Biochem. J. (1987) 246, 193–197 (Printed in Great Britain)

Characterization of α-amylase and pullulanase activities of *Clostridium thermohydrosulfuricum*

Evidence for a novel thermostable amylase

Hannes MELASNIEMI
Research Laboratories of the Finnish State Alcohol Company (Alko Ltd.), P.O. Box 350, SF-00101 Helsinki, Finland

Thermostable extracellular α-amylase and pullulanase activities of *Clostridium thermohydrosulfuricum* E 101–69 were characterized in a crude enzyme preparation. The activities responded similarly to temperature and pH, with optima at 85–90 °C and pH 5.6. The activities were stable at 65 °C, but were inactivated gradually in an identical manner at higher temperatures in the absence of Ca^{2+} and substrate. Ca^{2+} stabilized both activities similarly at high temperatures. Ca^{2+} also stimulated both activities, whereas EDTA reversed this stimulation. The activities were similarly inactivated at pH extremes. The two activities distributed in the same way during isoelectric focusing. The results suggest that the two activities are properties of the same protein, representing a novel, thermostable, amylase.

INTRODUCTION

Clostridium thermohydrosulfuricum (Hollaus & Sleytr, 1972; Wiegel *et al.*, 1979) is a thermophilic anaerobe capable of degrading starch and fermenting it to ethanol. Hyun & Zeikus (1985*a*,*b*,*c*) and Hyun *et al.* (1985) have investigated starch degradation by *C. thermohydrosulfuricum* E39. Hyun & Zeikus (1985*a*) characterized a pullulanase and a glucoamylase activity from cell extracts of strain 39E and reported that an α-amylase is not involved in starch degradation by this organism. Melasniemi (1987), on the other hand, reported the production by strain E 101–69 of α-amylase (EC 3.2.1.1), pullulanase (EC 3.2.1.41) and as yet unspecified α-glucosidase activities, which were found both extracellularly (in the medium) and associated with the cells. In the present paper are described some key properties of the extracellular α-amylase and pullulanase activities produced by *C. thermohydrosulfuricum* E 101–69. The activities responded similarly to all parameters tested, and produced the same isoelectric-focusing pattern, which suggest that both activities are functions of a single, novel, thermostable enzyme.

METHODS

Enzyme preparation

α-Amylase and pullulanase activities were characterized in a crude enzyme preparation (Melasniemi, 1987). Briefly, *C. thermohydrosulfuricum* E 101–69 was grown anaerobically in 2 litres of a complex medium with 2% (w/v) Zulkowsky starch (Merck) as the carbon source. Cells were removed by centrifugation (6500 *g* for 10 min), and solid $(NH_4)_2SO_4$ (505 g/l) was added to the supernatant. Material precipitated in 2 days at 4 °C was collected, dissolved and then dialysed against 20 mM-sodium acetate, pH 5.6. This crude enzyme preparation contained, in 50 ml: 0.42 g of protein, 600 k-units of α-amylase and 1850 k-units of pullulanase.

Enzyme assays

α-Amylase and pullulanase activities were assayed by measuring the reducing sugar released from pure amylose (Sigma, type III) and pullulan (Sigma) respectively. The standard assay contained 25 μl of diluted crude enzyme and 1 ml of 0.5% (w/v) substrate in 100 mM-sodium acetate, pH 5.6, containing 2 mM-$CaCl_2$, 0.1 mM-Na_2EDTA and 50 mM-NaCl. Reducing sugar was determined by the Nelson–Somogyi method (Nelson, 1944; Somogyi, 1952) after 15 min incubation at 85 °C. Samples were centrifuged (15000 *g*, 5 min) before dilution, and their A_{520} was read. The amylose was brought into solution by 1 M-NaOH and finally filtered through a membrane filter (Millipore RAWP; 1.2 μm pore size). One unit of α-amylase or pullulanase activity was defined as the amount of enzyme releasing 1 nmol of reducing sugar/min in the above assay, with anhydrous glucose (Merck) as a standard.

Isoelectric focusing

The crude enzyme was concentrated (3-fold) by ultrafiltration with a Centricon-30 micro-concentrator (Amicon), and 40 μl samples were applied on an agarose pI 3.5–5.0 isoelectric-focusing gel (0.1 cm × 11 cm) along with Pharmacia low-pI-calibration-kit standards. The gel had the following composition: Ampholine 3.5–5.0 (LKB), 1.9 ml; agarose IEF (Pharmacia), 0.3 g; sorbitol, 3.6 g; water, 27 ml. The gel was run on a Multiphor (LKB) flat-bed apparatus thermostatically controlled to 10 °C, with 1 M-NaOH and 0.05 M-H_2SO_4 as electrode solutions, for approx. 670 V·h with 2 W constant power. One crude enzyme lane was cut into 82 slices (each 1.15 mm) with a razor-blade slicer (Bio-Rad). Each slice was transferred to 400 μl of the buffer used in the enzyme assays and incubated for 10 min at 85 °C to dissolve the agarose. α-Amylase and pullulanase activities released were then assayed as described above, but with 40 μl samples and an incubation time of 45 min. One crude enzyme and one standard lane were stained with

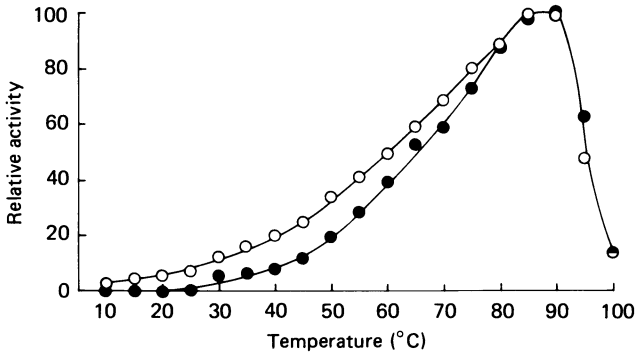

Fig. 1. Effect of temperature on the activity of α-amylase (●) and pullulanase (○)

Diluted crude enzyme (1.2 k-units of α-amylase/ml and 3.7 k-units of pullulanase/ml) was assayed for α-amylase and pullulanase as described in the Methods section, but at various temperatures. The highest activity is denoted as 100 in each case.

Coomassie Blue and scanned at 540 nm along the edge of the lanes with an Uvicon 820 (Kontron) spectrophotometer. The pH-gradient was measured by cutting one empty lane into 0.5 cm slices, eluting each of them with 2 ml of water and measuring the pH at 15 °C.

RESULTS

Fig. 1 shows the effect of temperature on the activity of *C. thermohydrosulfuricum* α-amylase and pullulanase. For the 15 min reaction times used, both activities had an optimum at 85–90 °C. At higher temperatures the activities declined very sharply, obviously because of thermal inactivation. Below the optimum temperature, the relative activity of pullulanase was somewhat higher than that of α-amylase.

The activities were stable at 65 °C in the absence of their substrates and Ca^{2+} for at least 2 h (Fig. 2), but they were gradually inactivated in an identical manner at higher temperatures. At 85 °C the inactivation occurred rapidly, and only some 30% of the activities was found after 10 min of incubation. Incubation for over 40 min at 85 °C destroyed both activities completely.

Ca^{2+} stabilized the activities equally at high temperatures (Fig. 3). More than 60% of both activities was still left after 2 h of incubation at 85 °C in the absence of substrate when 2 mM-Ca^{2+} was present. At 95 °C, however, addition of Ca^{2+} failed to protect either activity against inactivation. In the presence of 2 mM-Ca^{2+} and 0.5% substrate under the conditions of the standard assay, both reactions proceeded linearly for at least 45 min at 85 °C.

Fig. 4 shows pH-profiles of the α-amylase and pullulanase activities at temperatures of 85 and 60 °C. Both activities had an optimum at pH 5.6 at 85 °C. However, when the temperature was lowered to 60 °C, both activity profiles were broadened towards more acidic values, and had an optimum at pH 5.2. Although the activities had the same optima and the same pH-limits, the profile of α-amylase was somewhat broader inside these limits, especially at 60 °C.

The effect of pH on the inactivation of α-amylase and pullulanase activities at 70 °C in the absence of substrate

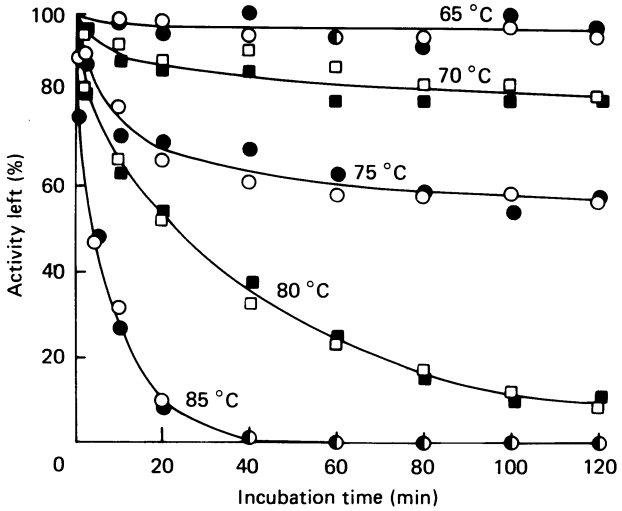

Fig. 2. Inactivation of α-amylase and pullulanase at different temperatures

Samples of crude enzyme were incubated with 100 mM-sodium acetate buffer, pH 5.6 (final concns.: α-amylase 0.8 k-unit/ml, pullulanase 2.4 k-units/ml and protein 0.5 mg/ml) at temperatures of 65–85 °C. Samples were withdrawn at the times indicated, and the remaining α-amylase and pullulanase activities were then determined as described in the Methods section, but with 50 μl samples. ■, ●, α-Amylase; □, ○, pullulanase.

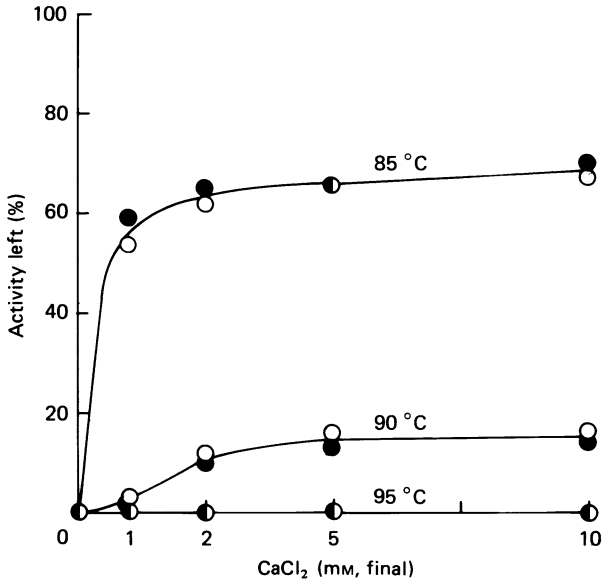

Fig. 3. Stabilization of α-amylase (●) and pullulanase (○) by Ca^{2+} against heat inactivation

Samples of crude enzyme were incubated with 100 mM-sodium acetate buffer, pH 5.6, containing various concentrations of $CaCl_2$ (final concns.: α-amylase 0.8 k-unit/ml, pullulanase 2.4 k-units/ml and protein 0.5 mg/ml) at temperatures of 85, 90 and 95 °C for 2 h. The remaining α-amylase and pullulanase activities were determined as described in the Methods section, but with 50 μl samples.

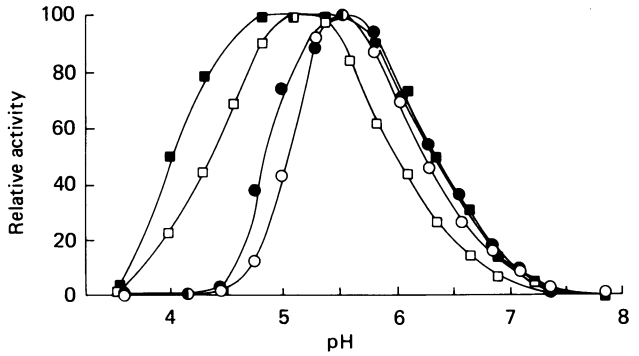

Fig. 4. Effect of pH on the activity of α-amylase and pullulanase at 85 and 60 °C

Diluted crude enzyme (25 μl; 1.2 k-units of α-amylase/ml and 3.7 k-units of pullulanase/ml) was added to 0.5% amylose or pullulan in 100 mM-sodium citrate buffer (1 ml) containing 50 mM-NaCl and 10 mM-$CaCl_2$ at various pH values. The tubes were incubated for 15 min at 85 or 60 °C, and reducing sugar released was then determined. The highest activity at each temperature is denoted as 100. The pH of the buffers used was measured at both temperatures. ■, ●, α-Amylase; □, ○, pullulanase. ○, ●, 85 °C; □, ■, 60 °C.

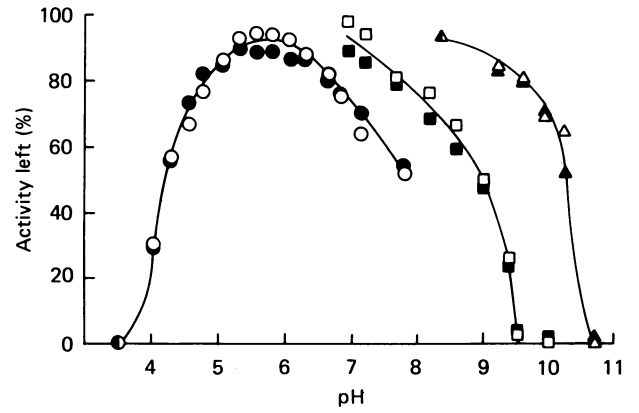

Fig. 5. Effect of pH on the inactivation of α-amylase and pullulanase

Diluted crude enzyme was added to sodium citrate, sodium phosphate or glycine/NaOH buffer at various pH values measured at 70 °C (final concns.: buffer 50 mM, α-amylase 1.2 k-units/ml, pullulanase 3.7 k-units/ml and protein 0.8 mg/ml). After 2 h of incubation at 70 °C, the remaining α-amylase and pullulanase activities were determined as described in the Methods section and compared with the activities of a sample kept at 0 °C in 100 mM-sodium acetate, pH 5.6, containing 2 mM-$CaCl_2$, 0.1 mM-Na_2EDTA and 50 mM-NaCl. ●, ■, ▲, α-Amylase; ○, □, △, pullulanase. ●, ○, Sodium citrate; ■, □, sodium phosphate; ▲, △, glycine/NaOH.

and Ca^{2+} is shown in Fig. 5. Neither activity was entirely stable at 70 °C, irrespective of the pH used. Both of them were effectively inactivated if the pH was lower than 4.5 with citrate buffer or higher than 10.5 with glycine/NaOH buffer. However, the stability of the activities was very dependent on the buffer used. Inactivation above the pH optimum occurred at

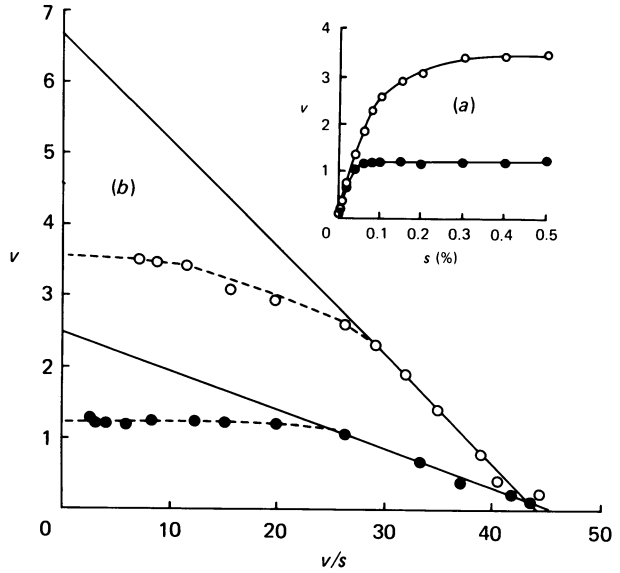

Fig. 6. Effect of concentrations of amylose and pullulan on α-amylase and pullulanase activities respectively

Diluted crude enzyme (1.2 k-units of α-amylase/ml, 3.7 k-units of pullulanase/ml and 0.8 mg of protein/ml) was assayed for α-amylase (●) and pullulanase (○) as described in the Methods section, but with different concentrations of the substrates. Reaction rate (v) is expressed as μmol of reducing sugar (as glucose) released/min, and substrate concentration (s) as % (w/v).

substantially lower pH values in citrate than in glycine/NaOH buffer, whereas phosphate buffer gave intermediate results (see Fig. 5). The addition of 10 mM-$CaCl_2$ made neither of the activities more stable towards acidic conditions in citrate buffer or towards basic conditions in glycine/NaOH buffer (results not shown).

α-Amylase and pullulanase were both activated by Ca^{2+}, this activation being reversed by EDTA (results not shown). Almost full activation was obtained in either case already with 0.2 mM-Ca^{2+}. Activities after full EDTA inhibition were about 40% of those obtained after full activation by Ca^{2+}, corresponding to about 2.5-fold activation by Ca^{2+} in both cases. Mg^{2+} stimulated neither activity.

The dependence of α-amylase and pullulanase activities on the concentration of their substrates, amylose and pullulan in the presence of saturating concentration of Ca^{2+} (2 mM), is shown in Fig. 6(*a*). Maximal activities were reached with concentrations of about 0.1% of amylose and 0.5% of pullulan. An Eadie–Hofstee plot of the data (Fig. 6*b*) gave values of apparent K_m of 0.055% for α-amylase and 0.15% for pullulanase, but also revealed that α-amylase activity followed Michaelis–Menten kinetics only up to a concentration of 0.04% amylose, and pullulanase activity did so only up to 0.08% pullulan. Because of substrate inhibition at higher concentrations, the observed reaction rates never rose above about half of the theoretical $V_{max.}$ values, and rates of half the practical maximum velocities were reached at about 0.02% amylose and 0.05% pullulan.

After isoelectric focusing of the crude enzyme, α-amylase and pullulanase activities were found in

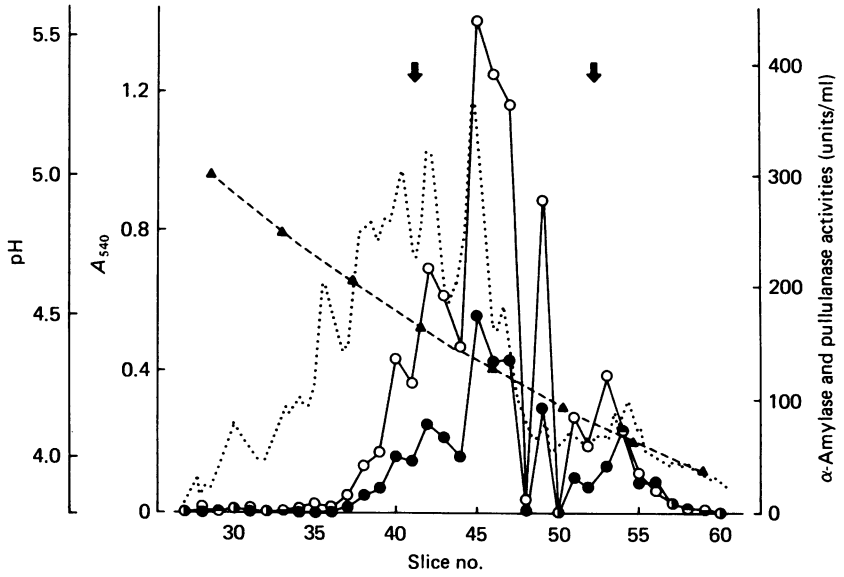

Fig. 7. Isoelectric focusing of crude enzyme

For details, see the Methods section. ●, α-Amylase; ○, pullulanase; ····, protein (A_{540}); ▲, pH. The arrows indicate the positions of the standard proteins soya-bean trypsin inhibitor (pI 4.55) and glucose oxidase (pI 4.15) contained in the Pharmacia low-pI calibration kit.

several peaks (Fig. 7), which were centred between the standards soya-bean trypsin inhibitor (pI 4.55) and glucose oxidase (pI 4.15) and formed the same pattern in both cases. The activities were largely associated with the major protein peaks. Highest activities relative to the amount of protein (A_{540}), however, were in either case associated with a minor protein peak located slightly above pH 4.2.

DISCUSSION

C. thermohydrosulfuricum E 101–69 produces an α-amylase activity (EC 3.2.1.1), which cleaves amylose to malto-oligosaccharides, and a pullulanase activity (EC 3.2.1.41), which cleaves the α-1,6 bonds of pullulan to produce maltotriose (Melasniemi, 1987). The temperature and pH optima (85–90 °C and pH 5.6) obtained in the present study for the extracellular α-amylase and pullulanase activities of *C. thermohydrosulfuricum* E 101–69 were the same as those obtained by Hyun & Zeikus (1985*a*) for the cell-bound pullulanase of strain E39. The only α-amylase reported to have a higher temperature optimum is that of *Bacillus licheniformis*, with its optimum at 92 °C (Madsen *et al.*, 1973). The temperature optimum of *C. thermohydrosulfuricum* pullulanase is appreciably higher than reported for any other pullulanase. *Bacillus acidopullulyticus* (Schülein & Højer-Pedersen, 1984) and *Bacillus sp.* (Norman, 1982) come next, with their temperature optima at about 60 °C. The pH optimum obtained is a fairly typical value for an α-amylase (Fogarty, 1983). The pullulanase of *B. acidopullulyticus* (Schülein & Højer-Pedersen, 1984) has its optimum slightly lower, at about pH 5.0, and that of *Klebsiella pneumoniae* (*Aerobacter aerogenes*) (Ohba & Ueda, 1973) is somewhat higher, at pH 6.0–6.5. Stabilities obtained in the present study for the unprotected extracellular activities were somewhat lower than those obtained by Hyun & Zeikus (1985*a*) for the cell-bound pullulanase, but so also were the protein concentrations used.

In a previous report (Melasniemi, 1987) it was shown that α-amylase and pullulanase activities of *C. thermohydrosulfuricum* E 101–69 were produced and repressed similarly. Depending on the carbon source used, most of the activities were found sometimes in the media and sometimes associated with the cells, but, regardless of their location, their relative proportions were always constant. In the present study the extracellular α-amylase and pullulanase activities responded similarly to every parameter tested, deviating from each other in excess of experimental error only in experiments (Figs. 1 and 4) where the parameters were tested in the presence of the appropriate substrate, amylose or pullulan. These observations suggest that a single protein is responsible for both the α-amylase and pullulanase activities of *C. thermohydrosulfuricum* E 101-69, and thus represents a novel, thermostable, amylase. In agreement with this idea, the two activities could not be separated by isoelectric focusing. Instead, the activities were resolved as several peaks having the same α-amylase/pullulanase activity ratio (Fig. 7). This means either that the enzyme occurs in several molecular species with different pI values, or that the exocellular enzyme is released to the medium from the cell wall/membrane of the organism (Melasniemi, 1987) in a rather tight association with other cell-wall/membrane proteins. The latter alternative is favoured by the fact that the activities are co-eluted on gel filtration of the crude enzyme (again in the same α-amylase/pullulanase ratio of approx. 1:3) with a minor part as an extremely high-M_r (> 10^6) aggregate (results not shown).

The first reports that fungal glucoamylases have both

α-1,4 and α-1,6 activities in a single enzyme (Tsujisaka *et al.*, 1958; Pazur & Ando, 1960; Pazur & Kleppe, 1962) appeared more than 20 years ago. The α-1,6 activity of some glucoamylases is quite large, as for *Cladosporium resinae* glucoamylase P (McCleary & Anderson, 1980). Several α-amylases have also been reported to show some α-1,6 activity with certain low-M_r substrates. *Thermoactinomyces vulgaris* α-amylase, which degrades pullulan by cleaving its α-1,4 bonds to produce panose (Shimizu *et al.*, 1978), can also cleave the α-1,6 bonds in isopanose and specific α-1,6 bonds in some other α-1,4;α-1,6-gluco-oligosaccharides (Fukushima *et al.*, 1982; Sakano *et al.*, 1982, 1983). Several other α-amylases, including the human salivary α-amylase, have also been reported to be able to cleave α-1,6 bonds in certain gluco-oligosaccharides (Sakano *et al.*, 1985). The amylase of *C. thermohydrosulfuricum* seems, however, to be the first α-amylase type of enzyme to be described that exhibits towards a polysaccharide substrate a prominent α-1,6 activity, which in this case is even more pronounced than its α-1,4 activity.

I thank Dr. John Londesborough and Dr. Roy Tubb for critically reading the manuscript, and Ms. Riitta Jussila for skilful technical assistance.

REFERENCES

Fogarty, W. M. (1983) in Microbial Enzymes and Biotechnology (Fogarty, W. M., ed.), pp. 1–92, Applied Science Publishers, Barking

Fukushima, J., Sakano, Y., Iwai, H., Itoh, Y., Tamura, M. & Kobayashi, T. (1982) Agric. Biol. Chem. **46**, 1423–1424

Hollaus, F. & Sleytr, U. (1972) Arch. Microbiol. **86**, 129–146

Hyun, H. H. & Zeikus, J. G. (1985*a*) Appl. Environ. Microbiol. **49**, 1168–1173

Hyun, H. H. & Zeikus, J. G. (1985*b*) Appl. Environ. Microbiol. **49**, 1174–1181

Hyun, H. H. & Zeikus, J. G. (1985*c*) J. Bacteriol. **164**, 1146–1152

Hyun, H. H., Shen, G.-J. & Zeikus, J. G. (1985) J. Bacteriol. **164**, 1153–1161

Madsen, G. B., Norman, B. E. & Slott, S. (1973) Starch/Staerke **25**, 304–308

McCleary, B. V. & Anderson, M. A. (1980) Carbohydr. Res. **86**, 77–96

Melasniemi, H. (1987) J. Gen. Microbiol. **133**, 883–890

Nelson, N. (1944) J. Biol. Chem. **153**, 375–380

Norman, B. E. (1982) Starch/Staerke **34**, 340–346

Ohba, R. & Ueda, S. (1973) Agric. Biol. Chem. **37**, 2821–2826

Pazur, J. H. & Ando, T. (1960) J. Biol. Chem. **235**, 297–302

Pazur, J. H. & Kleppe, K. (1962) J. Biol. Chem. **237**, 1002–1006

Sakano, Y., Hiraiwa, S., Fukushima, J. & Kobayashi, T. (1982) Agric. Biol. Chem. **46**, 1121–1129

Sakano, Y., Fukushima, J. & Kobayashi, T. (1983) Agric. Biol. Chem. **47**, 2211–2216

Sakano, Y., Sano, M. & Kobayashi, T. (1985) Agric. Biol. Chem. **49**, 3041–3043

Schülein, M. & Højer-Pedersen, B. (1984) Ann. N.Y. Acad. Sci. **434**, 271–274

Shimizu, M., Kanno, M., Tamura, M. & Suekane, M. (1978) Agric. Biol. Chem. **42**, 1681–1688

Somogyi, M. (1952) J. Biol. Chem. **195**, 19–23

Tsujisaka, Y., Fukumoto, J. & Yamamoto, T. (1958) Nature (London) **181**, 770–771

Wiegel, J., Ljungdahl, L. G. & Rawson, J. R. (1979) J. Bacteriol. **139**, 800–810

Received 5 December 1986/13 February 1987; accepted 14 April 1987