Novel oxygenation for lipopeptide production from *Bacillus* sp. GB16

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Abstract

A novel integrated method for increasing dissolved oxygen concentration in culture media has been developed. It involves adding hydrogen peroxide to the medium, which is then decomposed to oxygen and water by catalase and adding vegetable oil to the medium as antifoam agent and oxygen vector. A new apparatus for automated addition of hydrogen peroxide to the bioreactor to keep the dissolved oxygen concentration constant over the range 10-100% ± 5% was tested. A significant increase (over threefold) of cultivation time was obtained while the dissolved oxygen concentration remained stable (30% ± 5%). Therefore, use of corn oil mixed with Ca-stearate as oxygen vector and antifoam and hydrogen peroxide as oxygen source to control excessive foam that was generated by microorganism biosurfactant, GB16-BS produced at *Bacillus* sp. GB16 cultivation was appropriate for stable cultivation.

Introduction

The phenomenon of foaming in fermentations is an everyday occurrence for most fermentation technologies. On a small scale, it presents a problem in operation and process safety, and in process plants, foaming additionally jeopardizes economic viability. The effect of the variation of fermentation broth composition on foam coalescence behavior is difficult to evaluate, since several parameters can influence this process. The composition variation due to metabolites produced by the cells or dissolved proteins from dead organisms which have become liberated by cell lysis can have significant effects on the coalescence. Especially, excessive foam was generated by excretion into the broth of surface-active agents such as proteins, peptides, and fatty acids thatwere produced by

microorganisms (1, 2). The stripping of nutrients, products, and biomass (3, 4) into the foam can reduce productivity and, in extreme cases, prevent successful fermentation. Additionally, reactants and products accumulate in foam and may be lost from the reactor with escape of foam; the aeration-agitation rate may be limited unless foaming is controlled by some means. Both chemical and mechanical methods are available for foam control. Mechanical foam-control is preferable to chemical foam-control, avoiding problems such as the lowering of the mass transfer rate, blockage of the spinfilter mesh, reaction inhibition and toxicity, or adverse effects on separation and purification of products seen when foaming is controlled by the addition of antifoam agents (5, 6, 7, 8). However, mechanical methods of foam control include high operating costs, complicated designs, possible shear damage to the product or microorganisms, risk of disturbances to the unit operations, and their limited effectiveness (light foam, limited foaming). In addition, dynamic foam head may be decreased by mechanical shear when foam formation is not too excessive.

During aerobic and viscous fermentation, oxygen supply is very important because insufficient oxygen supply can lead to suboptimal productivities as well as products of low quality. Several methods have been used to try to enhance the gas-liquid oxygen transport, such as modifying reactor designs, using oxygen vectors, increasing the oxygen composition or using pure oxygen in the inlet gas, increasing the agitation or aeration, and so on. However, these methods retain the basic limitation of gas-liquid oxygen transport. Hence, they have been only partly successful and may not meet the requirement in large-scale bioreactors, and thus may make the systems partly anaerobic. In addition, often turbulence and shear associated with high rate mixing and aeration is incompatible with fragile cells. A biological approach to reactor aeration has been reported, involving *Gluconobacter oxydans* co-immobilized with the photosynthesizing algae *Chlorella pyrenoidosa*. Dihydroxyacetone productivity was enhanced 5.4-fold when compared with the pure culture. However, the oxygen production is limited and co-immobilization may cause inhibition or competition for the substrate. Another approach is to modify the medium in such a way that it dissolves more oxygen. Organic solvents, with the intention of increasing the solubility of both organic substrates and oxygen, can replace the water solution normally used. The addition of a non-aqueous liquid phase may provoke a significant increase in the transfer rate from the gas phase to the microorganisms without necessitating an increasing energy supply. This new approach is the strategy of oxygen-vectors and consists of the addition of a compound to the growth medium in

which oxygen has a high solubility. Examples include hemoglobin, perfluorochemicals and low viscosity silicone oils.

A method to overcome gas-liquid transport resistance and increase oxygen supply is by adding hydrogen peroxide in the fermentation system. Hydrogen peroxide can be converted to oxygen and water by enzyme catalase available from the culture itself. In this case, the oxygen molecule in liquid phase is ready to consumption by cells and hence gas-liquid oxygen transport resistance is not existent. Although it is commonly known that hydrogen peroxide is toxic to cells at high concentrations, a low enough concentration of hydrogen peroxide can not only increase oxygen supply and enhance product yield, but also influence the metabolism. On the other hand, the method of feeding hydrogen peroxide is simple, oxygen and water; do not create a pollution burden for the environment. More important, this method will not increase difficulties for separation and purification of product.

Vegetable oils have been used as antifoam and oxygen vector. They are easily available in agricultural products; being metabolizable, they can enhance product yields, and, therefore, they have an advantage over other chemical antifoam agents.

In previous study, oxygen request of *Bacillus* sp. GB16 by adjustment of the working volume in cultivation was very high. Therefore, enough oxygen supply was essential for cultivation of *Bacillus* sp. GB16. However, agitation and aeration rate increase or pure oxygen supply tried in existing method for DO control cause that encouraged foam creation. To control foam, added chemical antifoam also had an adverse effect on cell growth.

In this study, a novel oxygen supply method for increasing oxygen concentration in the culture broth and foam control has been developed to maximized GB16-BS production by *Bacillus* sp. GB16, cultivated in a jar fermentor.

Materials and Methods

Effect of Hydrogen Peroxide on Cell Growth

Bacillus sp. GB16 was cultured in 10 ml volumes in 100 ml flasks with various concentrations of hydrogen peroxide. And in order to reduce surface aeration effect on cell growth *Bacillus* sp. GB16 was cultured in 100 ml volumes in 100 ml flasks. The intermittent addition method also tested because of initial fully reversion hydrogen peroxide to water and oxygen.

Foam Stability Measurement

The sample solution (10 ml) was delivered into a 250 ml graduated cylinder and aerated at 100 ml/min, foam reached up to 100 ml. Immediately aeration was turned off and the decreased foam height (20 ml) was measured against time.

Antifoaming Activity Measurement

As in the Fig. 1, the 2-day culture supernatant of *Bacillus* sp. GB16 (1 ml) was poured into graduated measuring cylinder containing 9 ml distilled water to prepare 10% solution (v/v). A gas inlet connected with compressed air was installed in the center of the cylinder. A gas flow meter between gas inlet and compressed air was installed to control the feeding pressure of air. As feeding air at 100 ml/min, the increased foam height was measured against time.

Results and Discussion

Effect of Hydrogen Peroxide on Cell Growth

Bacillus sp. GB16 had catalase activity and decomposition of hydrogen peroxide was fast without additional catalase. However, due to the difficulty in the measurement of the effect on cell growth by sudden decomposition of hydrogen peroxide (working volume; 10, 100 ml in100 ml Erlenmeyer flasks) (data not shown) hydrogen peroxide was added intermittently into the medium during cultivation (0, 5, 10, and 15 h). Hydrogen peroxide showed inhibition effect on growth above 8.82 mM (final concentration). Moreover, cell was lysed at 88.2 mM (Fig. 2). The decreased growth rate is associated with superoxide ($O2^-$) and the accumulation of hydrogen peroxide, which is produced by high specific activities of NADH oxidase and pyruvate oxidase. In addition, cessation of cell growth and cell lysis was resulted from the damage to DNA and protein at 3% (w/v) hydrogen peroxide. However, novel oxygen supply by using oxygen that is generated by decomposition of hydrogen peroxide in culture broth is easier than previous aeration method when culture broth is highly viscous without gas-liquid film resistance. Therefore 0.03% hydrogen peroxide, which was below the toxic concentration, was used to supply the oxygen to cells.

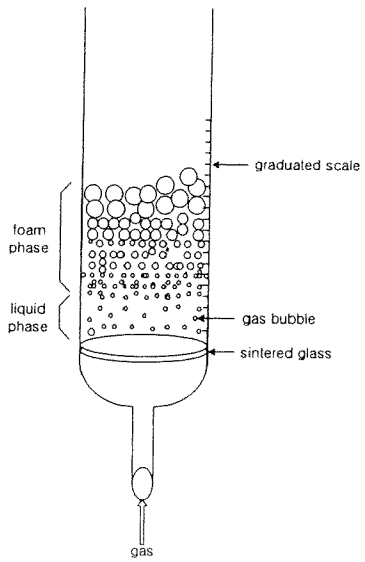

Fig. 1. Schematic diagram of foam generating apparatus for the measurement of foam stability and antifoam activity.

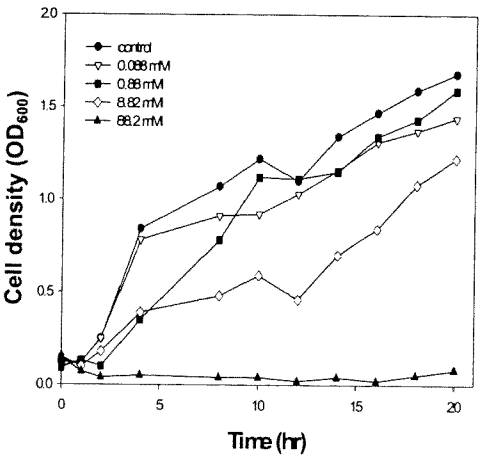

Fig. 2. Effect of intermittent addition of H_2O_2 on cell growth.

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