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Enhancement of glucose oxidase production by *Penicillium variabile* P16

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Effects of the polysaccharides alginate and locust bean gum, and oligosaccharides oligomannuronate (OM) and oligoguluronate (OG), on glucose oxidase (GOD) production by Penicillium variabile *P16 were studied. Small increases were observed when the cultures were supplemented with OG and OM blocks with an average degree of polymerization (DP) of approximately ten. With 200 mg l^{-1} OM blocks addition at 0 h, the increase reached 32.1% compared with the control; however, regardless of the time of addition, large increases (up to approximately 70%) in GOD production were obtained with 100 and, particularly, 200 mg l^{-1} of alginate-derived oligosaccharides (OG and / or OM blocks) with a DP of approximately seven. No significant influence was observed on mycelial biomass.* *© 1999 Elsevier Science Inc. All rights reserved.*

Keywords: *Penicillium variabile*; elicitation; glucose oxidase; oligosaccharides; alginate

Introduction

During the last decade, several groups have reported improvements in activity of cell cultures resulting from addition of small amounts of compounds commonly known as elicitors.[1–4] Oligosaccharides have been shown to act as a class of elicitors for bacterial,[5] fungal,[6] plant,[2,7,8] and animal[9] cell cultures.

Glucose oxidase (GOD, β-D-glucose:oxygen, 1-oxidoreductase, EC 1.1.3.4) catalyzes oxidation of glucose by molecular oxygen and is produced industrially as a by-product of the gluconic acid fermentation from *Aspergillus niger*. *Penicillium amagasakiense* and *Penicillium vitale* are also used for the industrial production of this enzyme.[10,11] Since the early 1950s glucose oxidase has been widely used in powdered egg manufacture[12] and paper test strips for diabetic patients.[13] A new application for GOD is its use in biosensors.[14,15] Over the last 40 years, many new applications have been developed. Glucose oxidase is produced by the fungi as a defense mechanism and has antimicrobial effects. It has been used as an ingredient of toothpaste.[16] This enzyme might also be a good alternative to traditional chemical and physical treatments in food preservation.[17,18]

While enhancement of metabolic activity by oligosaccharides in different plant and animal cell cultures has been reported, very little is published regarding fungal cultures. In a previous paper,[6] we reported enhancement of penicillin G, a secondary metabolite, in cultures of *Penicillium chrysogenum*. In this article, we investigate the enhancement of glucose oxidase production by cultures of *Penicillium variabile* P16. This strain was previously selected for its ability to exhibit extracellular GOD activity.[19] Alginate, locust bean gum, and oligosaccharides derived from alginate have been used as elicitors for the enhancement of the enzyme production.

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Materials and methods

Microorganism and culture medium

P. variabile strain P16 was used in this study. Cultures were maintained on malt extract agar (Oxoid Unipath Ltd., Basingstoke, U.K.) at 4–6°C and subcultured every month. A semidefined medium was used for growth and GOD production and contained (in g l^{-1} distilled water): $NaNO_3$, 5.0: KCl, 0.5; KH_2PO_4, 1.0; $FeSO_4 \cdot 7H_2O$, 0.01; and mycological peptone (Oxoid), 1.0. After sterilization (121°C for 20 min), $CaCO_3$ (sterilized separately at 121°C for 20 min) and a filter-sterilized (Millipore, Bedford, MA) membrane HA, 0.45 μm pore size, 47 mm diameter) glucose solution were added to a final concentration of 35 and 80 g l^{-1}, respectively. All chemicals, unless stated otherwise, were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Culture conditions

Erlenmeyer flasks (500 ml) containing 100 ml of the medium were inoculated with 5.0 ml of conidiospore suspensions (2×10^7 spores ml^{-1}). Cultures were incubated in an orbital shaker (200 rpm) at 28°C for 120 h. Culture samples were taken every 24 h and the mycelial growth followed by cell dry weight determination. Samples of culture supernatant were collected after centrifugation (3,000 *g* for 15 min) and were assayed for GOD.

Mycelial dry weight determination

Mycelial biomass collected after centrifugation (see above) was resuspended in 50 ml of deionized water and 6.0 N HCl was added to reduce the pH to approximately 1.0 and to dissolve the residual $CaCO_3$; the mycelial suspension was then neutralized with 2.0 N NaOH, filtered on preweighed Whatman GF/C discs, and dried overnight at 105°C.

Enzyme assay

GOD activity in the supernatants was determined spectrophotometrically by the method of Ciucu and Patroescu [20] as modified by Markwell *et al.* [21] Reaction mixtures (1.0 ml 1 M glucose, 0.5 ml 0.1% benzoquinone, and 0.45 ml 0.1 M Na-citrate buffer, pH 5.0) were preincubated at 25°C and the reaction started by adding 25–50 μl of enzyme solution (culture supernatant). The method is based on the enzymatic reduction of benzoquinone by hydroquinone measured by the rate of absorbance increase at 290 nm ($\epsilon = 2.31$ nM^{-1} cm^{-1}). One unit (U) of GOD activity is defined as the amount of enzyme which reduces 1.0 μmol benzoquinone ml^{-1} culture supernatant min^{-1}.

Preparation of oligosaccharides and determination of degree of polymerization (DP)

Oligosaccharides (oligomannuronates and oligoguluronates) were prepared from alginate as described before.[6] Sodium alginate (10 g); (BDH Ltd., Poole, Dorset, UK) isolated from *Laminaria hyperborea*, was partially hydrolyzed with 0.3 M HCl by heating at 100°C for 6 h. Homopolymeric blocks of mannuronate and guluronate remained intact as a residue which was removed by centrifugation (3,000 *g* for 30 min), washed, and resuspended in distilled water. Dilute NaOH (0.3 M) was added until all the residue dissolved. Sodium chloride was added to make a final concentration of 0.5% (w/v). Ethanol (2 volumes) was added and precipitated material was collected by centrifugation (3,000 *g* for 30 min). The precipitate was washed, redissolved in water, and the pH adjusted to 2.85 with 1 M HCl. A precipitate formed that consisted of oligoguluronate blocks, leaving oligomannuronate blocks in solution. The fractions were desalted, and then freeze-dried.

The degree of polymerization (DP) of prepared oligosaccharides (blocks) and the oligosaccharides present in the alginate was determined by size exclusion chromatography on a 1.0 cm i.d. × 88 cm Bio-gel P2 column (Bio-Rad Laboratories, Hercules, CA) equilibrated with 0.1 M NaCl. The elution was performed with 0.1 M NaCl at a flow rate of 0.25 ml min^{-1}. The mannuronic and/or guluronic acid content of the collected fraction (1.0 M) was determined by the carbazole assay.[22] The column was calibrated with the following standards: galacturonic acid (212.2 Da) di-galacturonic acid (370.3 Da) and tri-galacturonic acid (546.4 Da).

Effect of alginate, alginate oligosaccharides, and locust bean gum

Oligomannuronate (OM) and oligoguluronate (OG) blocks with DP ≈ 9–10 were prepared. The required amounts of OM, OG, alginate (unhydrolyzed), and locust bean gum were weighed and dissolved in water the pH of the mixture was adjusted with KOH to 6.5, and the solution was autoclaved at 121°C for 15 min. The solutions were added to the shaken flask cultures at 0, 24, or 48 h to form final concentrations of 50 (alginate only), 100, or 200 mg l^{-1}. Control cultures contained no alginate, locust bean gum or oligosaccharides.

Results

Shake flask experiments were performed at least in duplicate to study the effect of either polysaccharides such as alginate and locust bean gum, of oligosaccharides such as OM and OG and on extracellular GOD production by *P. variabile*. Results are reported in *Tables 1 and 2*. Polysaccharides at a concentration range of 100–200 mg l^{-1} were able to cause notable increases in the enzyme activity levels in the culture broths. On the contrary, little increase in the activity was observed after addition of similar concentrations of OM and OG blocks.

Table 1 Effect of alginate concentration and time of its addition on glucose oxidase production by *P. variabile* P16

Alginate (mg l^{-1})	Glucose oxidase activity (%)[a]		
	0 h[b]	24 h[b]	48 h[b]
50	0	5.1	0
100	41.2	30.2	11.7
200	51.2	35.4	NA[c]

[a]Glucose oxidase activity is given after 120 h of fermentation as percentage (%) of increase in comparison with that of the control
[b]Time (h) of alginate addition to the cultures
[c]NA, not available

Table 2 Effect of locust bean gum (100 mg l^{-1}) added after 24 h of fermentation on glucose oxidase production by *P. variabile* P16

	Fermentation time (h)			
	48	72	96	120
Glucose oxidase activity (%)[a]	10.6	10.9	33.2	29.5

[a]Glucose oxidase activity is given as the percentage (%) of increase in comparison with that of the control

Effect of alginate and locust bean gum on GOD production

Addition of 50 mg l^{-1} alginate after 0, 24, and 48 h of fermentation did not increase GOD production by *P. variabile*, thus results are not shown. On the contrary, the addition of alginate at higher concentrations to the cultures caused increases in the production of GOD in all the other cases (*Figures 1 and 2* and *Table 1*).

When the cultures were supplemented with 100 mg l^{-1} alginate, the highest increase in the enzyme production (7.10 U ml^{-1} GOD activity, 65.1% more than that of control) was observed in 72-h-old cultures complemented with alginate after 24 h. Although the GOD activity of these cultures gradually decreased over the next 48 h it was still 6.51 U ml^{-1}, 30.2% higher than the activity of the control culture.

Based on the results obtained from these experiments, a set of studies was performed by adding 200 mg l^{-1} alginate to the cultures after 0 or 24 h only (*Figure 2*). In both cases, GOD production was increased but higher increases were obtained from additions made at zero time. GOD production was highest (7.67 U ml^{-1} enzyme activity, 69.3% more than that of control) after 96 h of fermentation. Regardless of the time of alginate addition, GOD activities remained higher than those of the control for 72 h being 7.55 and 6.77 U ml^{-1} at the end of fermentation (35.4 and 51.2% of increase, *Table 1*). A gel permeation chromatography of alginate was performed; other than a peak corresponding to the void volume of the column (molecules with a DP more than 10), a sharp peak was eluted corresponding to oligosaccharides with a DP of approximately 7 (results not shown).

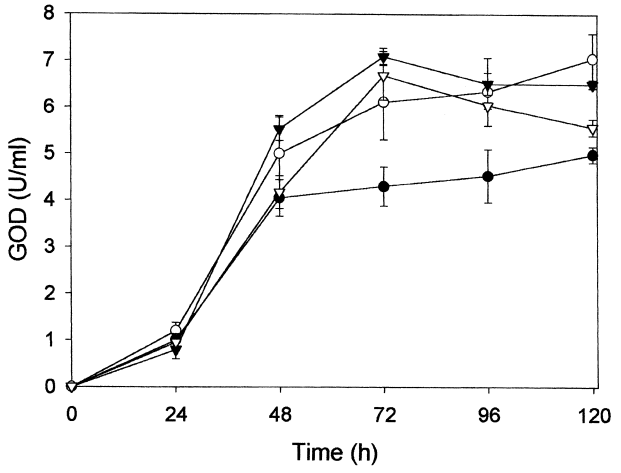

Figure 1 Effect of alginate (100 mg l^{-1}) added at different times during fermentation on glucose oxidase production by *P. variabile* P16. Time of polysaccharide addition: 0 (○), 24 (▼), and 48 (▽) h of fermentation. The control culture (●) contained no alginate. Values are the means of at lease two independent experiments; vertical bars represent standard deviations of the means. Glucose oxidase (GOD) activities are given in U ml^{-1} of culture supernanant (see Materials and methods)

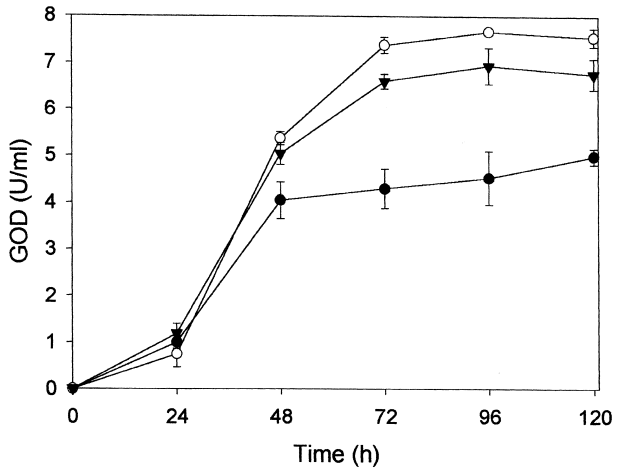

Figure 2 Effect of alginate (200 mg l^{-1}) added at different times of fermentation on glucose oxidase production by *P. variabile* P16. Time of polysaccharide addition: 0 (○) and 24 (▼) h of fermentation. Control culture (●) contained no alginate. Values are the means of at least two independent experiments; vertical bars represent standard deviations of the means. Glucose oxidase (GOD) activities are given in U ml^{-1} of culture supernatant (see Materials and methods)

The addition of alginate to cultures of *P. variabile* did not affect mycelial growth. No significant difference in final biomass (cell dry weight) was observed between the control and the supplemented cultures regardless of the concentrations and the time of addition of the supplements.

Locus bean gum (LBG) at concentrations of 50, 100, and 200 mg l^{-1} was added to the cultures after 0, 24, and 48 h (for each concentration). The highest enhancement of the enzyme production (6.04 U ml^{-1} of GOD activity, 33.2% more than that of control) was noticed after 96 h in samples taken from the cultures supplemented with 100 mg l^{-1} LBG after 24 h (*Table 2*). Other additions (50 and 100 mg l^{-1}) had not noticeable effect on production of GOD. In all experiments, no significant effect was observed on mycelial growth. The final cell dry weights were 6.3 $\pm$ 0.8 and 6.4 $\pm$ 0.7 g l^{-1} for cultures supplemented with 100 mg l^{-1} locust bean gum after 24 h and for the control, respectively.

Effect of OM and OG blocks on GOD production

OM and OG blocks with a DP of approximately 10

Table 3 Effect of 100 mg l^{-1} of oligomannuronate (OM) and oligoguluronate (OG) blocks added at zero time (0 h) and after 24 and 48 h of fermentation on glucose oxidase production by *P. variabile* P16

	Glucose oxidase activity (%)[a]		
Blocks	0 h[b]	24 h[b]	48 h[b]
OM	8.1	5.6	12.9
OG	12.7	9.3	7.8

[a]Glucose oxidase activity is given after 120 h of fermentation as percentage (%) of increase in comparison with that of the control
[b]Time (h) of locust bean gum addition to the cultures

were prepared. When 100 mg l^{-1} concentrations of each block were added to the cultures of *P. variabile*, in most cases, no significant enhancement was obtained in the GOD production (*Table 3*). The highest increases in GOD activity (12.7 and 12.9%) were observed when the cultures were supplemented with OG at the beginning of the fermentation and with OM after 48 h of fermentation. With 200 mg l^{-1} OM blocks addition at 0 h, a 32.1% increase in activity was noticed (*Figure 3*) when GOD production reached its highest (5.52 U ml^{-1} at 96 h of fermentation). No significant differences were observed on mycelial biomasses between cultures supplemented with oligosaccharide blocks and control cultures (results not shown).

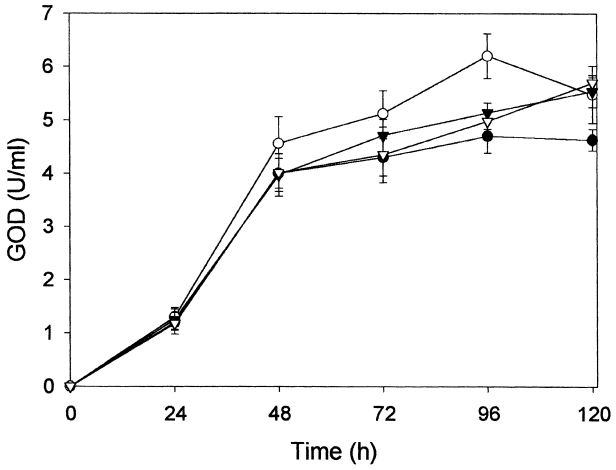

Figure 3 Effect of oligomannuronate (OM) blocks (200 mg l^{-1}) added at different times of fermentation of glucose oxidase production by *P. variabile* P16. Time of polysaccharide addition: 0 (○), 24 (▼), and 48 (▽) h or fermentation. Control culture (●) contained no alginate. Values are the means of at least two independent experiments; vertical bars represent standard deviations of the means. Glucose oxidase (GOD) activities are given in U ml^{-1} of culture supernatant (see Materials and methods)

Discussion

While elicitation of metabolites has been established in bacterial, plant, and cell cultures, very little information is available on the elicitation effect of fungal cultures. In a previous paper[6], we reported for the first time the enhancement of a secondary metabolite, penicillin G, production in cultures of *Penicillium chrysogenum* strain P2 using oligosaccharides OG and OM obtained from alginate. While changes in the physiological activities induced by alginate oligosaccharides have been established in reports on bacterial, animal, and in particular, plant systems,[5,9,23–25] relatively little information is available on the elicitation effect of intact alginate. Dörnenburg and Knorr[2,7] described the enhancement effect of microbial alginate on anthraquinone synthesis in *Morinda citrifolia* cultures; however, the authors observed no elicitation when LBG and alginate of plant origin were used. Also, enhancement of sesquiterpene synthesis in plant cell cultures of *Hyoscyamus muticus* has been reported.[26]

In this paper, we have investigated the effect of poly- and oligosaccharides on the enhancement of the production of GOD, an enzyme of commercial importance,[10,11,16] by *P. variabile* P16.

While enhancement of penicillin G yield has been detected after addition of OG and OM blocks with a DP of approximately ten [6] no considerable increase was observed in GOD activity (based on biomass cell dry weight) from *P. variabile* under similar elicitation conditions; however, the enhancement was significant (increases up to approx. 70%) after addition of alginate. This suggests that enhancement of metabolic activity in fungi by OM and OG blocks of the same DP is not generic. While elicitation occurs in the presence of these blocks, the degree of polymerization plays a very important role in the enhancement of the desired metabolites. The reason for this is not clear at present.

It is likely that the oligosaccharides must bind a receptor which is probably located on the cell membrane in order to enhance the production of GOD and other secondary products. The fact that only oligosaccharides of a specific size range exert this effect suggests that the receptor has a well-defined shape, akin to mammalian receptors, for instance, for mannose-6-phosphate. The intracellular consequences of binding to the receptor are initiated by the synthesis of cyclic AMP and the modulation of the activities of enzymes and control proteins, resulting in the activation of other enzymes probably activated allosterically by cAMP. Further studies are needed to elucidate the reasons for the change in the metabolism of the fungi.

In conclusion and in agreement with the suggestion made in our previous paper,[6] it seems that alginate and its oligosaccharides function as activators of defensive systems in *P. variabile*. In fact, GOD activity can play, in combination with other mechanisms of biocontrol, a significant role in antiobiosis in the soil environment:[27,28] the hydrogen peroxide enzymatically produced is cytotoxic for living organisms.[29] The growth of many bacterial and fungal species is thus inhibited in the

presence of GOD[30,31] which, e.g., killed microsclerotia of *Verticillium dahliae* both *in vitro* and in sterile soil.[28] The probable presence of alginate of microbial origin in carbohydrate-rich areas[32] might represent a biological signal to enhance GOD fungal production, thus increasing the competitiveness of such fungi.

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