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Novel application of oligosaccharides as elicitors for the enhancement of bacitracin A production in cultures of *Bacillus licheniformis*

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Abstract

This work reports, for the first time, oligosaccharides as elicitors enhancing production of bacitracin A in liquid cultures of *Bacillus licheniformis*. A 3^3 fractional factorial design was used to investigate optimal elicitation conditions. Mannan oligosaccharide moiety (MO), oligomannuronate (OM) and oligoguluronate (OG) were used as elicitors with concentrations of 100, 200 and 300 mg/L and addition times of 0, 12 and 24 h. There was enhancement of bacitracin A production with no significant change in biomass levels using the oligosaccharides as elicitors. Supplementation of *B. licheniformis* liquid cultures with mannan oligosaccharide moiety and oligomannuronate resulted in similar bacitracin A titres of up to 782 mg/L after 52 h. Response surface methodology, showed statistically significant effect on the cultures by the elicitor type ($P < 0.0001$) and concentration ($P < 0.005$). The final optimal condition for highest bacitracin A production was 100 mg/L of OG added to 24 h old cultures.

Overall, OG was found to be the most effective elicitor with bacitracin A yield significantly different compared to the control ($P < 0.5$). Yield improvements of 29%, 27% and 16% over the control were achieved with OG, MO and OM, respectively.

Keywords: *Bacillus licheniformis*; Oligosaccharides; Elicitors; Enhancement; Fractional factorial design; Bacitracin

1. Introduction

Bacillus licheniformis strains are widely distributed in the environment. The metabolic diversity of this organism has led to its exploitation in a variety of bio-industrial processes, including production of enzymes, antibiotics and fine chemicals [1,2]. Bacitracin produced by *B. licheniformis* and some strains of *Bacillus subtilis* is a polypeptide antibiotic [3,4] active against Gram-positive and some Gram-negative bacteria [3,5]. Bacitracin is also used as animal feed additive [6,7].

Bacitracin is composed of a mixture of similar analogues which differ by only one amino acid. Bacitracins A and B constitute 95% of the antimicrobial activity whereas bacitracin F, a nephrotoxic agent, does not show such activity and is an undesirable product of the fermentation [7].

Improvement in the yield of secondary metabolites of commercial importance is of prime significance to the bio-industry. Elicitation is the enhancement of secondary metabolites by addition of trace amounts of elicitors, which are molecules that trigger defence mechanisms in prokaryotic and eukaryotic systems resulting in metabolic changes [8,9]. Elicitation has been used as a technique for overproduction of bioactive compounds from plants for more than 30 years [10,11], but its use in enhanced production of antibiotics and other metabolites from fungi has been reported only in the last decade [8,12].

Elicitation studies in fungal cultures have focussed mainly on the use of oligosaccharides. In these cultures, the product yield enhancement varies depending on the degree of polymerisation, concentration and the time of addition of the elicitor [13,14]. Elicitors are added to the cultures in trace amounts (mg/L) and are assumed not to be used as carbon source [12,13]. However, the mechanism for elicitation in fungal cultures is still unknown. While the effect of carbohydrate elicitors on overproduction of metabolites in some fungal cultures has been reported [14,15], there are no publications on overproduction of secondary metabolites by these elicitors in bacterial cultures.

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This study investigates, for the first time, the effect of three different oligosaccharides as elicitors, their concentration as well as the time of their addition for the enhancement of bacitracin A produced by *B. licheniformis*. The optimal elicitation conditions were explored by response surface methodology to attain higher bacitracin A yields for its production at an industrial scale under the specified growth conditions.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were purchased from Sigma Chemical Co. (Poole, Dorset, UK), unless stated otherwise.

For quantitative and qualitative assays, analytical grade reagents were used. Reagents used for high performance liquid chromatography were HPLC grade.

2.2. Bacterial strain and bacitracin A production in M20 chemical defined medium

B. licheniformis NCIMB 8874 was obtained from Natural Collection of Industrial and Marine Bacteria, USA. *B. licheniformis* stock cultures (10^7 spores/mL) were kept in 20% glycerol at 80 °C in cryogenic tubes. One millilitre of thawed spore suspension was used to inoculate 100 mL sterile medium (M20) containing glutamic acid 20 g/L, citric acid 1.0 g/L, $NaH_2PO_4{\cdot}2H_2O$ 20.0 g/L, Na_2SO_4 0.5 g/L, $MgCl_2{\cdot}6H_2O$ 0.02 g/L, KCl 0.5 g/L, $CaCl_2{\cdot}2H_2O$ 0.01 g/L, $MnSO_4{\cdot}H_2O$ 0.01 g/L and $FeSO_4{\cdot}7H_2O$ 0.01 g/L. The pH of the medium was adjusted to 6.0 with 4 M NaOH prior to sterilisation. The culture was incubated at 37 °C in a rotary shaker (2 cm throw) at 200 rpm for 16 h. Ten millilitre lots of the growing culture was transferred into 500 mL shaken flasks (SF) containing 90 mL of M20 medium. Incubation was carried out at 37 °C on a rotary shaker at 200 rpm for 96 h.

2.3. Oligosaccharides preparation and addition of elicitors

Oligoguluronate (OG) and oligomannuronate (OM) were prepared by partial acid hydrolysis of sodium alginate as described by Asilonu et al. [9].

Mannan oligosaccharides (MO) were prepared by enzymatic hydrolysis of locust bean gum using gamanase enzyme mixture (Novozyme Ltd., Denmark) as described by Ariyo et al. [16].

For elicitation studies, sterile aliquots of MO, OG, and OM elicitors were added to each respective shaken flask at different times of addition: 0, 12, and 24 h to make final concentrations of 100, 200, and 300 mg/L in each flask. Control cultures without elicitor supplementation were used for comparison. A matrix of shaken flask experiments was devised using mathematical modelling generated by a full factorial 3^3 design.

Table 1
Experimental design factors and coded levels

Factor	Level (−1)	Level (0)	Level (+1)
Elicitor	MO	OM	OG
Concentration (mg/L)	100	200	300
Addition time (h)	0	12	24

2.4. Cell growth and pH

Growth of *B. licheniformis* cultures was monitored by measuring their optical density at 650 nm. The pH was also monitored throughout the course of fermentation.

2.5. Quantitative analysis of bacitracin A

Bacitracin A production was quantified using a high performance liquid chromatography (HPLC)-based method [17]. The HPLC system for analysis of bacitracin A concentration was composed of Dionex GS50 gradient pump, Dionex autoselect AS50 autosampler, Dionex PDA 100 photodiode array detector and Dionex STH 585 column thermostat.

The gradient elution system consisted of a Kromasyl reverse phase column C8 (5μ), (150 mm × 4.6 mm i.d.) (Phenomenex) maintained at 40 °C, where the flow-rate of the two mobile phases was set to 1.4 mL/min and the injection volume was 50 μL.

Bacitracin A was detected using UV light at 254 nm with an analysis time for each sample of 20 min. A calibration curve in the range of 0–1000 μg/mL was constructed using zinc bacitracin as standard. Fermentation samples beginning with the 24 h sample were analysed for the entire duration of the fermentation.

2.6. Fractional factorial experiment on the effect of type, concentration and addition time of elicitors on bacitracin A production

Previous elicitation studies on fungal systems have shown that the type of elicitor, the addition time and the concentration of the elicitor are key factors for the enhancement of secondary metabolites, hence these three main variables were tested for the optimization of elicitation conditions to enhance the most potent bacitracin A production using surface response methodology.

Optimal conditions (elicitor type, concentration of elicitor and the time of addition of elicitors to the cultures) for elicitation experiments were determined using response surface methodology based on a 3^3 fractional factorial design. The experimental conditions and set-up of the design are shown in Tables 1 and 2.

Experiments were carried out in triplicates. Based on response surface methodology the bacitracin A production can be described as a function of the test variable and the bacitracin A concentration was fitted to a second-order

Table 2
Experimental design (3^3 fractional factorial design)

Run	Coded			Uncoded		
	Elicitor	Concentration	Addition time	Elicitor	Concentration (mg/L)	Addition time (h)
T1	−1	−1	−1	MO	100	0
T2	−1	0	1	MO	200	24
T3	−1	1	0	MO	300	12
T4	0	−1	1	OM	100	24
T5	0	0	0	OM	200	12
T6	0	1	−1	OM	300	0
T7	1	−1	0	OG	100	12
T8	1	0	−1	OG	200	0
T9	1	1	1	OG	300	24

T (number) = number of treatment condition.