AIDIC *Conference Series*, Vol. 10, 2011
Editor Sauro Pierucci
DOI: 10.3303/ACOS1110013

ISBN 978-88-95608-58-7 ISSN 2036-5969

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 *Penicilliumsclerotiorum* 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 *Catharanthusroseus* 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 factors (abiotic elicitors) such as osmotic; heat and pH have been successfully applied for improvement of microbial metabolites (from pigments to antibiotics) by many researchers (Fiedurek, 1997; Nakata *et al*., 1999; Varela *et al*., 2004; Marova*et al*., 2004; Liang *et al*., 2009a). Red pigment production was reported to be improved by the alteration of pH in cultures of *Monascus purpureus* (Orozcoand Kilikian, 2008). Similar results in the same strain were described in solid state, with accumulation of pigments when exposed to osmotic stress. In *Streptomyces spp*. cultures, abiotic elicitors have been extensively applied for the encouragement of antibiotics biosynthesis. pH shock stress was reported to promote the antibiotic methylenomycin, kasugamycin and geldamycin production in cultures of *S.coelicolor, S. kasugaensis and S. hydroscopicus subsp. duamyceticus*, respectively (Hayes *et al*. 1997; Kim *et al*., 2000; Song *et al*., 2008).

Please cite this article as: De Vizio D., Valencia T., Reffatti P., Raina S. and Keshavarz T., (2011), Novel strategies for overproduction of microbial products, AIDIC Conference Series, 10, 111-118 DOI: 10.3303/ACOS1110013

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 *Penicillium spp.* cultures, the addition of biotic elicitors, oligoguluronate, oligomannuronate and mannan oligosaccharides, promoted an enhancement production of the antibiotic penicillin G (Ariyo, et al., 1998; Radman et al., 2004a) as well as the enzymatic biosynthesis of glucose oxidase (Petruccioloet *al*., (1999). Similar results involving an important oriental medicine, ganoderma extractives, was also described by the biotic elicitation research groups. Mannan oligosaccharides addition to *Ganoderma G991* cultures was reported to increase the productivity of the intracellular (IPS) and extracellular (EPS) polysaccharides and ganoderic acid (GA) (Ghorashi, 2004).

In bacterial system, the effects caused by biotic elicitors have been mainly reported in *Streptomyces spp*. and *Bacillus spp*. cultures (Sangworachat, 2006; Murphy *et al.*, 2007a). The presence of biotic elicitors in the *S. rimosus and S. coelicolor* cultures instigated an increase on the oxytetracycline and actinorhodin production (Sangworachat, 2006) whereas improvement caused by biotic elicitor in *B. licheniformis* was predominantly reported on the antibiotic production of bacitracin A (Murphy et al., 2007a).

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 and 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 in Gram-negative bacteria, modified or unmodified peptides in the majority of Gram-positive bacteria, complex γ -butyrolactone molecules and their derivatives in the genus Streptomyces. 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. Examples of these processes are the acyl homoserine lactones-induced productions of the antibiotic carbapenem in *Erwiniacarotovora*(Welch *et al.*, 2005) and the violet colour pigment in *Chromobacteriumviolaceum*(McClean *et al.*, 1997), the self-induced production of bacteriocins in *Lactobacillus acidophilus*(Barefoot *et al.*, 1994) and *Lactobacillus plantarum*(Diep *et al.*, 1995).

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 (Sorrentino et al., 2009; Raina et al., 2010) in response to the supplementation of liquid cultures by variety of quorum sensing molecules. In a previous work we reported oxylipins (linoleic acid-derived compounds) as signalling molecules in the ascomycete *Aspergillus terreus* where exogenous addition of linoleic acid induced the production of the secondary metabolite lovastatin. Transcriptional analysis revealed that linoleic acids are able to induce over-expression of the lovastatin biosynthetic genes (Sorrentino et al., 2009).

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 a previous work we reported oxylipins (linoleic acid-derived compounds) as signalling molecules in the ascomycete *Aspergillus terreus* where exogenous addition of linoleic acid induced the production of the secondary metabolite lovastatin. Transcriptional analysis revealed that linoleic acids are able to induce over-expression of the lovastatin biosynthetic genes (Sorrentino et al., 2009).
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 NaH_2PO_4 . $2H_2O$; 0.5 Na_2SO_4; 0.02 $MgCl_2$. $6H_2O$; 0.5 KCl, 0.01 $CaCl_2$. $2H_2O$; 0.01 $MnSO_4$. H_2O, and 0.01 $FeSO_4$. $7H_2O$. Sodium hydroxide was used to adjust the medium pH to 6.0 before sterilisation. The inoculum as spore suspension ($1x10^7$ 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% vv^{-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 ($1x10^7$ 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). Ten grams of sodium alginate were dissolved in 500 mL of distilled water by heating and

agitation. On complete dissolution 500 mL of warm 0.6 M hydrochloric acid was added. The solution was refluxed at 100°C for six hours, rapidly cooled down to room temperature and centrifuged at 4600 rpm (Sorvall legend RT, Thermo Scientific)for 30 min.

The supernatant was discarded and the precipitate was rinsed and re-suspended in 300 mL of distilled water. Sodium hydroxide (0.3 M) was added until all solid particles were dissolved. Sodium chloride was then added to make a final concentration of 0.5% (w/v). An equal volume of ethanol (99%) was added to the solution and allowed to stand overnight. The solution was centrifuged, the supernatant discarded and the precipitate was rinsed and re-suspended with 200 mL of distilled water. The pH was adjusted to 2.8 with 0.3 M of hydrochloric acid and the solution was centrifuged at 4600 rpm (Sorvall legend RT, Thermo Scientific)for 30 min. The precipitate (OG) was separated and freeze-dried. Products obtained were analysed by thin layer chromatography (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 (gL^{-1}): 30 sucrose; 2.6 ammonium tartrate; 0.4 $NH_4H_2PO_4$; 0.16 $(NH_4)_2SO_4$; 2.6 tartaric acid; 0.28 $MgCO_3$; 0.4 K_2CO_3; 0.07 $ZnSO_4.7H_2O$; 0.005 $CuSO_4.5H_2O$ and 0.06 $FeSO_4.7H_2O$. The medium pH was adjusted to 3.5 before sterilisation. The sterile medium (100 mL) was inoculated with 1 mL of spore suspension ($1x10^7$ spores per mL). The cultures were incubated at 26ºC 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).

Optical density and pH measurements:

The optical density (OD) of the *B. licheniformis* fermentation samples was read at 650 nm, where chemically defined medium was used as blank. Absorbance readings above 0.5 were diluted with sterile medium. The pH was also monitored.

Bacitracin A assay:

Bacitracin A production was quantified from fermentation broth samples using a High Performance Liquid Chromatography (HPLC) method (Pavli and Kmetek, 2001). The gradient elution system consisted of a C8 (5µ), 150 x 4.6 mm Kromasyl reverse phase column (Phenomenex) maintained at 40ºC, where the flow-rate of the two mobile phases was set to 1.4 mL min^{-1} and an injection volume of 20 µL.

Bacitracin A was detected under UV light at 254 nm. Zinc bacitracin (Sigma, UK) was used as a standard. Calibration curve was constructed using bacitracin concentration ranging from 0-1000 mg L^{-1}. The bacitracin A retention time under these conditions was approximately 11.31 min.

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

3.1 Bacitracin A overproduction

Based on our earlier optimization studies at SF scale (Murphy et al, 2007b), addition of OG at concentration of 100 mgL^{-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. Addition of elicitors to the cultures in SF and STR caused no significant difference (p>0.05) in pH and cell growth profiles between the elicited and the control cultures (data not shown). In all cases, the concentration (Figure 1) and the yield of bacitracin A was higher in the elicited cultures compared to the control. Moreover, the bacitracin A yield 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 (OG supplementation) 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	

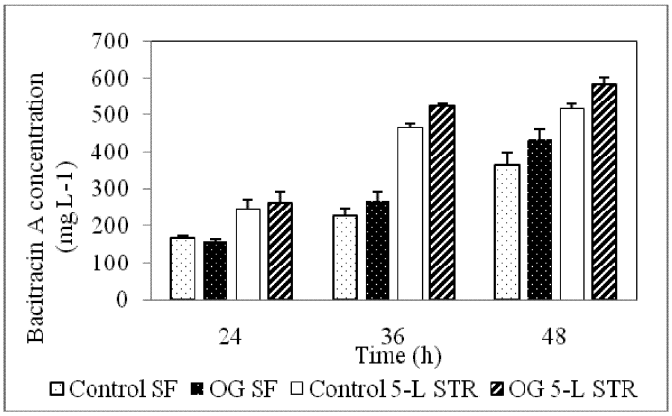

Figure 1: Bacitracin A concentration in the control and test cultures. Fermentations in shaken flasks (SF); Fermentations in bioreactors (STR).

3.2 Sclerotiorin overproduction

In order to investigate the role of □-butyrolactone molecules as signalling molecules produced by *P. sclerotiorum* IMI 104602 (Strain M), we tested the effect of these compounds on sclerotiorin production in *P. sclerotiorum* IMI 040574 (Strain S), a low sclerotiorin producer. Addition of 1.0% (v/v) spent medium containing □-butyrolactone molecules from Strain M to Strain S cultures 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) has the ability to restore sclerotiorin production in Strain S. Previous studies have highlighted that an essential criteria for the identification of a quorum sensing molecule is its capability to elicit an analogous response when added exogenously to the null mutant cultures as it would do when endogenously expressed by the producer organism(Winzer *et al.*, 2002). Therefore, the findings presented in this work suggest that □-butyrolactone molecules might act as signaling molecules in *P. sclerotiorum* for the production of the secondary metabolite sclerotiorin.

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 consumptoion 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 ($\mu g\ g^{-1}h^{-1}$) (120 – 168 h)	Yield (fold increase)	Sclerotiorin consumption rate ($\mu g\ g^{-1}h^{-1}$)(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.
P. chrysogenum has served as a model microorganism to study the effect of elicitation on the over production of penicillin G. Because of the established relationship between morphology, the production of secondary metabolite in fungal cultures and the assumption that production of penicillin G occurs at the tips of hyphae (Peñalva*et al.* 1998), Radman *et al.*(2004) investigated the impact of elicitation on the morphology of *P. chrysogenum*. The study suggested that the enhancement of penicillin G was due to the effect of elicitors on the morphology of the fungus, where hyphal tip number increased by 19%, 29% and 47% in oligoguluronate (OG), oligomanuronate (OM) and mannan oligosaccharide (MO) elicited cultures compared to the control cultures.
Recently, the effect of oligosaccharide elicitors has shown to change the intracellular calcium levels in fungal and bacterial cultures. Using a novel staining and quantification method (Fluo-4-AM and Cell trace calcein red-orange-AM), Nair et al. (2011) demonstrated that addition of oligosaccharide elicitors to the *P. chrysogenum* cultures causes an increase in the intracellular calcium levels. Concurrent with these findings, Murphy et al. (2011) using aequorin technology, challenged bacterial cultures with OG and MO which resulted in 11 and 7 fold increases in cytosolic calcium levels in *Escherichia coli* and 10 and 3 folds in *Bacillus subtilis*. This suggests that fungal and bacterial cultures sense external signals, such as elicitors, and such signals are transmitted through calcium transients to activate signalling pathways.
Signal transduction via elicitors has also been reported to have an effect at the transcriptional level. Nair and co-workers (2009) reported the over production of Penicillin G by elicitors in shaken flask and stirred tank reactors. The increase in antibiotic production in both systems was concomitant with the enhancement of penicillin G transcript copy number of the penicillin biosynthetic genes (*pcb*AB,*pcb*C and *pen*DE). Murphy et al. (2007a;2008) using quantitative PCR demonstrated that elicitors supplementation to *B. licheniformis* cultures not only altered the transcript levels of the antibiotic bacitracin biosynthetic genes (*bac*ABC) but of the genes involved in the export of the antibiotic out of the cell (*bcr*ABC). Although a great amount of data has been gathered in the quest for understanding the elicitor's mechanism of action, further investigation into the elucidation of elicitation mechanism is necessary.
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 a novel, easy and cheap alternative method for industrial-scale overproduction.

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