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ACTIVITY OF GULURONATE LYASE IN THE ENHANCED PRODUCTION OF BACITRACIN A IN THE LIQUID CULTURES OF *BACILLUS LICHENIFORMIS* NCIMB 8874

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ABSTRACT

This research reports for the first time the presence and the activity of guluronatelyase in the elicited (using oligoguluronate) liquid cultures of *Bacillus licheniformis* in the enhanced production of bacitracin A. Oligoguluronate (OG) prepared by partial acid hydrolysis of sodium alginate was added aseptically in concentrations ranging from 50 to 200 mg/L to cultures of *Bacillus licheniformis* (control cultures and cultures at 0hour (50 mg/L) and 24hour (200 mg/L)). Enhanced production of bacitracin A was observed at 32hour fermentation period with the highest production of bacitracin A obtained in the 0hour cultures (when 50 mg/L of OG was added at 0 hour) followed by the 24hour cultures to which 200 mg/L of OG was added. Results also showed, for the first time, that the enzyme guluronate lyase is produced in the cultures of *Bacillus licheniformis* when OG is added to the cultures and that the increase in the activity of this enzyme was concomitant with the bacterial growth, protein concentration of the cultures and the enhanced production of bacitracin A. The discovery of the presence of this enzyme (guluronatelyase) in the enhanced bacitracin A production from cultures of *B. licheniformis* could lead to potential applications of the methodology for large-scale production of bacitracin A in the future.

KEYWORDS: *Bacillus licheniformis*; Oligosaccharides; Oligoguluronate; Guluronate lyase; Elicitors; Bacitracin A; Overproduction; Cultures.

1. INTRODUCTION

The Gram-positive bacterium, *Bacillus licheniformis* is widely distributed in the environment. The ability of this bacterium to produce an array of metabolites has led to its utilization in different bio-industrial processes, including the production of antibiotics, animal feed additives, anti-tumor agents (levan) and enzymes (Dahech *et al*., 2012; Murphy *et al*., 2007a). The antibiotic produced by *B. licheniformis* is called bacitracin which is a polypeptide antibiotic active against most Gram-positive and some Gram-negative bacteria. There are three known types of bacitracin: bacitracin A, B and F. According to studies, bacitracinA and B (especially A) make up 95 % of antimicrobial activity while bacitracin F (a nephrotoxic agent) is not a desirable product of fermentation (Frøyshov, 1984; Hickey, 1964).

Elicitation involves enhancing the production of secondary metabolites by addition of trace amounts (mg/L^{-1} concentrations) of elicitors to microbial cultures. This process has been used in the enhanced production of bacitracin A in the cultures of *Bacillus licheniformis* (Asilonu *et al*., 2000; Radman *et al*., 2003). Elicitors are substances that can trigger defense mechanisms in microbial systems resulting in metabolic changes. Examples of elicitors are oligosaccharides such as oligoguluronate (OG), oligomanuronate (OM) and mannan oligosaccharide (MO). These elicitors are not added as carbon source or nutritive additives to the growth of microorganisms (Nair *et al*., 2005). The enhanced production of bacitracin A from the liquid cultures of *B. licheniformis* using different oligosaccharide elicitors has been studied. It was discovered that the concentrations of the different elicitors and their time of addition were critical in the enhanced production of bacitracin A. The mechanism behind the enhanced production of bacitracin A in the elicited culture of *B. licheniformis* is still being investigated.

It has also been reported that different enzymes are produced by *B. licheniformis* when different elicitors were added. For example, the addition of MO into cultures of *B. licheniformis* has been reported to induce an enzyme β-mannase, which degrades MO tomannose residues (Raffetti, 2012) revealed by measuring the activity of the enzyme. However, the activity of guluronatelyase when oligoguluronate is added to the cultures of *B. licheniformis* has not been studied. This researchaimed at investigating for the first time the presence andthe activity of guluronatelyase in the elicited (using oligoguluronate) liquid cultures of *B. licheniformis.*

2. MATERIALS AND METHODS

2.1. Strain and chemicals

Bacillus licheniformis NCIMB 8874 was obtained from the National Collection of Industrial and Marine Bacteria, USA. The chemicals were also purchased from Sigma Chemical Co. (Poole, Dorset, UK). Analytical grade of the reagents was used for all the quantitative and qualitative assays, while high performance liquid chromatography (HPLC) grade reagents was used for high performance liquid chromatography.

2.2. Maintenance and bacitracin A production media

Nutrient agar medium was used for the maintenance of the slants of the bacterium. Chemically defined M20 medium which contained L-glutamic acid 40.0 g/L, $NaH_2PO_4.2H_2O$ 40.0 g/L, Citric Acid 2.0 g/L, Na_2SO_4 1.0 g/L, KCl 1.0 g/L, $MgCl_2 \cdot 6.H_2O$ 0.4 g/L, $CaCl_2 \cdot 2.H_2O$ 0.02 g/L, $MnSO_4.H_2O$ 0.02 g/L and $FeSO_4.7H_2O$ 0.02 g/L was used for the growth of *B. licheniformis* and the production of bacitracin A. All solutions were prepared with distilled water and autoclaved at 121^oC for 15 minutes apart from L-glutamic acid and ferrous sulphate heptahydrate, which were sterilized by filtration (using a 0.2 µm syringe filter purchased from Fisher Scientific) and added separately. The pH of the medium (M20) was adjusted to 6.0 with 4M NaOH before sterilization.

2.3. Shaken flasks (SF) fermentation

The stock cultures on agar slants were grown for 7 days at 37 ^oC and were stored at 4 ^oC. Growth medium (80 mL of M20) was inoculated in SF with 2 mL of spore suspension (10^7 spores mL^{-1}) and incubated at 37 ^oC on a rotary shaker at 200 rpm for 16 hours. For SF fermentation, 10 mL (10 % of the working volume) of the 16-hour-oldculture was transferred into 500 mL flasks containing 90 mL of M20 medium. Incubation was carried out at 37 ^oC on a rotary shaker at 200 rpm for 72 hours. The SF studies were carried out in triplicate.

2.4. Oligoguluronate preparation from sodium alginate andelicitation conditions

Oligoguluronate (OG) was prepared bypartial acid hydrolysis of sodium alginate as described by Asilonu *et al*., (2000). TheOG was dissolved in HPLC water (in concentrations ranging from 50 to 200 mg L^{-1}) and sterilized at 121 ^oC for 15 minutes. The elicitorwas added aseptically to the cultures at 0 hours (50 mg L^{-1}) and24 hour (200 mg L-1) after inoculation with 10 mL of the inoculum. No elicitor was added to the control cultures.

2.5. Cell growth and pH measurements

The optical density (OD) of the fermentation samples was measured at 650 nm throughout the fermentation period, while chemically defined medium (M20) was used as blank. Absorbance readings above 0.5 were diluted with M20 medium. The pH of the medium was also monitored throughout the course of fermentation using the pH meter.

2.6. HPLC analysis of bacitracin A

The concentrations of bacitracin Aproduced in the cultures of *B. licheniformis* were quantified using the gradient HPLC method by Pavli and Kmetec (2001). The gradient elution system consisted of a C8 (5µm), 150 x 4.6 mm Kromasyl reverse phase column (Dionex) maintained at 40 ^oC, while 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 the standard and the calibration curve constructed using zinc bacitracin concentrations ranging from 0 to 1000 mg L^{-1}.

2.7. Quantification of the protein concentrationand enzymepurification

The quantification of the total amount of protein produced during the fermentation period inall the cultures following the addition of oligoguluronate (OG) was carried out using the standard Bradford protein assay method (Bradford, 1976). The purification of the enzyme (guluronate lyase) in the cultures of *B. licheniformis* was carried out according to the method described by Franklin *et al*. (1994) and Wenk (2007). Briefly, samples were centrifuged at 4 ^oC for 10 minutes at 1300 rpm. After centrifugation, the supernatant was transferred into an ice-cold glass beaker with a magnetic bead. Then, 8.0 g of ammonium sulphate ($(NH_4)_2SO_4$) was added slowly with stirring (small amount was added at a time and allowed to dissolve before further addition). The beaker was kept on the stirrer for 1hour precipitation to occur in the ice. After the precipitation, the samples were centrifuged at 10000 rpm for 15 minutes at 4 ^oC and the pellet containing the enzyme was re-suspended with 10 mL of lyase buffer (containing 2.62 g/L of NaH_2PO_4, 4.26 g/L of Na_2HPO_4 and 2.02 g/L of NaCl [pH 7.0]). The samples (re-suspended pellet with 10 mL lyase buffer) were transferred into dialysis tubes (MWCO-12-14000 Da. Medicell International Ltd) and the tubes were put into 250 mL glass beakers containing 220 mL of the lyase buffer each and magnetic beads. These were then left at room temperature for 1hour. After 1hour, the buffer was changed and left at room temperature for another 1hour. At the end of the last 1hour, the buffer

was changed again and the glassbeakers were transferred to the cold room and left overnight while stirring. After an overnight dialysis, samples weretransferred from the dialysis tubes into test tubes and stored at 5 ^{o}C until analyzed for enzyme assay (Franklin *et al*., 1994; Wenk, 2007).

2.8. Enzyme (guluronatelyase) assay

After enzyme purification, an aliquot of the sample (0.2 mL) was added to 2.0 mL of 0.1 % of oligoguluronate in 50 mM Tris-HCl buffer (pH 7.5) incubated previously for 10 minutes at 30 ^{o}C. The enzyme activity was measured using the spectrophotometer at 235 nm. One unit of the guluronatelyase activity was represented as an increase of 0.100 in the absorbance of the initial reaction time at 235 nm per minute. Specific activity of the enzyme (guluronate lyase) was expressed as units per milligrams of protein that was used (Miyazaki *et al*., 2001).

2.9. Statistical analysis

The statistical analyses were done using Graph Pad Prism Software Version 5.03 (Graph Pad Software, Inc. California, USA). The different conditions of the *B. licheniformis* cultures studied were compared using one-way analysis of variance (ANOVA). The conditions that showed significance were compared using Tukeys Multiple Comparison Test and levels of significance was considered at *p* values <0.05.

3. RESULTS

3.1. Cell growth and pH measurements

Cell growth and changes in the pH was monitored during the course of fermentation. As shown in figure 1, when the elicitor (OG) was added at different cultivation times (0 hour and 24 hours) to the liquid cultures of *B. licheniformis*, its addition did not cause any significant changes in the growth or pH profiles of the testcultures when compared to the control cultures in which no elicitor was added. However, a slight increase in the biomass concentration was observed in the growth of control cultures after 32 hours.

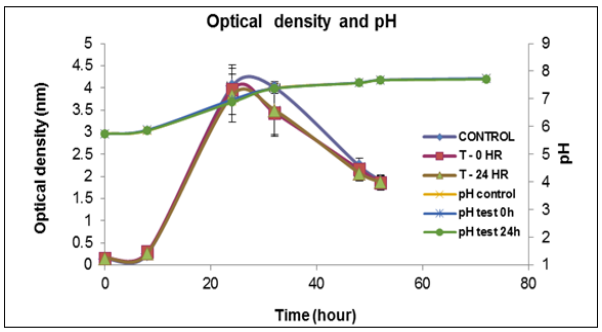

Figure 1: Biomass and pH profiles of B. *licheniformis* cultures in M20 medium throughout the course of fermentation. All experiments were carried out in triplicate.

3.2. Bacitracin A production by B. licheniformis culture

As illustrated in figure 2a, the maximum enhanced production of bacitracin A was obtained at 32 hour fermentation period in all the cultures (control, 0 hour and 24hours cultures). However, the highest production of bacitracin A was observed to be in the 0 hour cultures (when 50 mg/L of OG was added at 0hour), followed by the 24hours cultures (when 200 mg/L of OG was added at 24hour). Bacitracin A production in both the 0 hour and 24 hour cultures were observed to be higher when compared to the control cultures. After 32 hours, a decrease in the bacitracin A production was observed in all culture conditions.

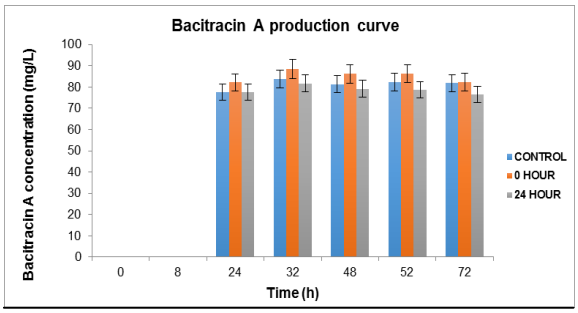

Figure 2(a): Production of bacitracin A by cultures of *B. licheniformis* (control cultures (no elicitor added)), 0 hour cultures (50 mg/L of elicitor added at 0 hour) and 24 hour cultures (200 mg/L of elicitor added at 24 hour). All experiments were carried out in triplicate.

3.3. Protein concentrations

Protein concentration was measured throughout the course of fermentation. As shown in figure 2b, in all the cultures (control, 0 hour and 24 hour), the increase in protein concentrations in the M20 medium began in the exponential phase of the growth of *B. licheniformis* and reached its peak at 32 hour. After 32 hours of fermentation, the protein concentration decreased and reached its lowest concentration at 72hour post inoculation for all culture conditions. The increase in the concentration of the protein was significantly different at $p<0.05$.

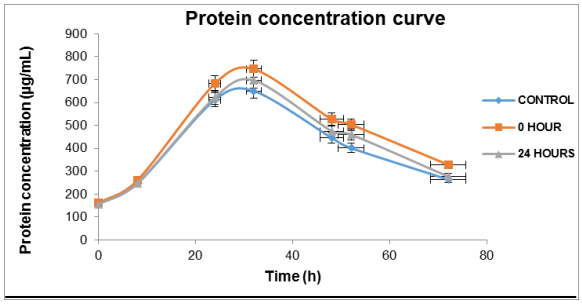

Figure 2(b): Protein concentrations in the cultures of *B. licheniformis* (control cultures (no elicitor added)), 0 hour cultures (50 mg/L of elicitor added at 0 hour) and 24 hour cultures (200 mg/L of elicitor added at 24 hour). Protein concentration was monitored throughout the course of fermentation. All experiments were carried out in triplicate.

3.4. Enzyme (guluronatelyase) activity

Following the enzyme purification, guluronate lyase activity was assayed by the method as described by Miyazaki *et al.* (2001). The enzyme activity was monitored from 24hours to 48hours of the period of fermentation. As indicated in figure 3, the peak activity of guluronate lyase was observed at 32hour fermentation time. The highest activity of the enzyme was observed in the 0 hour cultures, followed by the 24 hour cultures.

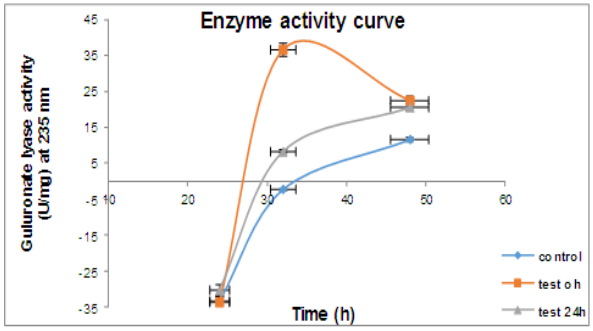

Figure 3: The activity of guluronatelyase in the cultures (control, test at 0hour and test at 24hour) of *B. licheniformis.* Maximum activity of guluronate lyase was observed at 32hour post inoculation time. All experiments were carried out in duplicate.

DISCUSSION

Earlier studies on the enhanced production of bacitracin A from the cultures of *B. licheniformis* have demonstrated that the use of oligosaccharide elicitors enhanced the production of bacitracin A when the elicitors were added at 0 and 24 hour (Murphy *et al.*, 2007b). It was also reported that the addition of oligoguluronate (OG) as an elicitor in a concentration of 100mg/L yielded maximum bacitracin A at 24 hour post inoculation (Murphy *et al.*, 2007a). The present study demonstrates that optimal bacitracin A production from the cultures of *B. licheniformis* was obtained when the elicitor (OG) was added at 0 hour and in a lower elicitor concentration of 50 mg/L. Thus, this study also confirms the previous findings of Murphy *et al*. (2007a) where optimal bacitracin A production requires low concentrations of OG.

In the present study, there was an overall increase of 6% in the level of bacitracin A produced in the 0 hour cultures with maximum production at 32 hour incubation period (at the stationary phase of the bacterial growth) after which the production of bacitracin A declined ($p<0.05$).

The decline in the bacitracin A production is associated with the increase in the pH of the cultures. It has been reported by investigators that the stability of bacitracin A in the cultures of *B. licheniformis* depends on the pH of the cultures. According to previous studies, when the alkalinity of the cultures of *B. licheniformis* increases, the production of bacitracin A decreases. This is because bacitracin A is converted to bacitracin F that is an undesirable by-product of fermentation (Konigsberg *et al*., 1961; Hickey, 1964; Flickinger and Perlman, 1979; Hanlon and Hodges, 1981).

In this work, the presence and activity of guluronatelyase on the enhanced production of bacitracin A was carried out for the first time. In order to gain a better understanding and optimizing the optimal time of enzyme production, protein concentration of the cultures was monitored throughout the course of fermentation. It was observed that the protein concentration of the cultures increased as the optical density and bacitracin A production increased and decreased after 32 hours of the fermentation period. Among these proteins is expected to be the guluronatelyase enzyme. Investigators have previously reported that different enzymes are induced by *B. licheniformis* when different elicitors were added. For example, the addition of mannan oligoguluronate (MO) into cultures of *B. licheniformis* has been reported to induce the enzyme, β-mannase that degrades MO to mannose residues (Raffetti *et al.*, 2012). In this study, the addition of oligoguluronate (OG) to the cultures of *B. licheniformis* was found to induce the activity of the enzyme called, guluronatelyase. This enzyme is said to have the molecular weight of 21 KDa (Osawa et al., 2004); that is why dialysis tubing that has a membrane pores of 12-14 Da. was used, in order to be sure that the enzyme was retained for analyses.

The maximum guluronatelyase activity was detected at 32 hour incubation time in the cultures that OG was added at 0 hour. This suggests that there is a relationship in the bacterial growth, the addition of elicitor (OG) to the cultures, protein concentration of the cultures, enzyme activity and the overproduction of bacitracin A. It was also observed in this study that the addition of the elicitor (OG) to the cultures of *Bacillus licheniformis* resulted in a wide range of biological responses (for example, the production of guluronatelyase, which degrades OG and enhances the production of bacitracin A. According to previous studies, as the elicitors (oligosaccharides) are added to the cultures of *B. licheniformis*, they recognize the elicitor receptors on the bacterial cell wall (cell wall of *B. licheniformis*) and enter the cell. On entry into the cell, they cause changes in the expression and phosphorylation state of the proteins, the increase production of enzymes and the enhanced production of bacitracin A (Tamerler *et al*., 2001). The results of this study therefore suggest that the activity of guluronatelyase is more when OG is added to the cultures of *B. licheniformis* than when it is not added. However, it also suggests that the elicitor may be added in trace amount, such as 50mg/L, in order to have an enhanced bacitracin A production. This is a novel finding in the investigation of the overproduction of bacitracin A from the cultures of *B. licheniformis* using oligoguluronate elicitor.

Enhanced bacitracin A production from the cultures of *B. licheniformis* was observed to occur when the OG was

added at lower concentration (50 mg/L) and addition time of 0 hour to the cultures. The discovery of the presence of guluronate lyase in the cultures of *Bacillus licheniformis* when OG is added could lead to potential industrial applications of the methodology for large-scale production of bacitracin A in the future.

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