

Application of Magnetic Field for Improvement of Microbial Productivity

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Continued attempt by the industry and research sectors to improve productivity of commercially viable microbial products fall into three general approaches including microbial-based (e.g. isolation, selection, and manipulation of microbes as higher producers), environmental-based (e.g. media development), and bioreactor/bioprocess-based studies.

Application of electromagnetic field to microbial cultures is a recent bioprocess-based technique. Current literature shows some effects on characteristics of microbial species (fungi and bacteria). These include enhancement of ethanol production capacity of *Saccharomyces cerevisiae*, citric acid and cellulase production by *Aspergillus niger* species and insulinase production by *Geotrichum candidum* after the cultures were exposed to electromagnetic field.

In this paper we report the application of electromagnetic field to cultures of *Bacillus licheniformis* to enhance productivity of bacitracin, a water-soluble branched polypeptide used as an antimicrobial agent against gram-positive and some gram-negative bacteria. Electromagnetic field was applied on cultures of *B. licheniformis* in stirred tank reactors (STRs) with working volume of 1.5 litres circulating into an in-house circulating and constructed magnetic field generator with low magnetic field intensity. The experiments were carried out both with and without pH control of the culture. Samples were assayed for bacitracin concentration to confirm the effects of electromagnetic field. The microbial growth and pH profiles were also monitored.

The results showed that circulation of culture at flow rate of 10 mL.min⁻¹ into magnetic field with 10 millitesla intensity leads to an increase in bacitracin concentration. The increase was higher when the pH of the culture was controlled compared to non-controlled culture. The highest percentage increase in bacitracin concentration was 36 % after 35 hours without pH control, while the highest bacitracin percentage increase obtained from the controlled culture under pH 7 exposed to electromagnetic field, was almost 89 % after 43 hours.

1. Introduction

1.1 Bacitracin

Bacitracin is a water-soluble, heat resistant (resists for 15 minutes at 100 °C) branched polypeptide, stable at pH range of 5-7 (Johnson et al., 1945). There are several types of bacitracin based on the molecular structure of the polypeptide. These molecules are differentiated by one amino acid in their structure. As described by Weinberg (1967), the most common types of bacitracin are bacitracin A and B, with Bacitracin A being the most active. This molecule needs the presence of metal ions for its antimicrobial activity. Bacitracin A has a thiazoline ring in its molecular structure and an amino group attached to its N terminal (Murphy, 2008). The nitrogen in its thiazoline ring and the adjacent amino group involve in chelation process of the metal and contribute to the stability of the thiazoline ring, inhibiting its oxidation and thus keeping the antimicrobial activity of bacitracin A (Stone and Strominger, 1971). Other types of bacitracin such as bacitracin F with low antibacterial activity are synthesized also during fermentation. Bacitracin F is produced by oxidation of bacitracin A under alkaline conditions. More precisely, under alkaline conditions, by deamination of the thiazoline ring, amino group is replaced by a carboxyl group (Reffatti, 2011).

Commercial bacitracin is available in the form of its zinc salt, which is more stable in dry state than bacitracin. It is very soluble in water and Methanol, slightly soluble in acetone, benzene and ether and insoluble in chloroform (Maynard, 1997). Its aqueous solution (both with and without buffer addition) is stable at pH range of 5-7 and rapidly loses its activity at pH values higher than 7. Also, it loses its activity at room temperature and can only be stored for up to four weeks at 4°C (Bond et al., 1949).

This antimicrobial product acts as an antibiotic by disrupting bacterial cell wall. It inhibits the reaction required for dephosphorylation of C55-isoprenyl pyrophosphate. This reaction is essential for synthesis of the lipid carrier responsible for peptidoglycan synthesis (Stone and Strominger, 1971). There have been several reports on the strains that bacitracin works primarily against. These include a range of gram-positive bacteria including *Staphylococcus* and *Streptococcus* spp. and some gram-negative enteric bacteria (Brewer, 1981).

Bacillus spp. including *B. licheniformis* and *B. subtilis* strains are the producers of this antibacterial compound. It is synthesized non-ribosomally by bacitracin synthase ABC (a multi-enzyme complex), which follows a thiotemplate mechanism from a protein template pathway (Konz et al., 1997). Bacitracin is used in pharmaceutical industry as medication for bacterial infections such as minor skin and eye infections and as a constituent of antibiotic sprays along with other antibiotics such as polymyxin and neomycin. It is widely used as animal food additive and acts as an important growth promoter in animal husbandry (Phillips, 1999).

There have been several reports on optimisation of bacitracin production, namely by optimisation of the medium and fermentation conditions (optimum temperature, oxygen levels and optimum pH). Also, the levels of bacitracin production have been shown to increase by elicitation (Murphy et al., 2007).

1.2 Effects of electromagnetic field

Electromagnetic field has been used widely in different areas of life sciences, for example in Magnetic Resonance Imaging (MRI) systems in radiology or electromagnetic therapy, where electromagnetic energy is used for treatment or diagnosis of a disease (Plonsey and Malmivuo, 1995). It has been under investigation also for its potential for cancer treatment (Williams et al., 2001). The application of electromagnetic field on microbial cultures has been under investigation as a potential means for improving industrial productivity. Electromagnetic field studies on microbial cultures have shown improvements in production of some microbial products. Citric acid and cellulase productions by *Aspergillus niger* spp. were enhanced by exposing the cultures to magnetic field. The production yield was shown to be dependent upon time of exposure and strength of magnetic field, which increased by increasing these parameters (Gao et al., 2011). There is evidence of increased insulinase production by *Geotrichum candidum* after application of low magnetic field, suggesting enhanced enzyme activity for the cultures grown under 7 mT magnetic field (Canli and Kurbanoglu, 2012). Also, studies on production of polyhydroxyalkanoates (PHAs) by activated sludge exposed to low intensity static magnetic field, have shown an increase of 28 % in the PHA yield (Xu et al., 2010). Furthermore, Perez et al. (2007) reported 17 % increase in overall volumetric productivity of ethanol by *Saccharomyces cerevisiae* when the culture was subjected to 20 mT magnetic field.

Effect of magnetic field on morphology, viability and growth of microbial species has also been investigated. The studies include increased growth of nitrite oxidising bacteria (Wang et al., 2012) and *E. coli* (Martirosyan et al., 2013), and increased viability of *E. coli* and *Pseudomonas aeruginosa* after magnetic field exposure (Segatore et al., 2012). Filipič et al. (2012) found that the activity of dehydrogenase enzymes of *Pseudomonas putida* and *E. coli* spp. were positively affected.

Other studies on several types of bacteria suggest that bacterial growth can be inhibited or stimulated depending on the strength and frequency of the electromagnetic field applied (Rodriguez Justo et al., 2006). Growth and sporulation of phytopathogenic microscopic fungi was reported to decrease under low intensity electromagnetic field (Nagy and Fischl, 2004). Inhan-Garip et al. (2011) reported decreased growth and morphological changes to gram positive and gram negative bacterial strains. Also, growth inhibition of *E. coli* by electromagnetic field due to cell membrane damage has been reported (Bajpai et al., 2012).

2. Materials and methods

2.1 Strain

Bacillus licheniformis NCIMB 8874 was purchased from National Collection of Industrial and Marine bacteria, UK.

2.2 Media and bacitracin A production from *B. licheniformis*

Nutrient agar was used for the maintenance of bacterial cultures.

Minimal medium (M20) was used for growth of *B. licheniformis* and bacitracin production.

The medium constituents were as follows:

L-Glutamic acid (20 g L⁻¹), Citric acid (1 g L⁻¹), NaH₂PO₄·2H₂O (20 g L⁻¹), KCl (0.5 g L⁻¹), Na₂SO₄ (0.5 g L⁻¹), MgCl₂·6H₂O (0.2 g L⁻¹), CaCl₂·2H₂O (0.01 g L⁻¹), MnSO₄·H₂O (0.01 g L⁻¹), FeSO₄·7H₂O (0.01 g L⁻¹).

All chemicals (Sigma Aldrich Ltd.) were added to de-ionized water and autoclaved at 121 °C for 15 min except for L-Glutamic acid and FeSO₄·7H₂O, which were filter sterilized separately using 0.2 µm filters into the sterile solution. The pH of the medium was adjusted to 6 by addition of 4 M NaOH solution.

2.3 Bioreactor Fermentation

Stock cultures on agar plates were sporulated overnight. Minimal growth medium (M20) was incubated on a rotary shaker (2 cm throw) at 37 °C, 200 rpm for 16 h, from 100 mL flasks into 500 mL flasks with 10 % of the total volume inoculum.

For bioreactor fermentation, 2 L bench-top stirred tank bioreactors (STRs) with 1.5 L working volume were inoculated with the above aerobic cultures.

In STR runs, each reactor had an outside circulation using a peristaltic pump. One of the STRs was attached to the magnetic field generator device (MFG), denoted as “the test STR”. The setup without the MFG system, is denoted as “the control STR” in this context.

Fermentation conditions were as follows: 10% working volume inoculum, temperature 37 °C, air flow rate 1 vvm, dissolved oxygen was kept above 30 % air saturation by change of the agitation rate between 200-600 rpm.

Foaming was controlled by an automated sterile anti-foam addition system. The antifoam used for this purpose was of organic nature (Antifoam 204, Sigma).

2.4 Magnetic Field Generator (MFG)

A magnetic field generator was attached to a stirred tank bioreactor. Microbial culture was circulated through the device with a peristaltic pump. The MFG, consisting of a cylindrical solenoid was attached to an AC/DC converter apparatus, which converted the alternating current to a direct current. Hence, the electromagnetic field used in this study was of static nature. A calibration curve for the strength of the magnetic field was generated using the corresponding flux density inside the solenoid to different electrical currents passing through. The measurements were taken by a gauss meter inserted inside the solenoid. The flux density in this study was set at 10 mT.

For the control reactor, run in parallel to the test run, the culture was circulated through silicone tubing by a peristaltic pump, in order to take into account any effect caused by the amount of time the culture was outside of the reactor.

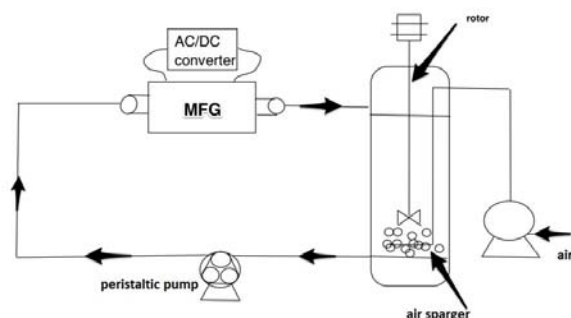


Figure 1. Schematic diagram of the “test” process design. The culture goes through silicone tubing into the glass tube inside the solenoid and gets exposed to magnetic field. The flow rate is set by the peristaltic pump and intensity of the magnetic field is set by the AC/DC converter device.

2.5 Growth curve

The growth profile of *B. licheniformis* was generated using optical density measurements at 650 nm.

2.6 Quantitative analysis of bacitracin

Bacitracin A concentration was determined using an optimized HPLC method, introduced originally by Pavli and Kmetec (2001). This method was based on a gradient elution reversed phase column chromatography system using a 5 µm C-8, 100 Å (150 x 4.6 mm ID) Kinetex column (Phenomenex Ltd., UK).

Two buffers, A and B, were used in the gradient elution system as described below:

Buffer A: Methanol – acetonitrile (1:1 v/v) – KH₂PO₄ (0.05 M, pH=6.0): (44:56 v/v).

Buffer B: Methanol – acetonitrile (1:1 v/v) – KH₂PO₄ (0.05 M, pH=6.0): (55:45 v/v).

Bacitracin detection program consisted of 20 minutes per each sample, at the optimum temperature of 40 °C, flow rate of 1.4 mL·min⁻¹, UV detection at 254 nm and 20 µL sample injection.

A standard curve was obtained by running known concentrations of zinc bacitracin standard solutions (1000, 800, 600, 400, 200, 100, 0 mg L⁻¹).

2.7 Statistical analysis

All experiments were carried out in duplicate and the statistical significance of results was determined by one-way analysis of variance (ANOVA) through calculation of standard deviation from the mean and use of standard error.

3. Results and discussion

3.1 Effect of Magnetic field on bacitracin production

Two 2 L STRs with 1.5 L working volume were used with outside circulation at a flow rate of 10 mL.min⁻¹. Electromagnetic field exposure started after 16 h from inoculation with magnetic field intensity of 10 mT, when the culture was at its late exponential phase. Two sets of experiments were performed, whereby the pH was not controlled during the course of fermentation in the first set, and it was kept below 7 by addition of 0.1 M HCL solution in the second.

Figure 2 shows the pH and growth profiles of *B. licheniformis* in the test and control cultures of both cases.

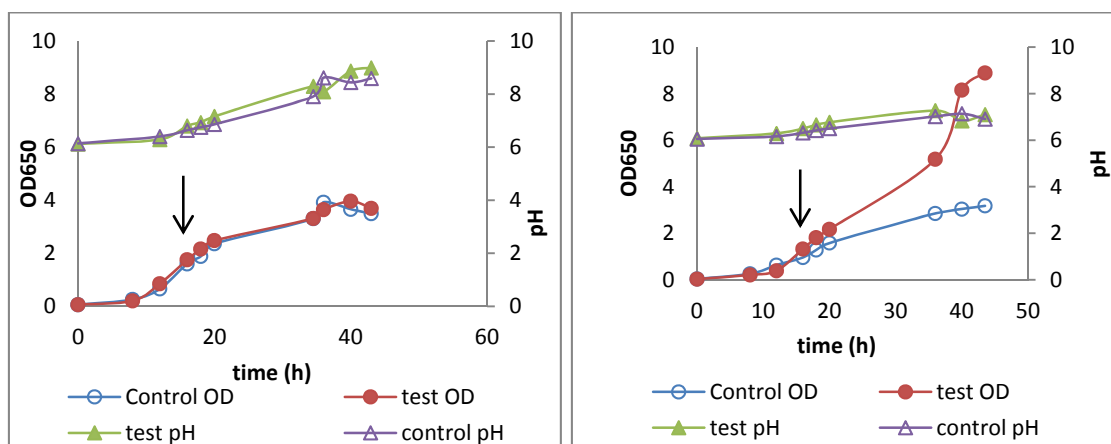


Figure 2. Growth curve in the control and test bioreactors based on optical density measurements at 650 nm (OD_{650}). Electromagnetic field application started at 16 h (indicated by arrows). Left: the STR runs without pH control; Right: the STR runs with pH levels kept below 7.

As shown in Figure 2, after the application of electromagnetic field at 16 h, cell growth did not change as pH increased during the course of fermentation. However, cell growth increased rapidly after almost two circulations through the device between 24 and 36 h, when the pH was controlled in the reactor.

The biomass increase under pH control, after application of electromagnetic field, is in line with previous studies with regards to increase in growth of microbial cultures. Wang et al (2012) reported enhanced growth of nitrite oxidizing bacteria. Also, growth increase has been reported for fungal cultures exposed to electromagnetic field (Nagy and Fischl, 2004, Segatore et al., 2012). The occurrence of this increase only with pH control can be due to the effect of electromagnetic field on the ions in the culture, promoting bacterial growth and a diauxic growth profile.

3.2 Effect of Magnetic field on bacitracin biosynthesis

Bacitracin A synthesis followed the same increasing trend in both reactors for both runs, with a higher rate in the test after magnetic field application in both cases. The effect started after 20 h from microbial inoculation, when the culture circulated for almost two rounds through the MFG.

As shown in Figure 3, bacitracin biosynthesis increased significantly in the culture medium by 36 % after 35 h of incubation in the uncontrolled pH conditions and 89 % after 43 h of incubation when the pH was controlled. The ANOVA test for both results show $p < 0.05$ specifying the significance of the results.

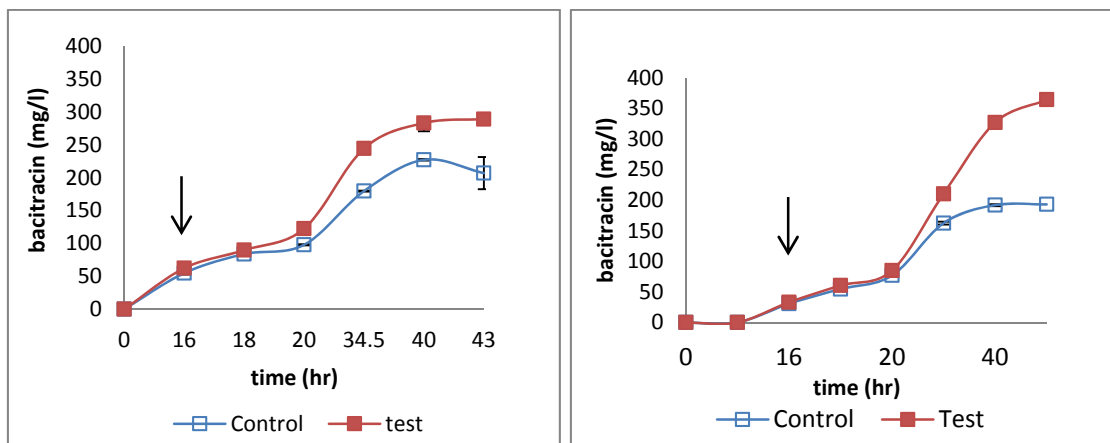


Figure 3. Bacitracin concentration in the STRs over time. Application of electromagnetic field started after 16 h of incubation (indicated by arrows). Left: pH levels increased during the course of fermentation; Right: pH levels kept below 7. These results are the outcome of duplicate measurements and the error bars show the standard deviation of the results from the mean.

Bacitracin loses its activity under alkaline conditions and higher concentrations of this product can be achieved in pH-controlled fermentation of *B. licheniformis* (Murphy, 2008). We found that the increase in bacitracin production was rather fast in the test run, with production rate of $125 \text{ mg L}^{-1} \text{ h}^{-1}$ compared to $80 \text{ mg L}^{-1} \text{ h}^{-1}$ in the run without pH control. Furthermore, the bacitracin concentration reached a maximum of 36 and 89 per cent in uncontrolled and controlled-pH runs, respectively. Hence, the application of electromagnetic field along with pH adjustment of the culture medium led to a more rapid bacitracin synthesis.

4. conclusions

The application of low intensity static electromagnetic field (10 mT) led to 36 % increase in bacitracin A synthesis by *B. licheniformis*. Since the alkalinity of the culture medium affects the concentration of bacitracin A, the pH was controlled and kept constant below 7, which resulted in notable increase (89 %) in bacitracin biosynthesis in cultures affected by the magnetic field.

The combined effect of electromagnetic field and pH control on the cell biomass was significant compared to uncontrolled pH conditions, in which cell biomass remained the same with or without magnetic field effect.

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