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Optimisation studies on multiple elicitor addition in microbial systems: *P. chrysogenum* and *B. licheniformis*

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Addition of oligosaccharide elicitors results in a series of coordinated events in microbial cells leading to changes in morphological and/or physiological responses in the microbial cultures. To expand the potential of elicitation in microbial systems from laboratory to industrial scale it is essential to establish the potential generic nature of the elicitors' effects and provide concrete reasons for the changes observed. Mannan oligosaccharides derived from locust bean gum, oligomannuronate and oligoguluronate from sodium alginate were used in this study. The effect of multiple addition of the same elicitor was investigated with a prospect that repeated addition would re-trigger the stimulation resulting in either maintenance of the antibiotic at high levels or enhancement of the production rate. Repeated addition of the same elicitor did not show any significant increase in the antibiotic concentrations compared to single elicitor additions. However, optimisation studies based on multiple additions of different elicitor types at different time and concentration showed increases of 74% and 34% in the penicillin G and 108% and 61% in the bacitracin A production compared to the control and single elicitor-supplemented cultures, respectively.

Introduction

Improvement of strains and cultivation procedures to obtain high yields has been one of the main objectives in industrial research. Strain improvement has been achieved by repeated rounds of random mutation and subsequent selective screening (Díez *et al.*, 1996). This classical approach is very powerful but has limited mutagenic potential and lacks specificity. The world demand for antibiotic production is ever increasing and one of the ways to supply this demand is to look for novel approaches to increase antibiotic production. One of the possible methods by which secondary metabolite production can be increased is through elicitation. An elicitor can be defined as a substance which, when introduced in trace amounts to a living cell system as a non-nutrient additive, can trigger certain morphological and/or physiological changes in the system. This phenomenon is termed as elicitation. Elicitors are classified as physical or chemical, biotic or abiotic and complex or defined depending on their origin and molecular structure (Radman *et al.*, 2003).

From early studies carbohydrates have been implicated in the overproduction of secondary metabolites in plant cell cultures. Elicitation studies in microbial cultures have focused mainly on the use of carbohydrates as elicitors. Carbohydrates are the building blocks of many of the structural polymers that give form to living cells and organisms, and they play important roles in the interactions of cells with one another as well as with their environment (Albersheim *et al.*, 1992). The extensive stereochemistry, multiple hydroxyl groups, oxygen atoms and accessible hydrophobic regions characteristic of glycosyl residues make oligosaccharides ideal ligands for precise interactions with recognition sites. However, only few oligosaccharides and polysaccharides have been screened for their elicitation effects. A fast screening method for characterization of a range of carbohydrates is needed for use in microbial cultures as effective and efficient elicitors. The use of oligosaccharides and polysaccharides as elicitors to enhance production of plant metabolites has been extended recently to different microbial cultures for overproduction of commercially useful by-products such as pigments (Nair *et al.*, 2005), antimicrobials (Ariyo *et al.*, 1997; Murphy *et al.*, 2007) and enzymes (Petruciolli *et al.*, 1999). Most of the commercially important secondary metabolites are produced by microbial cultures. The increased production through elicitation of the secondary metabolites from microbial cultures has opened up a new area of research which might have important economical benefits for the bio-pharmaceutical industry. It is clearly of practical as well as theoretical interest to seek the evidence of enhancement of secondary metabolites in microbial cultures.

In this work we have optimised the addition of multiple elicitors and studied the subsequent effect of addition at different times and concentrations on the antibiotic production in *P. chrysogenum* and *B. licheniformis* cultures.

Materials And Method

Microorganisms

Penicillium chrysogenum ATCC 48271 from American Type Culture Collection, Rockville, Maryland, USA and *Bacillus licheniformis* NCIMB 8874 from Natural Collection of Industrial and Marine Bacteria, UK, were used in this study.

Elicitor preparation

Three oligosaccharide elicitors were prepared. Mannan oligosaccharides (MO) were prepared from locust bean gum by enzymatic hydrolysis (Ariyo *et al.*, 1998). Oligomannuronate (OM) and oligoguluronate (OG) was prepared from sodium alginate by partial acid hydrolysis (Ariyo *et al.*, 1997).

Addition of elicitors

Oligosaccharide elicitors were dissolved in distilled water at the required concentrations and sterilised at 115°C for 15 min. No elicitor was added to the control culture. Experimental setup for the optimisation of the multiple elicitor addition studies in *P. chrysogenum* and *B. licheniformis* are presented in Table 1 and 2.

Table 1. Experimental setup for the optimisation of multiple elicitor addition for the enhancement of penicillin G in the cultures of *P. chrysogenum*

Flask Set	Elicitor I	Concentration (mg L^{-1})	Addition time (h)	Elicitor II	Concentration (mg L^{-1})	Addition time (h)
1 (Control)	-	-	-	-	-	-
2	MO	150	48	-	-	-
3	MO	150	48	MO	75	72
4	MO	150	48	MO	75	96
5	MO	150	48	MO	150	72
6	MO	150	48	MO	150	96
7	MO	150	48	OM	75	72
8	MO	150	48	OM	75	96
9	MO	150	48	OM	150	72
10	MO	150	48	OM	150	96

Table 2. Experimental setup for the optimisation of multiple elicitor addition for the enhancement of bacitracin A in the cultures of *B. licheniformis*

Flask Set	Elicitor I	Concentration (mg L^{-1})	Addition time (h)	Elicitor II	Concentration (mg L^{-1})	Addition time (h)
1 (Control)	-	-	-	-	-	-
2	OG	200	24	-	-	-
3	MO	100	0	OG	200	24
4	MO	150	0	OG	150	24
5	MO	200	0	OG	100	24
6	MO	150	0	MO	150	24
7	OG	100	0	MO	200	24
8	OG	150	0	MO	150	24
9	OG	200	0	MO	100	24
10	OG	150	0	OG	150	24

Culture conditions

P. chrysogenum: Two culture media were used: *Penicillium* Growth Medium and Penicillin Production Medium (Nair *et al*., 2005). Both media were adjusted to pH 6.5 with 2 M KOH before sterilization. Calcium carbonate was added as a buffering agent to a final concentration of 10 g L^{-1}. The growth medium was inoculated with spores at a final concentration of 10^6 spores mL^{-1}. The flasks were maintained at 26°C in a rotary shaking incubator at 200 rpm with a 2 cm throw for 48 h. For penicillin G production, 10% of the seed culture was added to the production medium and maintained at the same conditions as for growth. Phenylacetic acid was added to 24 h old cultures as a precursor for penicillin G synthesis and maintained at 1-1.5 g L^{-1}. Experiments were carried out in triplicate.

B. licheniformis: Chemical defined media was used for growth and bacitracin A production by *B. licheniformis* as decribed by Murphy *et al*. (2007). Growth Medium was inoculated with 1 mL of spore suspension (10^7 spores mL^{-1}) and incubated at 37ºC on a rotary shaker at 200 rpm for 16 h. For bacitracin A production, 10% of seed culture was used to inoculate the production medium, fermentation conditions were kept as described for the growth. Experiments were carried out in triplicate.

Assay

Penicillin G assay: Penicillin G and phenylacetic acid concentration in the culture broth were analysed by a gradient HPLC method (Adlard *et al.,* 1991) using a Phenomenex C8 (5 µm) column where the flow rate was set to 1.0 mL min^{-1} and the data measured at 220 nm. Standard calibration curve was constructed using sodium salts of penicillin G as standard.

Bacitracin A assay: Bacitracin A production was quantified by a gradient HPLC method (Pavli and Kmetec, 2001) using a Kromasyl Phenomenex C8 (5µm) column maintained at 40ºC. The flow rate was set to 1.4 mL min^{-1} and measurements were obtained at 254 nm. Standard calibration curve was constructed using zinc bacitracin as standard.

Biomass assay: Biomass production by *P. chrysogenum* was measured as cell dry weight per litre of culture broth. For determination of biomass in *B. licheniformis* cultures, optical density at 650 nm was used. Biomass assay was carried out in triplicate.

Results And Discussion

In this study repeated addition of the same elicitor from a single source was designed along with addition of different oligosaccharide elicitor derived from another source. The aim of this study was to investigate if repeated contact of the cells with the same or different elicitor would show an increase in the concentration or sustain the antibiotic production, compared to single addition and control cultures. The elicitors chosen, for the studies with *P. chrysogenum* were MO and OM. OG was not selected because it chelates with the calcium carbonate in the medium forming precipitates/complexes. Besides, previous studies have demonstrated that, MO at 150 mg L^{-1} had the highest increase in penicillin G production followed by OM and then OG; the latter two coming from the same source (Radman *et al.,* 2003). Following the experimental setup as shown in Table 1, multiple addition of the same elicitor (MO) derived from locust bean gum failed to show any significant increase in the penicillin G production compared to single addition and control cultures ($p>0.05$) (Figure 1). However multiple addition of the elicitor (OM) derived from sodium alginate, along with MO as the first elicitor showed significant increase in the penicillin G concentration ($p<0.01$). Maximum penicillin G concentration achieved at 144 h (1.7 g L^{-1}) was observed with OM at 75 mg L^{-1} added at 96 h as shown in Figure 2. As shown in Table 3, the percentage increase in penicillin G production in single elicitor added culture was 34% at 120 h compared to the control. This increase in production decreased gradually as the fermentation progressed. Whereas in Flask 8, after multiple elicitor addition, the percentage increase in penicillin G concentration compared to control was 58% at 120 h and continued increasing for another 24 h.

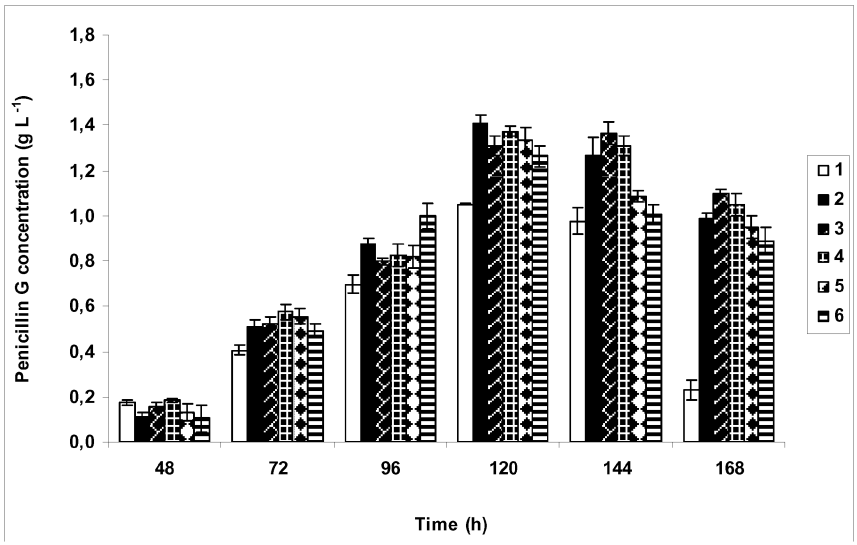

Figure 1. Effect of multiple elicitor addition (MO, MO) on penicillin G production. Flasks: 1 (no elicitors added), 2 (MO: 150 mg L^{-1}, 48 h), 3 (MO: 150 mg L^{-1}, 48 h; MO: 75 mg L^{-1}, 72 h), 4 (MO: 150 mg L^{-1}, 48 h; MO: 75 mg L^{-1}, 96 h), 5 (MO: 150 mg L^{-1}, 48 h; MO: 150 mg L^{-1}, 72 h), 6 (MO: 150 mg L^{-1}, 48 h; MO: 150 mg L^{-1}, 96 h).

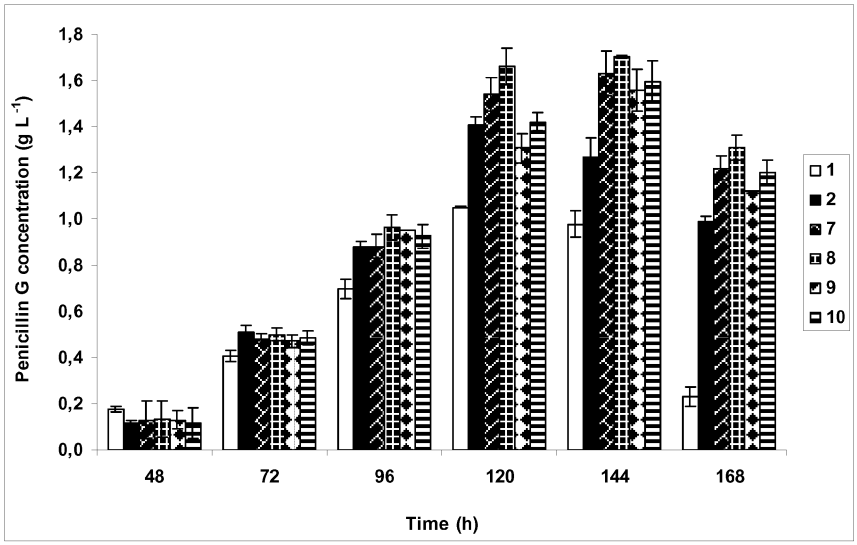

Figure 2. Effect of multiple elicitor addition (MO, OM) on penicillin G production. Flasks: 1 (no elicitors added), 2 (MO: 150 mg L^{-1}, 48 h), 7 (MO: 150 mg L^{-1}, 48 h; OM: 75 mg L^{-1}, 72 h), 8 (MO: 150 mg L^{-1}, 48 h; OM: 75 mg L^{-1}, 96 h), 5 (MO: 150 mg L^{-1}, 48 h; OM: 150 mg L^{-1}, 72 h), 6 (MO: 150 mg L^{-1}, 48 h; OM: 150 mg L^{-1}, 96 h).

In the case of bacitracin A, earlier optimisation studies investigating the best elicitation conditions for the enhancement of this antibiotic revealed that 24 h and 0 h were the addition times which resulted in the increase of bacitracin A production. It was also found that OG and MO were the most suitable elicitors under the conditions of this study (Murphy *et al*. 2007). For the reasons mentioned above, only 0 h, 24 h (addition time) and OG, MO elicitors were chosen as parameters for this study.

Maximum bacitracin A production was observed at 60 h for Flasks 1, 2, 3, 4, 5, 8 and 10 (Figure 3, Figure 4). Although multiple elicitor supplementation with the same elicitor (OG, Flask 6) increased the bacitracin A production after its addition, the maximum titre obtained at 60 h was not significantly different ($p>0.05$) compared to the control. In contrast, as observed in Figure 4, when elicitors OG followed by MO were added to the culture, the antibiotic production was not only significantly ($p<0.01$) enhanced reaching concentrations of 0.9 g L^{-1} and 0.8 g L^{-1} for Flask 7 and 8, respectively, but also the maximum bacitracin A concentration was reached 12 h earlier than in control cultures (Flask 1: 0.4 g L^{-1} at 52 h).

There was no significant difference ($p>0.05$) observed in the biomass between the control and elicited cultures in both microbial cultures tested.

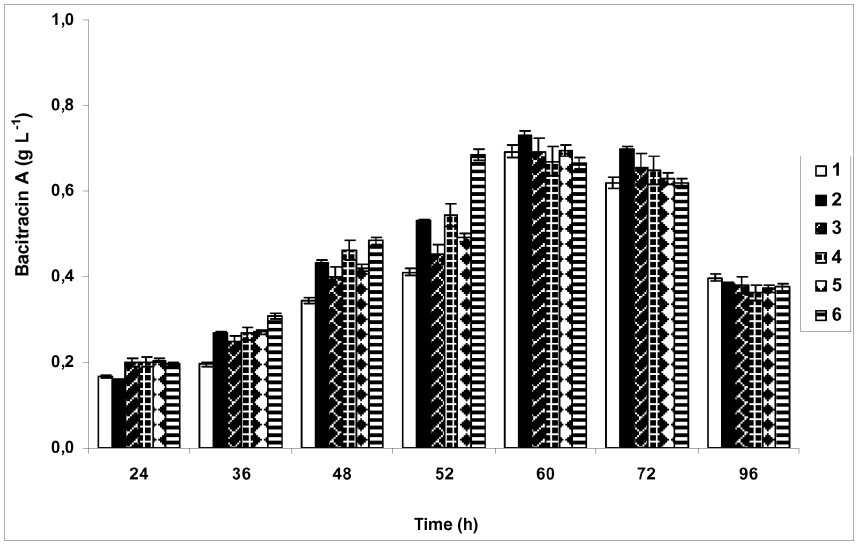

Figure 3. Effect of multiple elicitor addition (MO, OG) on bacitracin A production. Flasks: 1 (no elicitors added), 2 (OG: 200 mg L^{-1}, 24 h), 3 (MO: 100 mg L^{-1},0 h; OG: 200 mg L^{-1}, 24 h), 4 (MO: 150 mg L^{-1}, 0 h; OG: 150 mg L^{-1}, 24 h), 5 (MO: 200 mg L^{-1},0 h; OG: 100 mg L^{-1}, 24 h), 6 (OG: 100 mg L^{-1}, 0 h; MO: 200 mg L^{-1}, 24 h).

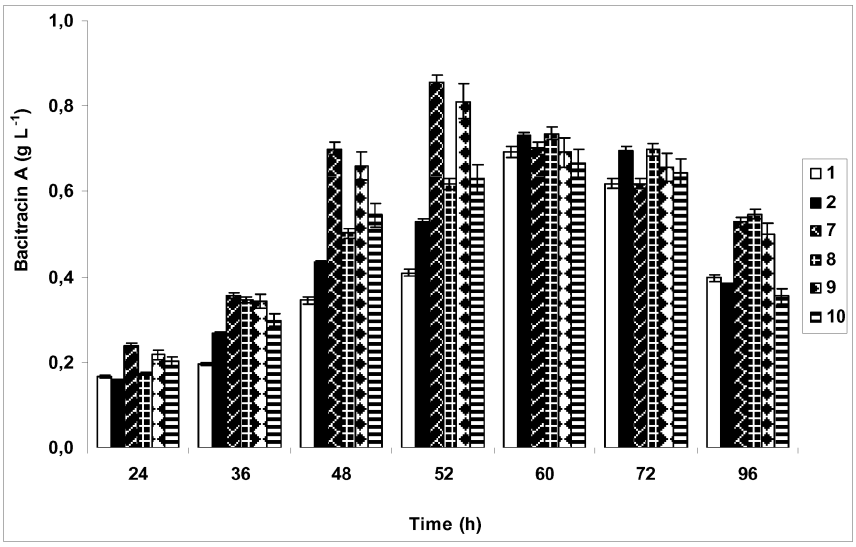

Figure 4. Effect of multiple elicitor addition (OG, MO) on bacitracin A production. Flasks: 1 (no elicitors added), 2 (OG: 200 mg L^{-1}, 24 h), 7 (OG: 100 mg L^{-1}, 0 h; MO: 200 mg L^{-1}, 24 h), 8 (OG: 150 mg L^{-1},0 h; MO: 150 mg L^{-1}, 24 h), 9 (OG: 200 mg L^{-1}, 0 h; MO: 100 mg L^{-1}, 24 h), 10 (OG: 150 mg L^{-1}, 0 h; OG: 150 mg L^{-1}, 24 h).

Antibiotic enhancement was achieved by both cultures (*P. chrysogenum* and *B. licheniformis*) upon addition of oligosaccharide-based elicitors. Increases on penicillin G and bacitracin A using the optimal single elicitor addition condition over the control were 33.9% and 29.5%, respectively. However, as shown in Table 3, higher enhancement of both studied antibiotics was achieved when two different elicitors were added at different time points.

Table 3. Percentage increases in antibiotic production during multiple elicitation addition fermentations.

	Percentage Increase (%)			
	Penicillin G		Bacitracin A	
Flask Set	120 h	144 h	52 h	60 h
2	33.9	29.9	29.5	5.6
7	--	--	108.7	1.2
8	58.3	74.2	--	--

These results relate with the hypothesis put forward for plant cells where cells respond to stimulation by elicitors, but behave refractory to second treatment with the same stimulus (Felix *et al.,* 1998). Investigation of this phenomenon in plant cell culture has established that repeated addition of elicitor causes desensitization of cells in a time and concentration-dependent manner.

This desensitization was not associated with increased inactivation of the stimulus or with the disappearance of high-affinity binding sites from the cell surface and thus appears to be caused by an intermediate step in signal transduction. In another experiment, cells did not respond when treated with a second dose of chitin fragments, although they still reacted to xylanase or ergosterol (Granado *et al.,* 1995). Reciprocally, when cells were treated with ergosterol, they were refractory to further stimulation with ergosterol but still responded to chitin fragments and xylanase (Granado *et al.,* 1995). These observations suggest a complex responsiveness of the system, thereby, indicating that the different chemoperception systems are desensitized in an independent manner.

In our study with microbial cells, it was observed that both cultures show similar behaviour to repeated and mixed elicitor addition. From the optimisation studies, MO (150 mg L^{-1}) added at 48 h followed by OM (75 mg L^{-1}) added at 96 h was the optimised condition for the enhancement of penicillin G production. While OG (100 mg L^{-1}) added at 0 h followed by MO (200 mg L^{-1}) added at 24 h was chosen for the enhancement of bacitracin A. The optimisation of multiple elicitor addition in microbial cultures leading to significant enhancement of a secondary metabolite production is reported for the first time. The work carried out so far suggests that application of elicitation can greatly contribute to future improvement in secondary metabolite production, which is crucial, given the economic benefits. This work can have an impact for larger scale production and the selection of this model system was used as a control for future application in different microbial systems.

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References

1. Adlard M.W., B.M. Gordon, T. Keshavarz, C. Bucke, M.D. Lilly, A.T. Bull and G. Holt, 1991, Biotechnol. Tech. 5, 121.
2. Albersheim, P., A. Darvill, C. Augur, J-J. Cheong, S. Eberhard, M.G. Hahn, V. Marfa, D. Mohnen, M.A. O'Neil, M.D. Spiro and W.S. York, 1992, *Acc. Chem. Res*. 25, 77.
3. Ariyo, T.B., C. Bucke and T. Keshavarz, 1997, Biotechnol. Bioeng. 53, 17.
4. Ariyo, T.B., C. Bucke and T. Keshavarz, 1998, FEMS Microbiol. Letters. 166, 165.
5. Díez, B., E. Mellado, M. Rodríguez, M. Fouces and J.L. Barredo, 1996, *Biotechnol. Bioeng.* 55, 216
6. Felix, G., M. Regenass and T. Boller, 1993, Plant J. 4, 307.
7. Felix, G., K. Baureithel and T. Boller, 1998, Plant Physiol. 117, 643.
8. Granado, J., G. Felix and T. Boller, 1995, Plant Physiol. 107, 486.
9. Murphy, T., R. Parra, R. Radman, I. Roy, A. Harrop, K. Dixon and T. Keshavarz, 2007, Enzyme Microb. Technol. 40, 1518.
10. Nair, R., R. Radman, I. Roy, C. Bucke and T. Keshavarz, 2005, AIDIC. 7, 239.
11. Pavli, V and V. Kmetec, 2001, J. Pharm. Biomed. Anal. 24, 977.
12. Petruccioli, M., F. Federici, C. Bucke and T. Keshavarz, 1999, Enzyme Microb. Technol. 24, 397.
13. Radman, R.,T. Saez, C. Bucke and T. Keshavarz, 2003, Biotechnol. Appl. Biochem. 37, 91.