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Regulation of the Production of Polygalacturonase by *Aspergillus niger*

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ABSTRACT. Synthesis of ethylene in static cultures as well as the effect of endogenous and exogenous ethylene on the synthesis of polygalacturonase by *Aspergillus niger* were determined. This strain produced maximum ethylene amounts when cultured at 30 °C for 3 d. The effect of adding ethylene precursors (citrate-cycle intermediates) on ethylene production was investigated. Best intracellular and extracellular polygalacturonase production was obtained with 2-oxoglutaric, pyruvic and fumaric acids, and with glutamic acid too. Addition of ethylene to the culture medium also increased the synthesis of polygalacturonase, although to a lower degree than when glutamic acid was added.

Ethylene regulates several aspects of plant growth, stimulating fresh fruit ripening (Liberman 1975), flower and leaf abscission by inducing synthesis and secretion of the mRNA of saccharide hydrolytic enzymes (Montaldi 1995) such as cellulases and pectinases, which degrade cell walls.

It is now known that ethylene is produced not only by plants but also by certain microorganisms (Chou and Yang 1973; Primose 1976; Smith 1976). Due to its association with citrus fruits, the production of ethylene by *Penicillium digitatum* (a well-known lemon pathogenic microorganism) has been widely studied, its culture conditions have been defined and biosynthetic pathways have been elucidated. When the fungus is cultured under static conditions, the synthesis of ethylene is regulated by glutamic acid while, when grown in a shaker, its synthesis is regulated by methionine (Chalutz and Lieberman 1977).

Plant pathogenicity is closely related to the synthesis of saccharide hydrolytic enzymes (pectinases and cellulases). *Aspergillus niger* (a nonpathogenic filamentous fungus) is an important producer of pectinolytic enzymes. Pectinases are inducible or constitutive enzymes that catalyze pectin breakdown. The regulatory mechanisms of pectinase synthesis, such as catabolic repression and induction, have been extensively studied (Aguilar and Huitron 1986; Maldonado *et al.* 1989*a*,*b*).

These and related enzymes are widely used in the industrial processing of fruit juice because of their ability to degrade pectin and related substances, which results in a markedly enhanced yield since the concomitant viscosity decrease in fruit pulp facilitates extraction, liquefaction, filtration and clarification processes (Kilara 1968; Brawman 1981; de Rezende and Felix 1999; Kolarova and Augustín 2001).

Here we examined the production of ethylene by the *A. niger* strain. In an attempt to get the maximum production of polygalacturonase we produced the effect by bubbling ethylene through the culture medium and adding some compounds of the citrate cycle as precursors of ethylene.

MATERIALS AND METHODS

Microorganism. Aspergillus niger strain MI299 was isolated from a rotten lemon (Maldonado *et al.* 1986). It was maintained by monthly transfers to Czapek agar slant tubes, incubated at 30 °C and stored at 4 °C.

Production medium contained in g/L: $NaNO_3$ 3, K_2HPO_4 1, KCl 0.5, $MgSO_4$ 0.5, polygalacturonic acid (PGA) 5; pH was adjusted to 4.5. The medium was sterilized for 15 min at 120 °C. An inoculum of 2×10^6 spores per mL was used, final concentration being 2×10^5; enzyme production was done on a orbital shaker (5 Hz, 30 °C, 1 d). The cultures were then supplemented with glutamic, pyruvic, succinic, 2-oxoglutaric, citric, and fumaric acids or with methionine, or bubbled with ethylene (1 L/min per L medium) for 1–7 min to obtain different gas concentrations in the medium.

When methionine was added, the inoculated medium was incubated at 30 °C in a shaker (5 Hz) for additional 2 d; when compounds of the citrate cycle or ethylene were added, the medium was cultured without shaking in an incubator at 30 °C for 2 d. At the end of that time, cultures were filtered (Schleicher & Schull no. 595 filter paper) and extracellular polygalacturonase (PG) activity was determined in the filtrates.

Extracellular PG assay. Five tenths mL of filtrate was added to a solution containing 2 mL 0.5 % PGA in 0.1 mol/L acetate buffer (pH 4.5). After incubation (37 °C, 1 h), reducing sugars were determined by the 3,5-dinitrosalicylic acid method (Miller 1959) using galacturonic acid as reference. Specific activity of PG is given in nkat/mL (release of 1 nmol/s galacturonic acid).

Intracellular PG assay. Mycelium was resuspended in 10 mL 0.2 mol/L phosphate buffer (pH 7.4), frozen and disrupted in a mortar. Finally, it was filtered through a 45-μm membrane. The filtrate was used to determine intracellular PG in the same way as extracellular one.

Ethylene determination. Erlenmeyer flasks containing inoculated production media were plugged with a stopper and sealed with paraffin. Then they were incubated at 32 °C for 3 d. Gas samples were obtained by using a syringe and needle, the presence of ethylene produced being determined as follows. The gas collected in the syringe was bubbled in 2 mL of bromine water. The decolorization produced by the addition of bromine (to the double bond of ethylene) confirmed the presence of ethylene (Shriner *et al.* 1998).

Biomass determination. The mycelium obtained after filtration was rinsed and dried at 90 °C until constant mass was reached.

Results are the mean of three different determinations.

RESULTS AND DISCUSSION

Production of ethylene by the culture reached a maximum when the fungus was cultured at 30 °C for 3 d. Under these conditions, the decolorization of the bromine water solution was observed immediately. In contrast, when incubation periods were longer or shorter than 3 d, decolorization took longer.

No changes in PG synthesis or in biomass production were observed when the fungus was cultured in a shaker with 0.3–15 mmol/L methionine. By the way, in *Penicillum digitatum* the production of ethylene by shaken culture in response to methionine seems to involve induction of an ethylene-synthesizing enzyme system(s) in the cells (Chalutz and Lieberman 1977).

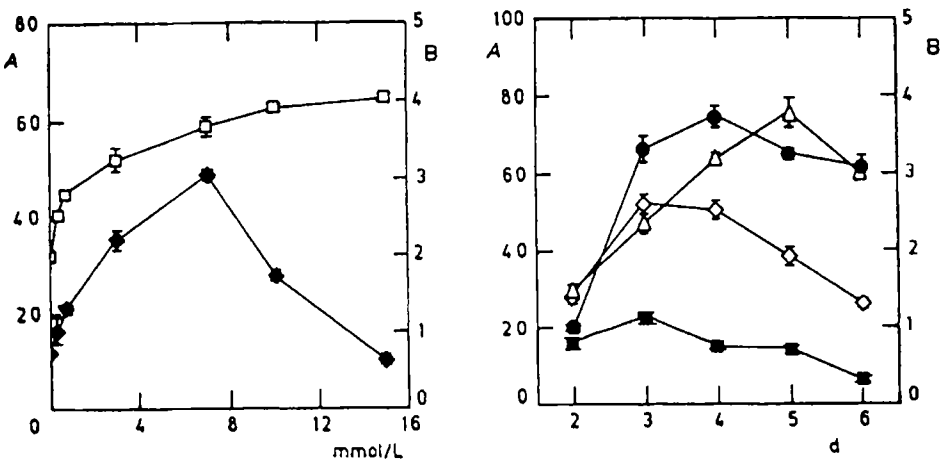

Fig. 1. *Left*: biomass production (B, g/L, □) and PG production (activity *A*, nkat/mL, ◆) at different concentrations of glutamic acid (mmol/L). *Right*: kinetics of growth (biomass B, g/L, ◇) and PG production (activity *A*, nkat/mL, △) with 7 mmol/L glutamic acid; without glutamic acid: growth (biomass B, g/L, ●) and PG (activity *A*, nkat/mL, ■).

In the presence of glutamic acid (Fig. 1 *left*), maximum PG production (49 nkat/mL) was reached at 7 mmol/L. On the other hand, only 13 nkat/mL was found in the control with no glutamic acid. Biomass values increased, but not as much as enzymic activity did. The specific production

reached with a 7 mmol/L concentration of glutamic acid was 13 μkat/g biomass, while that of the control (without acid) was 6 μkat/g.

The optimum conditions for enzyme production in the presence of 7 mmol/L glutamic acid were determined (Fig. 1 *right*); the incubation period for maximum production being found to be 3 d total.

Effect of citrate-cycle acids. From among the concentrations assayed (2.5; 5; 10 mmol/L), best results were obtained with 5 mmol/L, while with 2.5 and 10 mmol/L, PG production was similar to that of the control. Concentration of 5 mmol/L was therefore used to determine extra- and intracellular PG synthesis (Table I). Pyruvic acid caused a 2.28-fold increase, 2-oxoglutaric acid a 2.41-fold increase and fumaric acid a 2.06-fold increase in extracellular PG activity (with respect to the control without precursors). Intracellular PG activity with the above precursors at 5 mmol/L showed a similar tendency, significant increases being observed when 2-oxoglutaric or fumaric acid were added. It indicates that ethylene synthesized in the presence of the above precursors would favor the synthesis of the enzyme. These results are in agreement with Jacobsen and Ward (1968), who concluded that, in *P. digitatum*, ethylene biosynthesis is different from that observed in fruit and vegetable tissues.

Table I. Effect of citrate-cycle compounds (concentration, mmol/L) on extracellular and intracellular PG production (activity ± SE, nkat/mL)

mmol/L	Extracellular					Intracellular				
	pyruvate	succinate	2-oxoglutarate	citrate	fumarate	pyruvate	succinate	2-oxoglutarate	citrate	fumarate
0	17 (1.5)	17 (1.5)	17 (1.5)	17 (1.5)	17 (1.5)	27 (0.8)	27 (0.8)	27 (0.8)	27 (0.8)	27 (0.8)
5	39 (16)	25 (5.1)	41 (5.7)	24 (5.1)	32 (5.0)	27 (4.4)	20 (3.5)	41 (2.3)	25 (1.7)	34 (0.8)

Chow and Yang (1973) pointed out that 2-oxoglutaric acid is the dividing point at which the ethylene production pathway becomes differentiated from the citrate cycle. *A. niger* increases PG synthesis in the presence of glutamic acid, probably due to the increase in ethylene synthesis. These results agree with those found for *P. digitatum*, which utilizes glutamic or 2-oxoglutaric acid as immediate precursors of ethylene synthesis. On the basis of the results obtained in our experiments, a similar mechanism might be at work in the case of *A. niger*.

The increase in PG synthesis in the presence of glutamic acid or of certain compounds belonging to the citric acid cycle led us to investigate the response of the enzymic synthesis in the presence of exogenous ethylene, an event that could be of great importance for the industrial production of pectinolytic enzymes.

After the addition of ethylene to the culture medium, the highest PG production (29 nkat/mL) was obtained by bubbling the gas for 4 min and with an incubation period of 3 d, the enzyme production being 1.7 times greater. Comparison of the values obtained with exogenous ethylene and any of the compounds of the endogenous gas synthesis would lead to the conclusion that enzymic production is much more efficient when precursors such as glutamic acid are added to the culture medium. In the presence of this compound, enzymic activity was 49 nkat/mL, while in the culture medium with ethylene it was only 29 nkat/mL.

The same enzymic activity (17 nkat/mL) was obtained with all the compounds tested; it suggests that the changes in the enzyme content were due to the effect of the different compounds on enzyme synthesis.

When *A. niger* synthesizes ethylene, the enzymic induction mechanism is much more efficient than when ethylene is added to the culture medium. The mechanism of ethylene production is activated by the presence of certain compounds belonging to the citric acid cycle in a static culture. In the same way as in plants, the synthesis of PG increases in the presence of ethylene; it would be of commercial importance for the production of PG.

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