Production of an antimicrobial protein in a batch fermentor by Bacillus amyloliquefaciens MBL27

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Abstract
For the production of many biologically important enzymes, drugs and chemical compounds, microorganisms are used in industry. They can be easily cultivated in a fermentor due to their small size and producing higher yields. The production of broad spectrum antimicrobial protein from Bacillus amyloliquefaciens MBL27 was studied in a 3 L fermentor (Bioengineering, Switzerland) containing 2 L production medium. The study was carried out at optimum aeration rate (1.0 vvm), agitation rate (350 rpm), antifoam concentration (0.025% (w/v)) and glucose concentration [1.0% (w/v)], pH 7.0 and temperature 30°C.

The antimicrobial protein produced had 7400 AU/ml inhibitory activity against Staphylococcus aureus. This antimicrobial protein exhibited inhibitory activity towards a broad spectrum of wound and clinical pathogens including gram positive and gram negative organisms.

Keywords: Bacillus amyloliquefaciens, fermentor, antimicrobial protein, agitation.

Introduction
Scale-up is very important in process development. Due to difficulties in assessing the factors affecting the scale-up process during the cultivation, expanding a fermentation process from a lab-scale unit to a commercial one remains challenging. As a result, many large-scale fermentation processes give a lower yield than expected in the laboratory.1,8

The results of secondary metabolite production in shake flasks in aerobic condition cannot be extrapolated as indicators for possible performance in a fermentor. Both physical and biological factors are different in a fermentor and in a shake flask.

Moreover, in shake flask experiments, control over the reaction is extremely limited while in a fermentor, such controls are almost limitless.

The results obtained in a shake flask must be taken only as preliminary indicators necessary for successful industrial production. It must be verified in studies carried out in a fermentor. For full-scale production, it is important to devise a scale-up strategy adopting desired level of agitation and aeration rates (in fermentor), which would give comparable or better yields when compared to those obtained from shake flask study. This is necessary as it would enable to minimize the production cost and optimize the cost-effectiveness for the overall production process.

Microorganisms are exposed to varied environmental states in a large-scale fermentor. The submerged fermentation of microorganisms needs proper distribution of oxygen and nutrients for aerobic cultivation conditions. The vessel’s geometry and operating conditions like gas (i.e. air, nitrogen, oxygen) flow rates, pH, temperature and dissolved oxygen (DO) levels and agitation speed have to be monitored and controlled.

Among them, agitation and aeration are the most critical parameters used for process scale-up and play remarkable role in determining the productivity of the process2. Dissolved oxygen levels and its proper distribution in the fermentation medium are important for aerobic fermentation. The optimal operation conditions of fermentation process also closely depend upon cell’s morphological states, which in turn rely on the resistance of the organism to shear stress15,16.

Although, much work has been done on screening of antimicrobial substances producing microorganisms, these efforts have primarily been confined to culture medium and conditions in shake flask studies11-13. Little information is available about the optimum fermentation conditions in fermentor which is important for large-scale production of antimicrobials. Bacillus amyloliquefaciens MBL27, an AMP producing strain, is a potent strain that can be used in many industrial applications because of its broad inhibitory spectrum against many gram positive and gram negative pathogens and its fermentation condition in shake flask.17

Based on the preliminary information standardized from shake flask experiments, studies have been conducted in an in situ sterilizable 3 L fermentor with all controlled systems to facilitate scale up process in future experiments. The dissolved oxygen level during fermentation and the shear stress effect of the organism were also studied in addition.

Material and Methods
Culture medium: The composition of the production medium was formulated as follows in g/l (w/v): D-glucose, 10.0; K2HPO4, 2.0; triammonium citrate, 2.5; MgSO4, 7H2O, 0.2; MnSO4.7H2O, 0.2; surfactant (Antifoam 204, SIGMA) 0.25. The medium and fermentor along with all attachments were sterilized at 121°C for 20 min at 15 lbs prior to use.
**Inoculum preparation:** 0.1 ml of the culture from glycerol stock was inoculated in 10 ml nutrient broth and incubated in an incubator shaker at 200 rpm for 24 h. These cultures were then used to inoculate the fermentors. 1% (v/v) inoculum containing 2.2 × 10^6 cells/ml was used to inoculate the fermentation medium.

**Experimental set up:** A 3 L fermentor (Bioengineering, Switzerland) containing 2 L production medium was used to study the optimum aeration rate, agitation rate, antifoam concentration and glucose concentration. All the runs were performed in a temperature, pH, dissolved oxygen and stirring rate controlled in situ sterilizable fermentor (3 L). A connection to monitor allowed the following measurements and control measures to be obtained viz. pH, temperature, stirring speed, dissolved oxygen (DO) and foam control by means of an automatic addition process of an anti-foaming agent. Agitation was provided with a pair of six-bladed Rushton impellers and baffles were used to prevent vortex formation.

The required pH was controlled automatically by using sterilized 1N NaOH or 1N HCl solutions intermittently starting from the stage of inoculum through a peristaltic pump. Dissolved oxygen level was measured using a polarographic electrode (Ingold, Leicester, UK). Calibration (the percent of atmospheric oxygen) was performed with air-saturated medium (100%) and nitrogen-saturated medium (0%) after sterilization. The fermentation medium was continuously aerated for almost 100% oxygen content using compressed air supplied initially at 1.0vvm (air volume per broth volume per min) and the dissolved oxygen level was maintained above 20% throughout the experimental run. The temperature of the fermentation broth was monitored by temperature probe and it is controlled by circulating cooling water.

Only the values of the parameters under investigation were changed in the predetermined range of previous experiments while the other parameters were fixed at their optimum values. Sterility of the fermentation system was checked microscopically.

![Figure 1: Time course of AMP production by *B. amyloliquefaciens* MBL27 in a 3 L fermentor](image-url)
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Results

Effect of incubation period on AMP production: Batch cultivation of *B. amyloliquefaciens* MBL27 was carried out in 3 L benchtop fermentor with 1.0% (w/v) glucose, agitation speed of 350 rpm and at an aeration rate of 1.0 vvm. Biomass was assessed by optical density, concentration of consumed glucose and inhibitory activity of the AMP produced measured as a function of time and the results are shown in figure 1.

The samples were withdrawn at regular intervals from the fermentation medium under aseptic conditions and microscopically studied for cell morphology and for possible contamination. The inhibitory activity of the AMP produced was also studied by well diffusion assay. Glucose utilization was also studied from the samples collected by phenol-sulphuric acid method. It is clear from figure that AMP production was growth associated and both bacterial growth (OD₆₀₀ 5.4) and AMP production reached a maximum after 17 h of fermentation (7400 AU/ml against *S. aureus*). After 17 h, both biomass and AMP production decreased proportionately. But maximal activity and bacterial growth were observed only after 36 h of incubation in the shake flasks experiments.

The dissolved oxygen decreased due to the increasing oxygen demand for growing the culture. To guarantee sufficient oxygen supply, oxygen was kept at above 20% by rising the agitation speed. Glucose utilization pattern also showed that biomass production was proportionate to glucose consumption and glucose concentration in the growth medium was decreased from 10 g/l to 1.51 g/l after 17 h of fermentation.

Effect of glucose concentration: The influence of glucose concentration on AMP production was studied to maximize AMP production. The results are presented in figure 2.

![Figure 2: Effect of different concentration of glucose on AMP production by *B. amyloliquefaciens* MBL27 in a 3 L fermentor](image-url)
It is clear from the figure that maximum growth and AMP production were observed when 1.0% (w/v) glucose was added to the fermentation medium. At 1.5 - 2.0% (w/v) glucose concentration, there was not much variation in the activity to that at 1.0% (w/v) concentration. But 0.5% (w/v) glucose concentration did not support both biomass and AMP production effectively. Therefore, 1.0% (w/v) glucose was used for further studies.

**Effect of agitation speed:** Since agitation speed is a very important in the fermentation process, the effect of agitation speeds on AMP production the fermentations was carried out at the constant temperature of 30°C and at an aeration rate of 1.0 vvm varying only the agitation speed (250, 350 and 450 rpm). The results are given in figure 3.

From the figure, it is clear that AMP production increased almost at the same rate at 250 and 350 rpm initially. But at 450 rpm, AMP production started early. However, after 15 h of incubation, AMP production rate showed a variation with a much higher production level at 350 rpm (7400 AU/ml against *S.aureus*). At 450 rpm the production and the bacterial growth were slightly lowered (6900 AU/ml against *S.aureus* and OD$_{600}$ of 4.4, respectively) compared to 350 rpm. The increase in agitation rate produces higher shear stress in the broth which may cause a decrease in the growth of shear-sensitive microorganisms as well as the antimicrobial activity (Figure 4).

![Figure 3: Effect of different agitation speed on AMP production by *B.amyloliquefaciens* MBL27 in a 3 L fermentor](image-url)
Figure 4: SEM micrographs of the effects of various agitation speeds on morphology of *B. amyloliquefaciens* MBL27 cells a) 250 rpm b) 350 rpm c) 450 rpm

Figure 5: Effect of different concentration of antifoam on AMP production by *B. amyloliquefaciens* MBL27 in a 3 L fermentor

Antifoam concentration (w/v)
- ■ 0.025%
- ▲ 0.05%
- □ 0.1%
Effect of antifoam concentration: To study the influence of antifoam agent on AMP production, various concentrations of antifoam agent (Antifoam 204, SIGMA) are added to the production medium (0.025, 0.05 and 0.1% w/v) and the run was performed at an agitation speed of 350 rpm and an aeration rate of 1.0 vvm. The results are presented in figure. 5. It was observed that AMP production was maximal (7400 AU/ml against S.aureus) when the concentration of antifoam agent was 0.025% (w/v). At higher concentration above 0.025% (w/v), bacterial growth and the AMP production reduced proportionately.

Effect of aeration level: The effect of aeration rate was investigated with a constant agitation of 350 rpm and the results are shown in figure. 6. The inhibitory activities of the AMP produced for aeration rates of 0.5, 1.0, 1.5 and 2.0 vvm were 6900, 7400, 7000 and 6900 AU/ml respectively. It was found that the upper limit of the aeration rate for this fermentor was about 2.0 vvm. Over this value, a broth overflow was created inevitably.

The aeration rate directly influences the oxygen supply which in turn might affect the bacterial growth and AMP level during the fermentation. Since aeration rate of 1.0 vvm provided the best AMP production in terms of inhibitory activity, it was selected for subsequent studies.

![Figure 6: Effect of different aeration level on AMP production by B.amyloliquefaciens MBL27 in a 3 L fermentor](image-url)
Discussion

*Bacillus amyloliquefaciens* MBL27 isolated from dairy waste was able to produce AMP with broad antimicrobial activity against wide range of both gram positive and gram-negative organisms. This includes certain wound pathogens which also makes it a very important property to combat infection and used in wound healing studies. The results obtained in batch flask experiments for AMP production were experimentally validated by conducting experiments in a fermentor with all process control systems.

Time profile experiments showed that AMP reached a maximum after 17 h of cultivation after which the bacterial growth decreased. DO also drastically dropped to 20%. Similar findings were observed by Wu et al\(^1\) where thuringiensin production by *B.thuringiensis* reached a maximum of 5.0 g/l at 15-17 h of cultivation.

The AMP production reached a maximum activity of 7400 AU/ml against *S.aureus* at 17 h under 350 rpm. The activity of the AMP at 250 rpm was lower than that at 350 rpm. This may be due to incomplete mixing and/or oxygen transfer resistance resulting in localized oxygen toxicity at the lower agitation rate. A higher agitation speed increased the DO level and dispersion of macromolecules in the medium. This might have contributed to the greater growth and better AMP production. However, the shearing effect induced by the higher agitation speed on the cells may contribute negatively towards bacterial growth and AMP production. Similar findings were also reported by Wu et al\(^1\) and Potumarthi et al\(^1\) during the cultivation of *B.thuringiensis* and *B.licheniformis* respectively.

Crognaile et al\(^2\) also observed shear stress on *Streptomyces mobaraensis* in bench top reactor at higher agitation rates. Flickinger and Perlman\(^3\) observed that the length of time decreased from 36 h at 200 rpm to 14 h at 400 rpm on bacitracin production by *B.licheniformis*.

The dissolved oxygen concentration profiles were different under different aeration rates. At a low aeration rate of 0.5 vvm, dissolved oxygen shortage was observed for most of the time. In contrast, dissolved oxygen concentrations were above 30 and 60% saturation at the higher aeration rates of 1.5 and 2.0 vvm respectively. DO concentration of above 20% saturation is necessary to satisfy oxygen demand of *B.amyloliquefaciens* MBL27 and to achieve high level of antimicrobial protein production. There was no significant difference in AMP production at aeration rates of 1.0 – 2.0 vvm. Since more power would be needed for a higher aeration level, 1.0 vvm was considered to be the optimum level in this study. This significant difference may also be due to excessive foaming and broth overflow created because of vigorous air sparging at higher aeration levels.

Broth overflow is created because the upper limit of aeration rate for this fermentor was about 2.0 vvm only. The stripping of nutrients, products and biomass into the foam can reduce productivity and in extreme cases, impairs or prevents successful fermentation. Feng et al\(^4\) observed similar findings for maximum β-mannanase production by *B.licheniformis* where the production was minimum at low aeration rate of 0.5 vvm (170.6 U/ml) and maximum of 208.2 U/ml was achieved at 1.0 vvm.

Vigorous agitation and aeration may lead to severe foaming causing unstable and inefficient fermentor operation. The reduction in the activity of the AMP production on increasing the antifoam concentration from 0.025 - 0.1% (w/v) may be due to the decrease in the mass transfer efficiency. At low concentrations, these substances markedly decrease mass transfer efficiency\(^8\) while at high concentrations, opposite may occur\(^9\). Matar et al\(^10\) also used Antifoam 204 (SIGMA) to eliminate foaming.

Conclusion

The investigation on the production of AMP using a fermentor under optimal conditions showed that *B.amyloliquefaciens* MBL27 produced 7400 AU/ml of inhibitory activity against *S.aureus* after 17 h of incubation in the presence of 1.0% (w/v) glucose as the carbon source at 350 rpm, 1.0 vvm aeration level, pH 7.0 and temperature 30°C.

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References


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