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Effect of mannan and alginate oligosaccharides on production in bioreactors of penicillin G and its biosynthetic intermediates

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Abstract - The addition of alginate and mannan oligosaccharides to *Penicillium chrysogenum* was examined to determine their effects on the production of penicillin G and biosynthetic intermediates. Addition of oligosaccharides to cultures changed the accumulation pattern of the secreted intermediates of penicillin biosynthesis, δ-(L-α-aminoadipyl)-L-cysteinyl-α-D-valine (ACV) and isopenicillin N (IPN) when compared to the control cultures. The culture age at which the oligosaccharides affect penicillin G biosynthesis was determined. After addition of mannan oligosaccharides at 24 hours, the maximum increase in specific productivity of penicillin G compared to the control culture was 130% measured between 96 and 120 hours.

Key words: biosynthesis, elicitation, fermentation, penicillin G production, intermediates, *Penicillium chrysogenum*.

INTRODUCTION

The production of secondary products by plant cell cultures may be increased by the addition of elicitors or enhancers of various kinds among which oligosaccharides are prominent (Walker-Simmons *et al*., 1983; Darvill *et al*., 1992; Fry *et al*., 1993). Recently, similar phenomena have been demonstrated in fungal cultures producing commercially used secondary metabolites (Ariyo *et al*., 1997; Petruccioli *et al*., 1999).

Trace amounts (~100 mg l^{-1}) of oligosaccharides can cause increased yields of the desired products in some fungal cultures. The enhancement in productivity could be due to an increase in product accumulation during a very short period of exposure of the culture to the oligosaccharides, or due to longer and sustained induction time of the desired product by the oligosaccharides as demonstrated in plant systems (Eilert *et al*., 1984).

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We have studied the effect of oligosaccharides on production of penicillin by the filamentous fungus *Penicillium chrysogenum*. The yield of penicillin G increases after addition of oligosaccharides to the cultures in shake flasks (Ariyo *et al*., 1997). In this work, the effect of alginate and mannan oligosaccharides was investigated by comparing penicillin G production together with the accumulation profiles of its intermediates, δ-(L-α-aminoadipyl)-L-cysteinyl-α-D-valine (ACV) and isopenicillin N (IPN) in the fermenter cultures. The intermediates, ACV and IPN, were found to have relatively low levels in the cultures supplemented with the mannan oligosaccharides which produced the greatest enhancement of penicillin G synthesis.

MATERIALS AND METHODS

Strains and culture medium. *Penicillium chrysogenum* strain P2 was maintained on solid agar slopes of glycerol and molasses medium (Ariyo *et al*., 1997). Semi-defined media were used for growth and penicillin production. The growth medium contained g l^{-1}: sucrose, 20; lactose, 10; mycological peptone, 5; $(NH_4)_2SO_4$, 13; KH_2PO_4, 3; $Na2SO_4$, 0.5; EDTA, 0.55; $MgSO_4.7H_2O$, 0.25; $CaCl_2.2H_2O$, 0.05; $FeSO_4.7H_2O$, 0.25; Mn $SO_4.4H_2O$, 0.02; $ZnSO_4.7H_2O$, 0.02; and $CuSO_4.5H_2O$, 0.005. The pH of the growth medium was adjusted to 6.8 with KOH before sterilization. The semi-defined production medium was as above but contained 100 g of lactose and 1 g of mycological peptone per litre. For penicillin G production phenyl acetic acid (PAA) was added to 24 hour old cultures to make a final concentration of 1.5 g l^{-1}. PAA level was kept between 0.5 and 1.5 g l^{-1} in the fermenter cultures by intermittent addition of a concentrated solution when necessary.

Inoculum preparation. Spore suspensions of *Penicillium chrysogenum* P2 were inoculated into 2 l flasks containing 20 % vol/vol sterile semi-defined growth medium to give a final concentration of 5×10^5 spores ml^{-1}. The flasks were then incubated at 26 ^oC in an orbital shaker at 200 rpm for 48 hours to prepare inoculum for fermentation in stirred tank reactors (STRs).

Fermentation. Fermentations were carried out in 5 l STRs (Inceltech, Reading, UK) with 4 l working volume. The reactors were baffled and culture was agitated by two Rushton turbine impellers located 12 cm apart on the drive shaft. Air was sparged into the culture at a flow-rate of 0.5 volume of air per volume of culture per minute and the stirrer speed was set at 600 rpm. The pH of the culture was maintained between 6.7-6.9 by the controlled addition of 2M aqueous ammonia and 2M sulphuric acid as required and the temperature was kept at 26 ^oC. Foaming was controlled by addition of 1 ml of antifoam (Henkel-Nopco Foamaster TDB-1) per litre of the medium before sterilization. *In situ* sterilization of the medium was carried out at 121 ^oC for 15 minutes.

The medium in the STR was inoculated aseptically with a 48 hour old inoculum culture of *Penicillium chrysogenum* P2 (10% vol/vol). Samples were removed at regular intervals and filtered for dry cell weight analysis. Filtrates were kept at 0 ^oC for later analyses.

Addition of alginate and mannan oligosaccharides. Oligomannuronate (OM) and oligoguluronate (OG) blocks with an average degree of polymerization (DP) of 10 and mannan oligosaccharides (MO) with an average DP of 7 were prepared as described previously (Ariyo *et al*., 1997). The hydrolysis products were analyzed by thin-layer chromatography and the mannan oligosaccharides were separated by gel-filtration chromatography. *Penicillium chrysogenum* cultures were supplemented with 25 mg oligosaccharide ml^{-1} at 48 hours of growth in semi-defined production medium.

Analysis of samples for growth, penicillin G, ACV and IPN. Dry cell weight (DCW) was determined by collecting 20 ml samples which were filtered through reweighed Whatman No. 1 filter paper, washed thoroughly with water and then dried at 80 ^{o}C to a constant weight. Penicillin G, the precursor tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) and isopenicillin N (IPN) concentrations in each culture were determined by HPLC (Adlard *et al*., 1991). The equipment used was a Perkin Elmer HPLC system consisting of a series of 4 pumps and a programmer and an LC1-100 integrator. The mobile phase contained acetonitrile and 0.03 M KH_2PO_4 buffer pH 5.5. The method involved the use of a gradient elution profile. Two replicates were used in all experiments which were repeated at least twice. The assay results are averages with less than 10% deviation from each test experiment.

RESULTS AND DISCUSSION

The addition of oligosaccharides to submerged cultures of *Penicillium chrysogenum* at very low concentrations had varying effects on the biosynthesis of penicillin G. Differences were found also in the concentration of penicillin intermediates.

The profiles of penicillin G and its intermediates, ACV and IPN are shown for each of the control and oligoguluronate (OG), oligomannuronate (OM) and mannan oligosaccharides (MO) supplemented cultures in Figures 1-4, respectively. Fermentation profiles of biomass concentration and dissolved oxygen tension (% DOT air saturation) are not included; they were found to be similar in all of the cultures whether or not the oligosaccharides were added.

The average specific penicillin G production rates (q_{pen} = [dP/dt] * [1/X], where dP = difference in product concentration taken at two points, dt = time difference taken at the two points, X = average of biomass concentration in dry cell weight relevant to the time points considered) during the control and the oligosaccharides supplemented fermentations are divided into different periods and are shown in Table 1. Compared with the control fermentations, the cultures with added oligosaccharides showed longer periods of penicillin production and also higher specific productivity of penicillin G.

In the control fermentations, the ACV level in the cultures increased steeply similar to penicillin G production within the 48-72 hours. At the later stages, the concentration increased with a slower rate with increasing penicillin G concentration (96-144 hours). In terms of specific productivity of penicillin G, the highest productivity was achieved within the 48-96 hours (Table 1). IPN concentration in the cultures increased almost linearly from the beginning of fermentation

TABLE 1 – Average specific penicillin G productivity (q_{pen}; mg g^{-1} h^{-1}) of the fermentation cultures

Fermentations	q_{pen} (mg g^{-1} h^{-1}) 48-96 h	Increase (%)	q_{pen} (mg g^{-1} h^{-1}) 96-120 h	Increase (%)	q_{pen} (mg g^{-1} h^{-1}) 120-144 h	Increase (%)	q_{pen} (mg g^{-1} h^{-1}) 144-192 h	Increase (%)
Control	1.15	–	0.95	–	0.80	–	~0	–
OM added	1.65	44	1.79	88	1.00	25	0.75	–
OG added	1.60	39	1.12	18	1.20	50	0.60	–
MO added	1.63	42	2.19	130	1.02	28	0.51	–

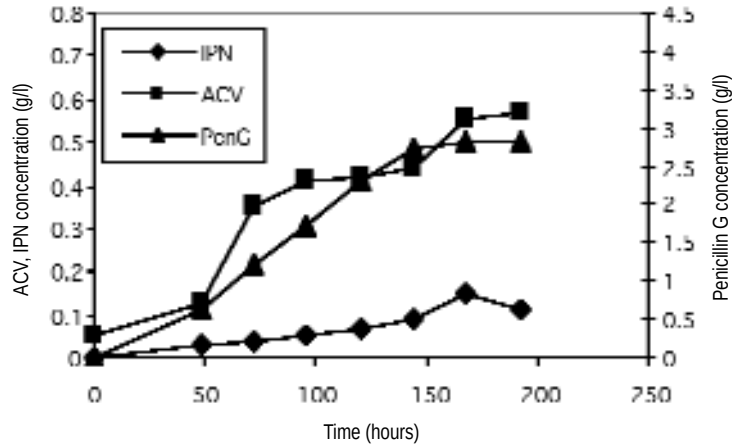

FIG. 1 – Penicillin G and its bio-intermediates production in control cultures.

to 144 hours. After 144 hours, a steeper increase was observed for both ACV and IPN accumulation leading to a slower rate of increase in ACV pattern and a decline in IPN. Penicillin G concentration stayed almost constant, although there was an increase in both ACV and IPN accumulation patterns after 144 hours. Control cultures contained the highest concentration of IPN at the end of the fermentations (Figure 1) compared to the OM, OG and MO block supplemented fermentations. Only in the control cultures, the production rate of penicillin G declined to zero between 144-192 hours.

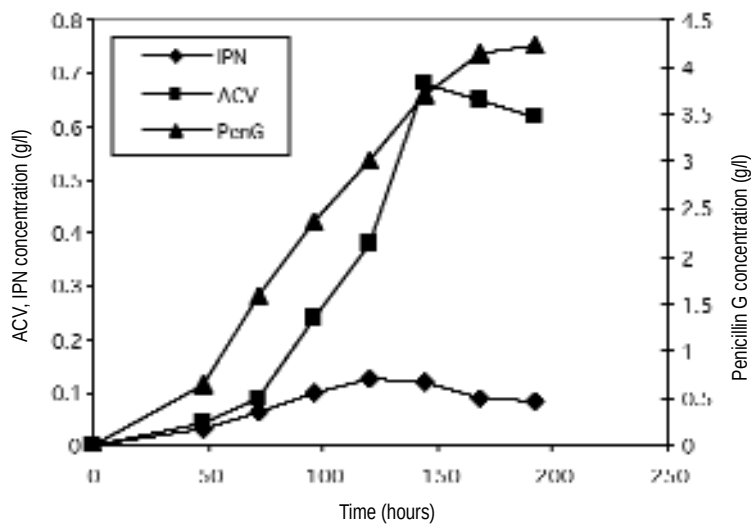

FIG. 2 – Effect of OG on penicillin and its bio-intermediates production.

In the fermentations with OG block addition (Figure 2), penicillin G, ACV and IPN concentrations steadily increased until 120 hours, then IPN level was found to be almost constant although there was still sharp increases in both penicillin G and ACV levels until 144 hours. Between 120-144 hours, the highest increase in the specific penicillin G productivity (50%) was achieved compared to the control culture (Table 1). After 144 hours, both ACV and IPN accumulation started to decrease until the end of the fermentations, although there was still a slight increase in penicillin G production.

In the fermentations with OM block addition (Figure 3), penicillin G, ACV and IPN levels increased until 144 hours, although there was a slower rate of increase in IPN level between 96-120 hours. Within this period, specific penicillin G productivity reached its highest value for this run at 1.79 mg g^{-1} h^{-1} and 88% increase was obtained in the productivity of penicillin G compared to the control (Table 1). Penicillin G concentration continued to increase in this fermentation until the end of the run although the concentration of both of the intermediates decreased.

In the fermentation with MO block addition (Figure 4), ACV concentration reached a maximum of only 0.2 g l^{-1} at 180 hours. This was the lowest ACV concentration achieved during all the control, OM and OG block added fermentations. The IPN concentration reached a similar value with ACV peaking at a concentration of 0.2 g l^{-1} on an earlier time than ACV, 120 hours, before decreasing to zero by the end of the fermentation. The specific penicillin G productivity achieved the peak value of 2.19 mg g^{-1} h^{-1} and the highest increase in the productivity was 130% compared to control cultures between 96-120 hours where both IPN and ACV concentration accumulated steadily in the cultures.

The biosynthetic pathway of penicillin is almost completely elucidated (Nuesch *et al*., 1987; Martin and Liras, 1989; Martin *et al*., 1995; Martin *et al*., 1999). The first enzymatic reaction involved in biosynthesis of all naturally occurring penicillins is the formation of the tripeptide L-aminoadipyl-L-cys-

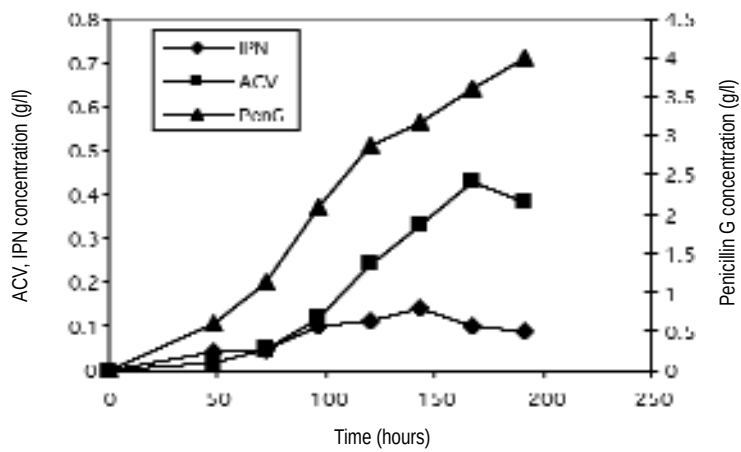

FIG. 3 – Effect of OM on penicillin and its bio-intermediates production.

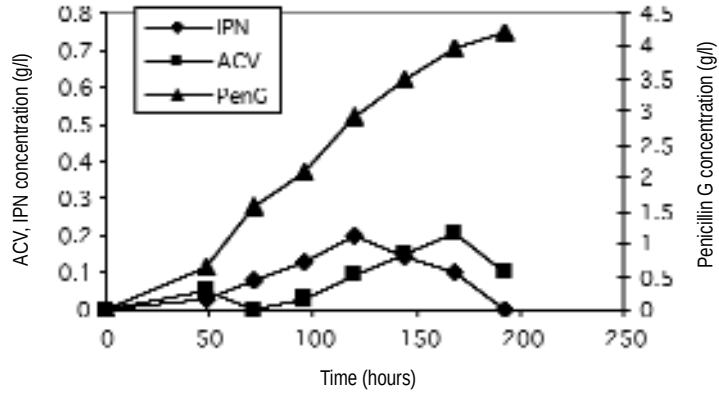

FIG. 4 – Effect of MO on penicillin and its bio-intermediates production.

teinyl-D-valine (ACV). ACV is formed in an ATP-dependent reaction by the sequential condensation of L-cysteine, L-valine and L-α-adipic acid. The next step in penicillin biosynthesis is the cyclization of ACV by isopenicillin N synthase (IPNS) to isopenicillin N (IPN). The differences we observed in the accumulation of ACV and IPN intermediates between the control and the oligosaccharide added cultures, lead us to speculate a mechanism of enhancement by which the oligosaccharides change the biosynthetic pathway through affecting ACV and IPN levels and their rate of production. However, the detailed mechanism(s) by which the oligosaccharides affect enzyme activity remain to be clearly identified. It can be assumed that the oligosaccharides, or breakdown products from them, interact with the enzymes ACVS and IPNS, with signals that control the activity of the enzymes or with systems that control the synthesis of the enzymes. The mechanism by which the oligosaccharides affect the activity of the enzymes will depend on the communication between the oligosaccharides and the fungal cells. In order for the oligosaccharides to control the activity of the enzymes directly they must enter the cells and the same is true if the oligosaccharides work by interacting with DNA or RNA. To act in a "hormone-like" manner (that is trigger a metabolic cascade) the oligosaccharides need not enter the cells but must be recognized by a binding site on the cell membrane: the enzyme activity would then be altered by phenomena activated by adenosine-3′, 5′-cyclic adenosine monophosphate (cAMP). In this regulatory system four basic steps mediate altered cell metabolism: (1) recognition of the external signal (hormone-receptor binding); (2) transduction of the signal (activation of adenyl cylase); (3) transmission of the signal (via cAMP and protein kinase); and (4) metabolic response of the cell (the presence of active key enzymes). The cAMP system has been reported to exist in animals (Rasmussen and Goodman, 1977) and higher plants (Dieter, 1984) but whether a comparable system occurs in filamentous fungi is not known. The molar concentration at which the oligosaccharides are effective can give indications as to which type of mechanism is most likely: at micro-molar concentrations or lower the interaction is likely to be at the mem-

brane surface or with nucleic acids; for direct interaction with enzymes a higher concentration may be necessary.

In this study, alginate oligosaccharides (OM and OG blocks; DP 10) were effective in *Penicillium chrysogenum* cultures at 25 μg ml^{-1} (14 mM) and mannan oligosaccharides of DP 7 were also effective at 25 μg ml^{-1} (20 mM). Given that the oligosaccharides were effective at μM concentrations, it could be suggested that the oligosaccharides may be interacting with the cells in a "hormone-like manner".

The enhanced overproduction of commercially important metabolites using small amounts of oligosaccharides is potentially valuable for biotechnological and pharmaceutical industries. The extension of the use of such compounds will help to further exploit their potential in the production of commercial products.

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