PL in filter-sterilized fluid from PELF culture lost almost one-half its activity at the routine growth

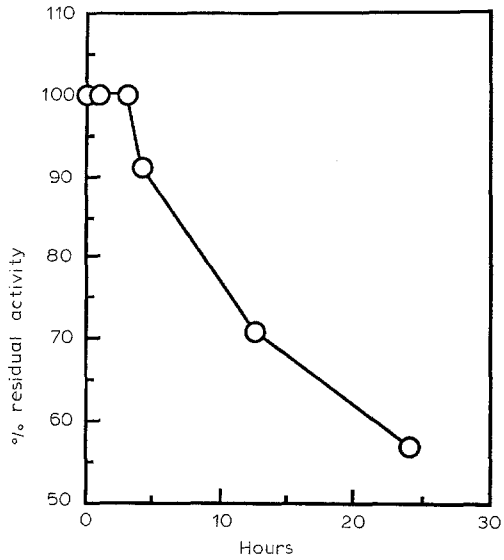

Fig. 5. PL inactivation in sterile culture fluid shaken at 53 °C.

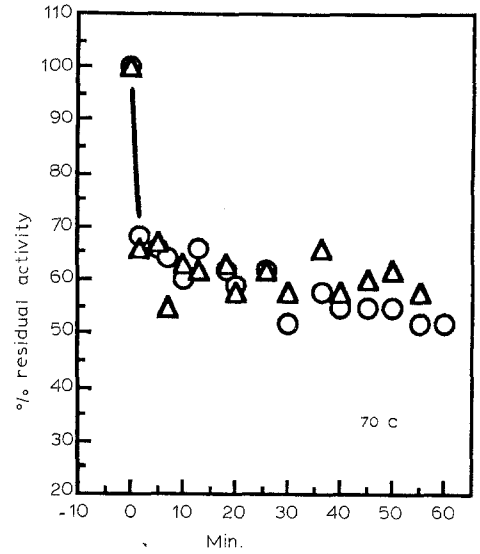

Fig. 7. Biphasic inactivation of PL in culture fluid at 70 °C; symbols: control (no Ca, ○), fluid with 1 mM Ca (△). Points are cumulative data from two separate experiments.

temperature of 53 °C (Fig. 5). At 60 °C, it was inactivated at the rate of about 30% per h but was stabilized by the addition of calcium (Fig. 6). This stabilizing effect of calcium prompted an experiment to determine whether calcium could prevent PL inactivation during growth. Inclusion of 1 mM $CaCl_2$ in the medium resulted in a 15% increase in maximal PL levels, but this small increase was accompanied by a proportional increase in total extracellular protein. Therefore it seemed probable that the increased PL was a result of stimulated growth rather than enzyme stabilization.

Some of the *T. curvata* extracellular enzymes, when held at slightly alkaline pH, undergo thermal activation at 65–70 °C which results in activity increases of 50–70% [13,28]. However, the PL was rapidly inactivated in a biphasic manner at 70 °C, pH 8. Inactivation constants were estimated to be 0.084 for the rapid phase and 0.002 for the slow phase. The presence of calcium had no apparent effect on either the rate or the shape of the inactivation curve (Fig. 7).

The possibility was considered that the rapid loss of

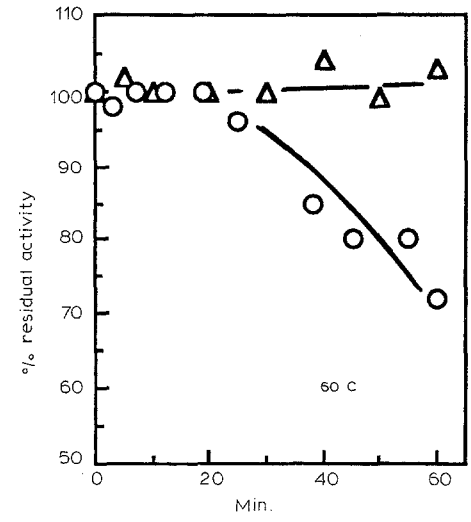

Fig. 6. PL stabilization in culture fluid by calcium at 60 °C; symbols: control (no Ca, ○), fluid with 1 mM Ca (△).

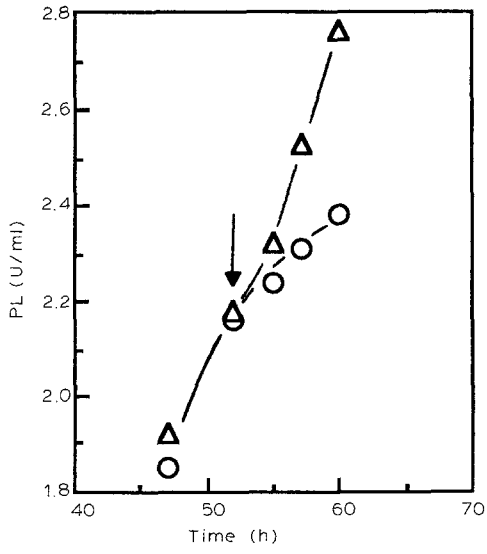

Fig. 8. Increase in PL accumulation in culture by addition of 5×10^{-5} M PMSF (△) compared to control (○).

PL activity in crude culture fluid was due to factors other than thermal denaturation. *Thermomonospora* species have recently been shown to produce extracellular serine proteases [5,14]. To determine whether proteolysis was a factor in PL inactivation, PMSF was added to 2-day cultures. The effectiveness of this inhibitor was limited by its short half-life under culture conditions (about 30 min at pH 8 [16]) and by its toxicity. When added at 0.05 mM, the inhibitor caused an average of 30% decrease in the protein secretion rates. Despite its toxicity, the rate of PL accumulation in PELF culture fluid was increased for several hours (Fig. 8). The protection afforded by the inhibitor was limited to a single addition since higher PMSF concentrations caused cessation of protein secretion. Therefore, within a day after addition, most of the increase in PL was lost.

In previous studies [19,26] exoenzymes produced by *T. curvata* on PELF exhibited different physical and

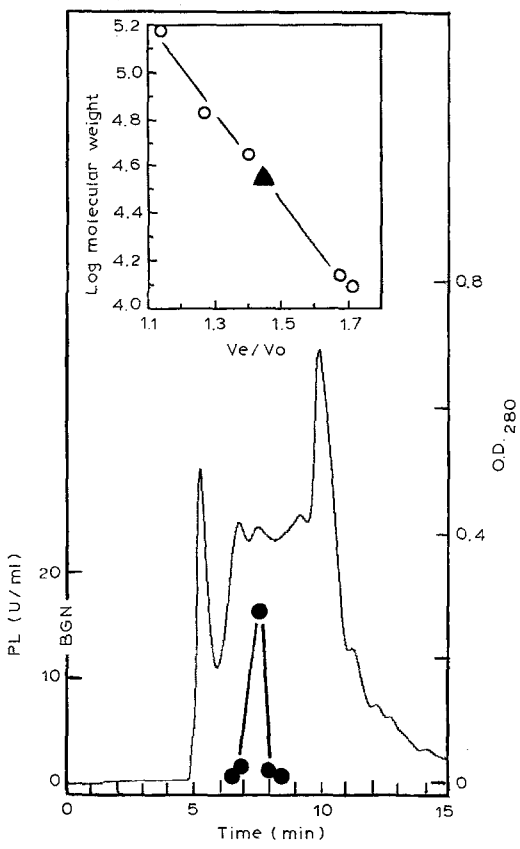

Fig. 9. HPLC size exclusion elution profile of PL activity (●) compared to plotter-integrator tracing of OD_{280}. Fraction volume and flow rate were 0.5 ml and 1 ml/min, respectively. Void volume (V_o) was 5.2 ml. Inset depicts column calibration curve using the molecular weight standards alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), ribonuclease (14 kDa) and cytochrome *c* (12.4 kDa). Position of PL on standard curve indicated by (▲).

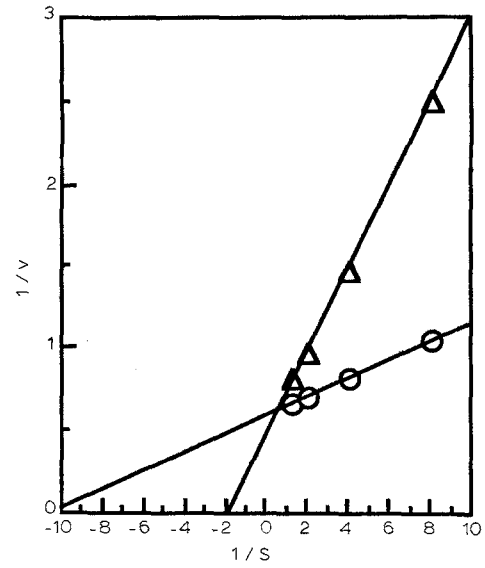

Fig. 10. Double reciprocal plots for determination of K_m values for PL produced on purified pectin (○) and on PELF (△) as sole carbon source. The substrate was citrus pectin with concentrations expressed in percent (w/v).

kinetic characteristics than those produced on purified substrates. Since the PL produced during growth on PELF also appeared to be sufficiently different than that produced on purified pectin, some characterization was done to compare it to the PL produced earlier on purified pectin [25]. A size exclusion HPLC fractionation of the concentrated exoproteins from a PELF-grown culture indicated that PL activity resided in protein(s) with an estimated molecular weight of 35 kDa (Fig. 9). The 5.9-fold purification of the PL rendered it so unstable that further purification was not attempted. The half-life of the partially purified enzyme at 60 °C, pH 8, was about 7 min. Furthermore, its affinity for pectin was reduced compared to that of the PL purified from cultures grown on purified pectin. Calculations from the double reciprocal plot shown in Fig. 10 yielded K_m of 0.54% for the PL from PELF culture compared to 0.09% for the PL produced on pectin.

DISCUSSION

The Thermomonosporaceae establish themselves as dominant populations in heating masses of hay and grain, municipal solid waste and other complex cellulosic substances [8,10,20,23]. Therefore, PELF is similar to the complex substrates available in their natural environment. The availability of pectic inducer derived from the fiber limits PL biosynthesis; less than 3% of the total exoprotein biosynthesis is devoted to PL production (based on the specific activity of the purified PL [25]). The

major portion of the exoprotein appears to consist of the multicomponent cellulase complex which is induced only by cellulose or its degradation product, cellobiose [29]. Gusek et al. [15] have shown that *T. fusca* produces high levels of its heat-stable serine protease only when grown on cellulosic substrates. Addition of small amounts of cellobiose during growth on purified pectin causes immediate cessation of PL biosynthesis in *T. curvata* and rapid loss of the enzyme already accumulated in the culture fluid [25]. Whether this loss is a direct result of proteolytic degradation is yet to be proven. *T. curvata* produces very high extracellular protease levels during growth on PELF [5]; the addition of PMSF in the present study temporarily sustained a higher rate of extracellular PL accumulation than in the controls lacking the inhibitor. If proteolytic activity controls PL accumulation during later growth on PELF, it would be interesting to determine whether it inactivates it directly or modifies the enzyme in a limited manner so that the modified form is unstable under culture conditions. The latter alternative seems more probable since the PL produced on PELF is considerably smaller (35 kDa) than the enzyme produced on purified pectin (56–59 kDa [25]) and the affinity for substrate was six-fold lower. The PL produced on PELF appears to be thermolabile and is inactivated in a biphasic manner, suggesting the possibility that two forms exist, one much more labile than the other. To explore the possibility of a role for proteolytic activity in this regard, we are in the process of determining the factors which induce protease production in *T. curvata* and the characteristics of the protease(s), including action on other exoenzymes potentially important in bioconversion processes.

ACKNOWLEDGEMENTS

This study was supported by the U.S. Army Research Office and the South Carolina Energy Research and Development Center.

REFERENCES

1 Albersheim, P. and U. Killias. 1962. Studies relating to the purification and properties of pectin transeliminase. Arch. Biochem. Biophys. 97: 107–115.
2 Bateman, D.F. 1976. Plant cell wall hydrolysis by pathogens. In: Biochemical Aspects of Plant-Parasite Relationships (J. Friend and D.R. Threlfall, eds.), pp. 79–103, Academic Press, London.
3 Bernfeld, P. 1955. Amylases, alpha and beta. Methods Enzymol. 1: 149–154.
4 Bernier, R.F. and F.J. Stutzenberger. 1989. β-glucosidase biosynthesis in *Thermomonospora curvata*. MIRCEN J. Appl. Microbiol. Biotechnol. 5: 15–25.
5 Bernier, R.F., M. Kopp, B. Trakas and F. Stutzenberger. 1988. Production of extracellular enzymes by *Thermomonospora curvata* during growth on protein-extracted lucerne fibres. J. Appl. Bacteriol. 65: 411–418.
6 Bonner, M.A. and F.J. Stutzenberger. 1988. Cell surface changes in *Thermomonospora curvata* during cellulase induction and repression. Lett. Appl. Microbiol. 6: 59–63.
7 Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72: 248–254.
8 Cross, T.M. and M. Goodfellow. 1973. Taxonomy and classification of the actinomycetes. In: Actinomycetales: Characteristics and Practical Importance (G. Sykes and F.A. Skinner, eds), pp. 11–112. Academic Press, London.
9 Dale, B.E. 1983. Biomass refining: protein and ethanol from alfalfa. Ind. Eng. Chem. Prod. Res. Dev. 22: 466–472.
10 Fergus, C.L. 1964. Thermophilic and thermotolerant molds and actinomycetes of mushroom compost during peak heating. Mycologia 56: 267–284.
11 Fogarty, W.M. and C.T. Kelly. 1983. Pectic enzymes. In: Microbial Enzymes and Biotechnology (W.M. Fogarty, ed.), pp. 131–182. Applied Science Publishers, London.
12 Food and Agriculture Organization of the United Nations. 1981. Agriculture: toward 2000. Rome.
13 Glymph, J.L. and F.J. Stutzenberger. 1977. Production, purification and characterization of α-amylase from *Thermomonospora curvata*. Appl. Environ. Microbiol. 34: 391–397.
14 Gusek, T.W. and J.E. Kinsella. 1987. Purification and characterization of the heat-stable serine protease from *Thermomonospora fusca* YX. Biochem. J. 246: 511–517.
15 Gusek, W., D.B. Wilson and J.E. Kinsella. 1988. Influence of carbon source on production of a heat stable protease from *Thermomonospora fusca* YX. Appl. Microbiol. Biotechnol. 28: 80–84.
16 James, G.T. 1978. Inactivation of the protease inhibitor phenylmethylsulfonyl fluoride in buffers. Anal. Biochem. 86: 574–579.
17 Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227: 680–684.
18 Lamed, R., J. Naimark, E. Morgenstern and E.A. Bayer. 1987. Specialized cell surface structures in cellulolytic bacteria. J. Bacteriol. 169: 3792–3800.
19 Lupo, D. and F. Stutzenberger. 1988. Changes in endoglucanase patterns during growth of *Thermomonospora curvata* on cellulose. Appl. Environ. Microbiol. 54: 588–589.
20 Niese, G. 1959. Microbiological research on the question of self-heating of organic matter. Arch. Microbiol. 34: 285–318.
21 Sebo, J. 1975. Press-extraction of green plants for separation of the cell sap and the fiber fraction. In: Symposium on the Enzymatic Hydrolysis of Cellulose (M. Bailey, T.M. Enari and M. Linko, eds.), pp. 63–75, Finnish National Fund for Research and Development, Aulanko.
22 Simantov, A. 1982. World supply and demand of food proteins. In: Food Proteins (P.F. Fox and J.J. Condon, eds.), pp. 35–50. Elsevier Applied Science Publishers, London.
23 Stutzenberger, F.J. 1971. Cellulase production by *Thermomo-*

nospora curvata isolated from municipal solid waste compost. Appl. Microbiol. 22: 147–152.

24 Stutzenberger, F.J. 1972. Cellulolytic activity of *Thermomonospora curvata*: nutritional requirements for cellulase production. Appl. Microbiol. 24: 77–82.

25 Stutzenberger, F.J. 1987. Inducible thermoalkalophilic polygalacturonate lyase from *Thermomonospora fusca*. J. Bacteriol. 169: 2774–2780.

26 Stutzenberger, F.J. 1988. Production and activity of *Thermomonospora curvata* cellulases on protein-extracted lucerne fibers. Appl. Microbiol. Biotechnol. 28: 387–393.

27 Stutzenberger, F.J. Kaufman and R.D. Lossin. 1970. Cellulolytic activity in municipal solid waste compost. Can. J. Microbiol. 16: 553–560.

28 Stutzenberger, F.J. and D. Lupo. 1986. pH-dependent thermal activation of endo-1,4-beta-glucanase in *Thermomonospora curvata*. Enzyme Microb. Technol. 8: 205–208.

29 Vaughn, S.R., R.M. McDonald, P.E. Donnelly, N.A. Hendy and R.A. Mills. 1984. The biomass refinery as a route to fuel alcohol from green crops. In: Sixth International Alcohol Fuels Technology Symposium. 3: 467–473. Ottawa, Canada.

30 Wood, W.E., D.G. Neubauer and F.J. Stutzenberger. 1984. Adenosine 3′,5′-cyclic monophosphate levels during induction and repression of cellulase biosynthesis in *Thermomonospora curvata*. J. Bacteriol. 160: 1047–1054.