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Enhancement of chrysogenin production in cultures of *Penicillium chrysogenum* by uronic acid oligosaccharides

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Abstract

Additions of 50 to 100 μg of acid-hydrolysed alginate oligosaccharides ml^{-1} and enzyme-hydrolysed pectin oligosaccharides to 24- to 48-h cultures of *Penicillium chrysogenum*, ATCC 9480, led to enhanced production of chrysogenin by over 30 to 40% in shaken flasks and bioreactors. Some of the oligosaccharides also promoted biomass formation but were not used as a carbon source.

Introduction

Several polysaccharides and oligosaccharides, in small concentrations, have differing effects on both eukaryotic and prokaryotic organisms. One of these effects is the ability of oligosaccharides to induce or enhance the formation of metabolites in cell cultures. Oligosaccharides that have biological functions but are not used as carbon or energy source are termed oligosaccharins (Albersheim *et al.* 1983) and have been studied intensively in recent years.

Elicitation is a means to induce or enhance the production of secondary metabolites, especially from plant cell cultures. Oligosaccharides have been used to elicit phytoalexins (Eilert 1987) and pigments from plant cell cultures (Suvarnalatha *et al.* 1994). They have also been reported to enhance growth and differentiation in red and green macroalgae (Polne-fuller *et al.* 1993). Effects of some polysaccharides and oligosaccharides on animal cells have also been reported (Skjak-Braek & Espevik 1996). However, little has been published on oligosaccharide elicitation of secondary metabolites in fungal cultures. In our previous article (Ariyo *et al.* 1997), we reported the enhancement of benzylpenicillin production by some oligosaccharides in submerged fungal cultures. In this article, we report the enhancement of chrysogenin by alginate and pectin oligosaccharides in fungal cultures of *P. chrysogenum*.

Chrysogenin is a yellow pigment produced by strains of *Penicillium chrysogenum*. It is very soluble in acetone, ether, ethyl alcohol, ethyl acetate and several other organic solvents but not in light petroleum and water. It is strongly L-rotatory, having a specific rotation of $[\alpha]_{540}$ of $-762°$ and $[\alpha]_{579}$ of $-570°$ (Cluttterbuck *et al.* 1932). Chrysogenin has not been well characterised since its description in the early 1930s. However, the producer of the pigment, *P. chrysogenum* is a well-studied industrial fungus, thus it is a good model to study pigment enhancement by oligosaccharides in fungal cultures.

Materials and methods

Microorganism and growth

Penicillium chrysogenum, ATCC 9840 was grown on semi-defined *Penicillium* growth medium (GM) which contained (g l^{-1}): $(NH_4)_2SO_4$, 13 $CaCl_2$, 0.05; $CuSO_4$, 0.005; EDTA, 0.55; $FeSO_4$, 0.25, mgSO_4, 0.25; $MnSO_4$, 0.02; Na_2SO_4, 0.5; KH_2PO_4, 3; $ZnSO_4$, 0.02, lactose, 10; mycological peptone (Oxoid), 5; and sucrose, 20.

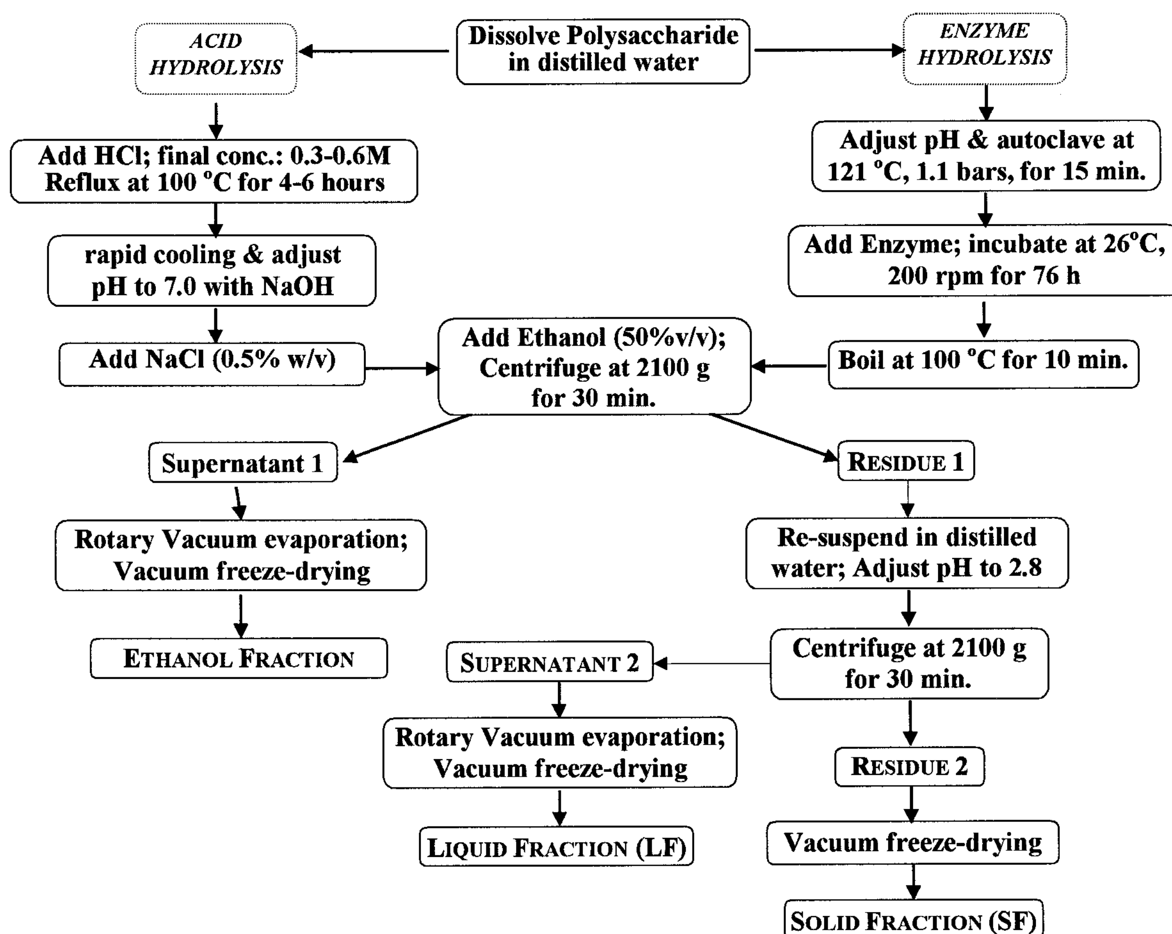

Fig. 1. Flow chart for production of oligosaccharides from sodium alginate and pectin polysaccharides.

Semi-defined production medium (PM) contained the same amount of salts as the growth medium with the addition of lactose, 100; mycological peptone (Oxoid), 1 and sucrose, 10 g l^{-1}. All the salts except $FeSO_4$, and the organic components were autoclaved together. KOH (2M) was used to adjust the pH of the growth medium to 6.5 and the production medium to 6.8. $FeSO_4$ was filter-sterilised and added to the media prior to inoculation.

Culture conditions

Inoculum growth

Inocula for shaken flask and fermentation cultures were grown in 100 ml lots of sterile GM in 500-ml flasks. Flasks were inoculated with a spore-suspension (10^6 spores ml^{-1} of medium). Incubation was at 26 °C with shaking at 200 rpm for 24 h.

Pigment production culture

Two-l bioreactors were sterilised while containing only the salts of semi-defined production medium. Separately sterilised carbon sources and filter-sterilised $FeSO_4$ were added aseptically to the bioreactors before inoculation with 15 ml inoculum. Two bioreactors with the same configuration were run in parallel with working volumes of 1.5 l. The pH was automatically maintained between 6.8 and 6.9 with 2 M NH_4OH and 2 M H_2SO_4. The dissolved oxygen tension (%DOT air saturation) was automatically controlled above 30% with stirrer speed ranging from 400 to 600 rpm. The concentration of chrysogenin and biomass were assayed daily for 6 days.

Preparation of oligosaccharides

Oligosaccharides were produced by partial acid or enzyme hydrolysis using the flow chart (Figure 1). Al-

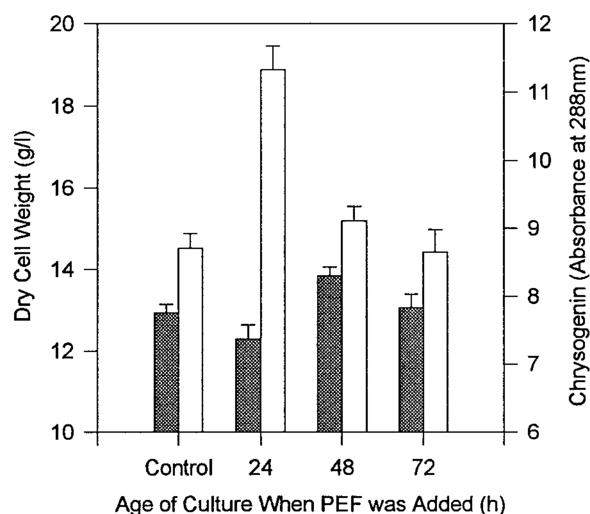

Fig. 2. Effects of PEF addition time on production of biomass and chrysogenin by ATCC 9480 in shaken flasks. A 24-h inoculum was used to inoculate the production flasks. Control flask contained no PEF. Chrysogenin and biomass were assayed after 140 h of growth in PM. □ Chrysogenin absorbance; ▩ Dry cell weight (g l^{-1}).

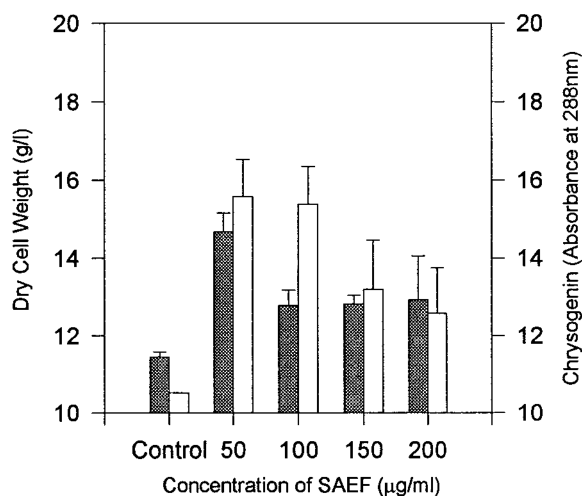

Fig. 3. Effects of SAEF on production of biomass and chrysogenin by ATCC 9480. SAEF was added after 72 h of growth in production shaken flasks. Chrysogenin and biomass were assayed after 140 h of growth in PM. □ Chrysogenin absorbance; ▩ Dry cell weight (g l^{-1}).

ginate oligosaccharides were prepared by a modified partial acid hydrolysis adapted from the method described by Haug *et al.* (1966). This modified method generated three fractions of oligosaccharides instead of two oligomers. Pectin oligosaccharides were produced by partial enzyme hydrolysis from pectin using

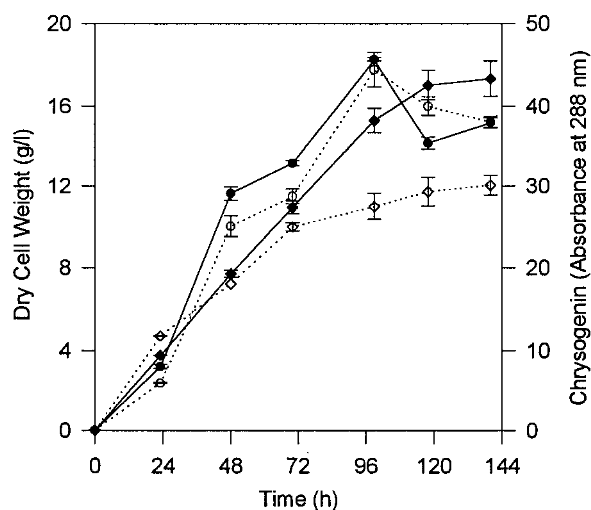

Fig. 4. Effect of PEF on biomass and pigment production by ATCC 9480 in bioreactor. PEF was added to bioreactor at the beginning of the fermentation. The inoculum was grown in a shaken flask using GM for 24 h before transfer to the bioreactors. ○ Control culture dry cell weight (g l^{-1}), ● PEF supplemented culture dry cell weight (g l^{-1}), ◇ Control culture chrysogenin absorbance, ◆ PEF supplemented chrysogenin absorbance.

the enzyme, pectinase (Pectinex 3×, Novo Industri A/S Copenhagen, Denmark).

Addition of oligosaccharides to cultures in PM

This is shown in Figure 1.

Oligosaccharides fractions

Three oligosaccharide fractions were produced from each of the two polysaccharides sodium alginate and pectin. The fractions derived from sodium alginate were designated as ethanol fraction of acid-hydrolysed sodium alginate (SAEF), soluble (water) fraction of acid-hydrolysed sodium alginate (oligomannuronate, OM) and insoluble fraction of acid-hydrolysed sodium alginate (oligoguluronate, OG). Three oligosaccharides were also derived from pectin by partial enzyme hydrolysis. They were designated as ethanol soluble fraction of enzyme-hydrolysed pectin (PEF), soluble (water) fraction of enzyme-hydrolysed pectin (PSF) and insoluble fraction of enzyme-hydrolysed pectin (PIF).

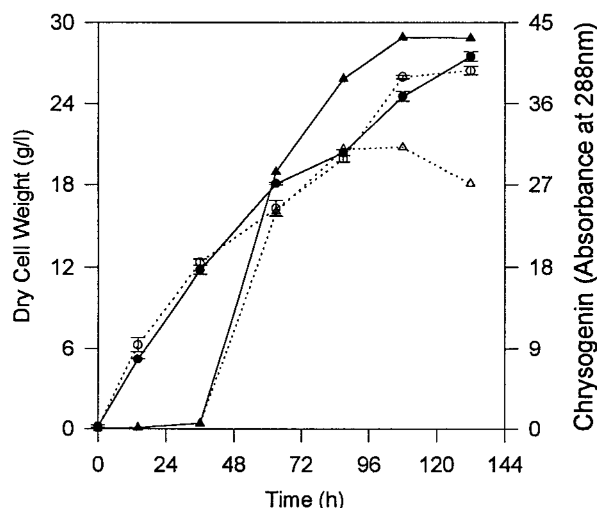

Fig. 5. Effect of OM on biomass and pigment production by ATCC 9480 in bioreactor. OM was added to bioreactor after 24 h of growth in the bioreactor. The inoculum was grown in a shaken flask using GM for 24 h before transfer to the bioreactors. ○ Control culture dry cell weight (g l^{-1}), ● OM supplemented culture dry cell weight (g l^{-1}), ◊ Control culture chrysogenin absorbance, ♦ OM supplemented chrysogenin absorbance.

Assays

Biomass

Cell dry weight (DCW) was determined gravimetrically passing 10 ml of broth samples through a pre-weighed filter paper (Whatman No. 1) and washing thoroughly with distilled water. The mycelia on the filter papers were weighed after drying to constant weight and the weight of the mycelia determined by difference. Biomass assays were carried out in duplicates.

Chrysogenin

Samples of filtered broth (1 ml) were added to 9 ml of ethanol and centrifuged at 2100 *g* to remove the precipitate. The supernatant was measured at 288 nm. Supernatants having absorbencies greater than 0.6 were diluted with ethanol before spectrophotometric measurement. Reference used for pigment assay was a reserved portion of the complete production medium removed from the bioreactors before inoculation. The reference was treated as the broth samples before use.

Results and discussion

Shake flask studies

Preliminary experiments showed that four oligosaccharides, SAEF and OM from sodium alginate, PEF, and PSF from pectin, showed best enhancement effects on chrysogenin production. Unhydrolysed polysaccharides did not have any appreciable enhancement effect on biomass and chrysogenin production by the strain at the concentrations and conditions studied. The best conditions at which these oligosaccharides had optimal enhancement of the metabolites in *P. chrysogenum* were studied. The conditions were the age of the culture at which the elicitors were added and the concentrations of the elicitors needed for optimal effect.

The best culture age at which SAEF was added to the *Penicillium* fermentation and the optimal concentration of the elicitor needed for best enhancement of chrysogenin were determined to be 72 h and 50 μg ml^{-1} respectively. OM at 100 μg ml^{-1} was added after 48 h (Ariyo *et al.* 1997) to the bioreactors. The oligosaccharide PEF, from pectin, had its greatest effect when added to 48-h cultures at 50 μg ml^{-1}. PSF had its optimal effect at concentration of 100 μg ml^{-1} added after 48 h. Compared on fraction-to-fraction basis, the oligosaccharides derived from pectin by partial acid hydrolysis had similar effects (on biomass, and pigment production) to those derived from pectin by enzyme hydrolysis. The results of pectic oligosaccharide, ethanol fraction of enzyme hydrolysed pectin (PEF) produced by enzyme hydrolysis and ethanol fraction of acid hydrolysed sodium alginate (SAEF) produced by acid hydrolysis are presented in Figures 2 and 3. In all shaken flask studies, production of chrysogenin and biomass were assayed after 140 h of growth in PM.

All results are presented as averages of duplicate samples. Repeat experiments confirmed reproducibility of the results.

PEF enhanced the production of chrysogenin in culture of ATCC 9480 when it was added at the beginning of the production culture (i.e., using 24-h old inoculum) to PM. Addition of the oligosaccharide to PM inoculated with spores at the beginning of the production culture did not enhance chrysogenin production. Later addition of PEF also affected on production of biomass by ATCC 9480.

Bioreactor studies

Optimal culture parameters determined from shaken flask experiments were used for bioreactor studies. Two oligosaccharides OM and PEF were selected for further studies. Results of the bioreactor fermentation of the two oligosaccharides are shown in Figures 4 and 5. Compared with the control cultures, the oligosaccharides enhanced the biosynthesis of the yellow pigment chrysogenin by *P. chrysogenum* (ATCC 9480). OM and PEF enhanced pigment concentration in the test culture by over 40% above control culture. The enhancement by OM was achieved only through increased production without concomitant increase in biomass (dry cell weight) by the end of fermentation (Figure 5). However, the oligosaccharide PEF enhanced biomass production between 24 to 96 h of the fermentation run, thus pigment production was enhanced via an initial increase in biomass.

Results of this study showed that the effect of the oligosaccharides was proportional to their concentration up to an optimal concentration. Further increase in the concentration of the elicitor had negative effect on biomass and/or secondary metabolite production. Similar results on effects of elicitor concentration on secondary metabolites have also been observed in plant cell cultures (Marero *et al.* 1997). In our study, optimal elicitor concentrations for enhancement of biomass and chrysogenin were not necessarily the same. Production of biomass was most affected within the concentration range of 50–100 μg ml^{-1} (SAEF). Above this range there was a deleterious effect on biomass and consequently chrysogenin production.

The importance of time of addition of elicitors has been studied in several plant cultures and it has been found that different cell systems require different times at which they express response to elicitors (Marero *et al.* 1997). In our study, the addition of the oligosaccharide to the culture at the beginning of fermentation (flasks were inoculated with spores) did not have detectable effect on secondary metabolism. The effects of the oligosaccharides were observed only when the cultures were 24 to 72 h old. The optimal time of addition of the oligosaccharides was mainly within the range of 24 to 48 h. Addition at 24 h enhanced more biomass production and pigment formation compared to later additions. Elicitors added outside a specific time 'window' do not have the desired effects and could have inhibitory effects on primary and/or secondary metabolism (Marero *et al.* 1997).

The mechanisms for elicitation by these oligosaccharides are unknown in fungi. However, in our experiments the enhanced rate of pigment production in elicitor-supplemented cultures was concomitant with faster utilisation of the carbon source and increase in carbon dioxide evolution rate compared with the control cultures. In the test culture supplemented with PEF, the rate of carbon utilisation was 2.95×10^{-2} g g^{-1} h^{-1} compared to 1.36×10^{-2} g g^{-1} h^{-1} in the control culture. The rate of pigment production was 1.22×10^{-2} absorbance units g^{-1} h^{-1} in control culture compared to 2.43×10^{-2} absorbance units. g^{-1} h^{-1} in elicitor supplemented culture (Figure 4) over a period of 117 h (23 to 137 h).

The mechanistic events leading to the enhancement of chrysogenin by the elicitors can only be speculated at this stage. A possible mechanism is that elicitors act via transduction pathways by binding to specific surface receptors and their effect transduced via other molecules which affect metabolic regulation of the pigment (Braun & Walker 1996).

Elicitation has been used to enhance production of pigment in plants (Suvarnalatha *et al.* 1994). The overproduction of commercially important metabolites using small amounts of elicitors has potential for biotechnological and pharmaceutical industries. The results of this study show that elicitors can be applied to improve production of secondary metabolites such as pigments in fungal processes to fully exploit their use in the production of commercial products. As legislation on replacement of synthetic additives by natural products in the formulation of food and pharmaceutical goods is becoming more stringent, potential application oligosaccharins as elicitors of natural pigments in microbial cultures for enhanced production of natural pigments could become a viable and attractive alternative.

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