

Novel Strategies for Overproduction of Microbial Products

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Biotechnology and pharmaceutical industries are in continual quest for the discovery of novel products and the enhancement of productivity of value products to retain their global competitiveness. Traditional methods employed to achieve these goals include microbial strain selection, culture improvement, media development, and, bioreactor and process design. These methods, however, suffer from severe drawbacks such as the long time required for successful outcomes, high expenses and, in many cases, low success rate.

Over the last fifteen years we have introduced two novel strategies for overproduction of industrially desirable microbial products. These strategies are based on microbial response to the microbes in their vicinity (quorum sensing) and, to their surrounding environment (elicitation).

In all these cases, enhancement of productivity was established with several fold increases: in the elicitation work, addition of oligosaccharide (oligomannuronate and oligoguluronate blocks) resulted in a 50% increase in penicillin G yield for *Penicillium chrysogenum*. While *Bacillus licheniformis* cultures supplemented with oligoguluronate resulted in over 19% and 11% increase in bacitracin A yield in the shaken flasks and bioreactors respectively. In quorum sensing studies addition of spent medium from *Penicillium sclerotiorum* IMI 104602 (strain M) - a high producer of multicolic acid- to *P. sclerotiorum* IMI 040574 strain S) - a low producer of multicolic acid- led to a 6.4-fold increase in sclerotiorin yield.

We suggest that exploitation of microbes' communications to other microbes (self-community) and to the environment may provide new industrial opportunities for improved productivity of microbial biomolecules.

1. Introduction

Environmental abiotic and biotic stress factors have been proved to effect variety of responses in microbes. Elicitors, as stress factors, induce or enhance the biosynthesis of secondary metabolites added to a biological system. They are classified into various groups based on their nature and origin: physical or chemical, biotic or abiotic (Radman, et al., 2003). Initial studies on elicitation of secondary metabolites were carried out on plant cells (e.g. jasmonic acid supplemented *Catharanthus roseus* cultures had an increase in the specific yields of serpentine, ajmalicine, tabersonine, and lochnericine; Shanks and Bhadra, 1998) and extended, over the years, to bacteria, animal cell cultures and filamentous fungi. Abiotic stress (abiotic elicitors) imposed by

pH improves pigment production by *Monascus purpureus* (Orozco and Kilikian, 2008) and antibiotic production by *Streptomyces spp.* (Kim, et al., 2000). Traditionally carbohydrates have been used as carbon sources in fermentation processes. They have also been used widely in small amounts (mg L^{-1}) as elicitor molecules in bacterial and fungal fermentations for overproduction of commercially important secondary metabolites.

In one approach to improve production, we investigated the effect of carbohydrate biotic elicitors (oligosaccharides, oligomannuronate, oligoguluronate and mannan oligosaccharides) on variety of fungal systems: *Penicillium spp.* (Ariyo, et al., 1997), *Ganoderma spp.* (Ghorashi, 2004), *Corylopsis spp.* (Vanhulle, et al., 2007) and bacterial cultures: *Streptomyces spp.* (Sangworachat, 2006), *Bacillus spp.* (Murphy, et al., 2007a) for production of antibiotics, enzymes, pigments and changes in morphology.

In another approach we used quorum sensing phenomenon. Quorum sensing is the inter-cell communication between cells through the release of chemical signals when cell density reaches a threshold concentration (critical mass). Under these conditions, they sense the presence of other microbes; change their own genetic expression to find advantage over their competitors for survival. This process, investigated for more than 30 years, was first discovered in Gram-negative bacteria, and then in Gram-positive bacteria and dimorphic fungi. The quorum sensing signals differ in different microbial systems; examples are acyl-homoserine lactones, modified or unmodified peptides, complex γ -butyrolactone molecules and their derivatives. A number of physiological activities of microbes (e.g. symbiosis, competence, conjugation, sporulation, biofilm formation, virulence, motility and the production of various secondary metabolites) is regulated through the quorum-sensing.

While research into the quorum sensing process has been continuing with an impressive pace, the activity is limited to research at bench scale mainly in biomedical areas. However, as the range of quorum sensing-affected physiological activities show, there is great potential for the use of this communication process for industrial exploitation.

Filamentous fungi are a main microbial source for production of pharmaceutical and biotechnological products. However, until recently, very little was reported in the literature regarding quorum sensing phenomena in these fungi. We explored, for the first time, the possibility of overproduction of fungal metabolites (Raina et al., 2010) in response to the supplementation of liquid cultures by variety of quorum sensing molecules.

Bacillus licheniformis is widely present in the environment. Its metabolic diversity has resulted in its use for production of enzymes, antibiotics and fine chemicals. Bacitracin produced by *B. licheniformis* is a polypeptide antibiotic active against Gram positive and some Gram-negative bacteria. Bacitracin is also used as animal feed additive.

Sclerotiorin synthesized by *Penicillium sclerotiorum* is a phospholipase A2 inhibitor and has been classified as an octaketide. Sclerotiorin has also been studied for its cholesterol ester transfer protein (CETP) inhibitory activity and recently extracts from *Penicillium sclerotiorum* have been studied for their activity against methicillin resistant *Staphylococcus aureus* (MRSA).

In this paper, we report overproduction of antibiotics using the two methods, elicitation and quorum sensing. We describe enhancement in the concentration of bacitracin in

response to supplementation of *Bacillus licheniformis* cultures with oligomanuronate and overproduction of the fungal antibiotic sclerotiorin in cultures of *Penicillium sclerotiorum* (strain S) through the addition of spent medium from a culture of *Penicillium sclerotiorum* (strain M) (quorum sensing-based procedure).

2. Materials and Methods

2.1 Microbial strains

Bacillus licheniformis NCIMB 8874 was obtained from Natural Collection of Industrial and Marine Bacteria, USA. This strain was used for bacitracin elicitation studies.

Penicillium sclerotiorum IMI 104602 (Strain M) and IMI 040574 (Strain S) were obtained from CABI Biosciences UK Centre, Surrey, United Kingdom. These strains were used for quorum sensing studies.

2.2 Chemicals and reagents

All chemicals used in this study were obtained from Sigma-Aldrich Company Limited, Dorset, United Kingdom unless stated otherwise. Analytical grade reagents were used for quantitative and qualitative assays and HPLC grade reagents were used for high performance liquid chromatography assays.

2.3 Media and growth conditions

B. licheniformis NCIMB 8874 was grown in a defined medium (no carbohydrates as carbon source). The medium contained (gL⁻¹): 20 glutamic acid; 1 citric acid; 20 NaH₂PO₄ · 2H₂O; 0.5 Na₂SO₄; 0.02 MgCl₂ · 6H₂O; 0.5 KCl, 0.01 CaCl₂ · 2H₂O; 0.01 MnSO₄ · H₂O, and 0.01 FeSO₄ · 7H₂O. Sodium hydroxide was used to adjust the medium pH to 6.0 before sterilisation. The inoculum as spore suspension (1x10⁷ spores mL⁻¹) was added to 100 mL the sterile defined medium and incubated at 37°C, 200 rpm for 16 h. Aliquots of the culture at exponential phase were transferred into shaken flasks (SF) or fermenters (5 L FerMac 360 Stirred tank reactor, STR). Shaken flasks (500 mL) contained 90 mL of the defined medium. Incubation was carried out at 37°C at 200 rpm for 96 h. Growth of *B. licheniformis* cultures was monitored by measuring their optical density at 650 nm. For STR experiments, the defined medium was inoculated with a 10% v/v⁻¹ of inoculum to make 4 L total. The temperature was 37°C and the stirrer speed was between 300-600 rpm to keep %DOT above 30% air saturation.

For production of sclerotiorin and study of the effect of the spent medium on overproduction of this compound, a 2.5 L Stirred Tank Reactor (2.5 L STR, FerMac360, Electrolab Ltd., UK) with 1.5 L of sterile potato dextrose broth was inoculated with spores (1x10⁷ mL⁻¹) of *P. sclerotiorum* Strain S. Two bioreactors were run simultaneously as control and test. The airflow rate and temperature were set at 1.0 vvm and 27°C respectively. The % DOT (air saturation), pH, and temperature were monitored throughout the fermentation. The stirrer speed was increased gradually from initial 120 rpm to 250 rpm during the course of fermentation. Samples were assayed every 24 h for sclerotiorin production, pH and carbohydrate utilization.

2.4 Preparation of supplements and product assay

Oligoguluronate elicitor (OG): OG was prepared by acid hydrolysis from sodium alginate as described by Ariyo et al. (1997).

Sterile aliquots of OG, were added to the test SFs and STRs at 24 h for a final concentrations of 100 mg L⁻¹. Control cultures without addition of OG were used for comparison.

Spent medium (containing multicolic acid): For preparation of the spent medium for use as a supplement for production of sclerotiorin by *P. sclerotiorum* Strain S, *P. sclerotiorum* Strain M was grown for 8 days in a medium containing (g L⁻¹): 30 sucrose; 2.6 ammonium tartrate; 0.4 NH₄H₂PO₄; 0.16 (NH₄)₂SO₄; 2.6 tartaric acid; 0.28 MgCO₃; 0.4 K₂CO₃; 0.07 ZnSO₄.7H₂O; 0.005 CuSO₄.5H₂O and 0.06 FeSO₄.7H₂O. The medium pH was adjusted to 3.5 before sterilisation. The sterile medium (100 mL) was inoculated with 1 mL of spore suspension (1x10⁷ spores per mL). The cultures were incubated at 26°C and 150 rpm (2 cm throw) for 9 days. The culture broth was then filtered through a Whatman filter paper (No.1). The cell free broth was subsequently filter sterilised using a 0.2 µm cellulose acetate membrane filter and added as the spent medium at 48 h to the test cultures of *P. sclerotiorum* Strain S (1.0 % v/v).

Bacitracin A assay: Bacitracin was quantified by gradient HPLC.

Sclerotiorin assay: Quantification of sclerotiorin was carried out using an HPLC method (Weng et al. 2004).

Total carbohydrate assay: Carbohydrate content of the culture broth was quantified using the phenol-sulphuric acid method (Chaplin 1994).

3. Results and Discussion

Bacitracin A overproduction: Based on our earlier optimization studies at SF scale (Murphy et al, 2007b), addition of OG at concentration of 100 mg L⁻¹ to the *B. licheniformis* cultures was carried out in the bioreactors after 24 h of culture growth. Control bioreactors runs were carried out under the same conditions as the test runs but without OG supplementation. The results of the SF and STR fermentations were compared. In all cases, the concentration (Figure 1) and the yield of bacitracin A was higher in the elicited cultures compared to the test. Moreover, the yield increase in the bioreactor runs was higher than the SF runs and the time to reach the maximum production was decreased (Table 1). This suggests that scale-up runs under controlled bioreactor conditions could further increase the productivity.

Table 1: Bacitracin A yield in the controlled and test conditions. Fermentations in shaken flasks (SF); Fermentations in bioreactors (STR).

	SF		STR	
	Control	Elicited	Control	Elicited
Time of max production (h)		48		42
pH at max	8.01	8.13	8.30	8.30
Bacitracin A Yield (mg g ⁻¹)	168.9	201.5	287.9	321.4
Yield % increase		19.3		11.6

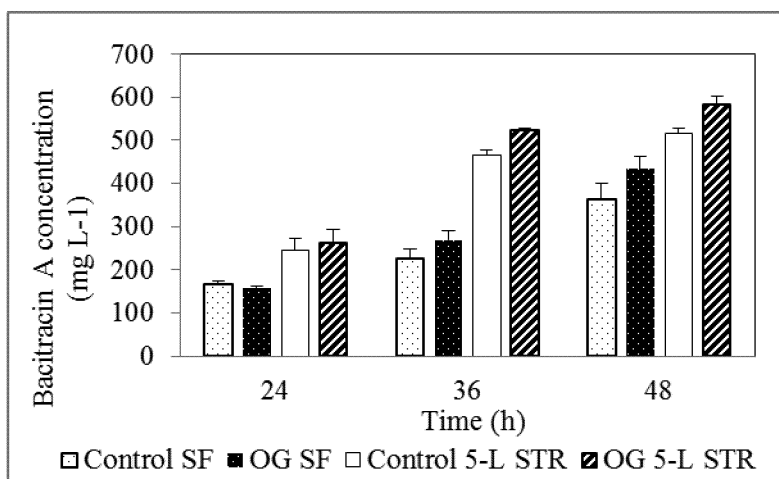


Figure 1: Bacitracin A concentration in the control and test cultures. Fermentations in shaken flasks (SF); Fermentations in bioreactors (STR).

Sclerotiorin overproduction Addition of spent medium containing γ -butyrolactone molecules from strain M to *P. sclerotiorum* IMI 040574 (Strain S) resulted in 6.4-fold increase in the yield of sclerotiorin at 168 h post-inoculation (Table 2). The difference between the biomass concentration of the test and control cultures was not significant ($p > 0.05$) (data not shown). The difference in carbohydrate consumption rate was also insignificant ($p > 0.05$). The results suggest that the spent medium from *P. sclerotiorum* (strain M) containing multicolic acid (and related derivatives) may be involved in quorum sensing process in the filamentous fungus *P. sclerotiorum*. The effects are similar to those of biotic elicitors as the concentration of multicolic acid and its derivatives in the spent medium used were small (data not shown) and the concentration and rate of total carbohydrate consumption between the control and test cultures were similar.

These results carry potential promise for use in pharmaceutical and biotechnology industry where microbial communication may be used for the overproduction of commercially desirable bioproducts.

Table 2: Sclerotiorin maximum production rate (120 – 168 h), maximum carbohydrate consumption rate and yield fold increase in the test and control cultures of *P. sclerotiorum* Strain S. The test cultures were supplemented with spent media from *P. sclerotiorum* Strain M in volumes of 1.0 %v/v at 48 h post inoculation.

	Sclerotiorin production rate ($\mu\text{g g}^{-1}\text{h}^{-1}$) (120 – 168 h)	Yield (fold increase)	Sclerotiorin consumption rate ($\mu\text{g g}^{-1}\text{h}^{-1}$)(0 – 72 h)
Control	38.8	--	0.140
Test	252.4	6.4	0.151

While the results of the two systems for elicitation and quorum sensing show notable increases in the productivity of the target products, our studies on other systems (data

not shown) suggest that there are no generic biotic elicitors or quorum sensing molecules to fit all microbial strains. The time of supplementation, the concentration and the chemical structure of the molecules are essential in reaching satisfactory results. Our investigation into the mechanism of these enhancers of productivity has revealed activity at molecular level covering gene expression and, recently, changes in intracellular calcium levels and protein phosphorylation.

The minimal requirements for the cultures when biotic elicitors and quorum sensing molecules are used and the high increases in the productivity of the desired products make these molecules suitable potential sources to be exploited as alternative methods for industrial-scale overproduction.

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