BIOTECHNOLOGY TECHNIQUES
Volume 10 No.7 (July 1996) p.495-500
Received 7th May.

ENHANCEMENT OF MAGNETIC PARTICLE PRODUCTION BY NITRATE AND SUCCINATE FED-BATCH CULTURE OF *MAGNETOSPIRILLUM* SP. AMB-1

Tadashi Matsunaga*, Noriyuki Tsujimura and Shinji Kamiya††

Department of Biotechnology, Tokyo University of Agriculture & Technology, 2-24-16, Naka-cho, Koganei-shi, Tokyo 184, Japan
* To whom all correspondence should be addressed
†† R & D Center, TDK Co., 2-15-7, Higashi-Ohwada, Ichikawa-shi, Chiba 272, Japan

SUMMARY

Magnetospirillum sp. AMB-1 is a magnetic bacterium, which is capable of growing under air atmosphere. This bacterium was employed to make bacterial magnetic particles (BMPs). AMB-1 only makes BMPs during logarithmic growth phase under anaerobic conditions. Since it requires nitrate as a nitrogen source, control of nitrate concentration in the medium was necessary. The fed-batch culture of AMB-1 was carried out by adding nitric acid and succinate as nitrogen and carbon source respectively. One liter of AMB-1 culture produced 0.34 g of dry cells and 4.5 mg of BMPs. BMP production by AMB-1 cultivated in the fed-batch culture was found to be seven times higher than that cultivated in the batch culture.

INTRODUCTION

Magnetic bacteria which orientate and swim along geomagnetic fields have been found in freshwater and marine sediments. A magnetic bacterium, *Magnetospirillum* sp. AMB-1, capable of growing aerobically has been isolated (Matsunaga et al., 1991). Because it can form colonies on agar plates, this strain has been used for the genetic analysis of magnetite formation (Nakamura et al., 1995). An anaerobic sulphate-reducing magnetic bacterium RS-1, has also been isolated (Sakaguchi et al., 1993). These bacteria contain BMPs in the

size range of 50 ~ 100 nm (Matsunaga and Kamiya, 1987b). These BMPs form alignments of 10 to 30 particles which disperse very effectively in aqueous solutions because they are covered with stable lipid membranes. For this reason BMPs have been used for enzyme, antibody and DNA immobilization (Matsunaga, 1991; Matsunaga et al., 1987b; Matsunaga et al., 1993; Takeyama et al., 1995). BMPs have also been introduced into blood cells by cell fusion (Matsunaga and Kamiya, 1987a) and by phagocytosis (Matsunaga et al., 1989). Blood cells containing BMPs can be manipulated magnetically.

An aerobically grown cells of AMB-1 produce magnetic particles. When nitrate and succinate were employed as nitrogen and carbon sources respectively, 2.6 mg magnetite was obtained from an one liter batch culture of AMB-1 (Matsunaga et al., 1990).

In this paper, we describe fed-batch culture of magnetic bacterium AMB-1, with added nitric acid and succinate in order to enhance the production of bacterial magnetic particles for biotechnological application.

MATERIALS AND METHODS

Chemicals. All reagents for medium preparation were purchased from Kanto Chemical Co. (Tokyo, Japan) or Wako Pure Chemical Industries (Osaka, Japan). Other reagents were commercially available or laboratory grade materials. Deionized and distilled water was used in all procedures.

Cultivation of Magnetic Bacterium. *Magnetospirillum* sp. AMB-1, which has been previously described,(Matsunaga et al., 1991) was originally isolated from the sediment of a pond at Koganei in Tokyo. It was grown anaerobically at 26 °C in a modified magnetic spirillum growth medium (MSGM, pH 6.75) (Blakemore et al., 1979). One liter of this modified MSGM contained 10 ml of Wolfe's vitamin solution, 5 ml of Wolfe's mineral solution, 2 ml of ferric quinate solution (0.27 g of $FeCl_3$ and 0.19 g of quinic acid in 100 ml water), 0.68 g of KH_2PO_4 , 0.12 g of sodium nitrate, 0.05 g of sodium thioglycollate, and 0.74 g of succinic acid. The pH of the medium was adjusted to 6.75 with NaOH solution. The medium was autoclaved and sterilized. Anaerobic conditions were achieved by sparging the culture with argon gas. The cell concentration was determined using a hemacytometer. Batch culture was carried out in a 50 ml or 5 liter flask, containing 40 ml or 4 liter of medium.

Nitrate concentration was determined by ion chromatography (Shim-pack IC-A3, Shimadzu, Kyoto, Japan).

Fed-batch Cultures. Fed-batch culture was carried out in a 4 liter fermentor (MODEL PC-5, ABLE, Tokyo, Japan). An inoculum of 40 ml of cell culture grown to exponential phase in a 50 ml flask, was transferred to the fermentor which contained 4 liter of modified MSGM. For culture agitation, a four-blade disc turbine impeller was used. The agitation rate was maintained at approximately 100 rpm and the culture temperature was kept constant at 26 °C. The pH of the culture was kept at 6.75 using nitric acid or HCl. When the succinate concentration decreased under 6.3 mM, succinate 1M solution was added.

BMP Isolation. Approximately 10^{12} cells were suspended in 20 ml of HEPES buffer (pH 7.0), and disrupted by three passes through a French Press at 1300 kgf cm^{-2}. These disrupted cells were treated with an ultrasonic disrupter (UR-200P, Tomy Seiko, Tokyo, Japan) operated for 5 min at 0 °C. This procedure was repeated five times. BMPs were collected from the sonicated cell fraction using a samarium-cobalt (Sm-Co) magnet (18x11x14 mm) that produces an unhomogeneous magnetic field (0.4 T on the surface of the magnet, with an average gradient of 0.2 T cm^{-1}). The magnet was placed at the bottom of the tube, attaching all BMPs to it, and then the supernatant was removed.

RESULTS AND DISCUSSION

Cell Growth, BMP Production and Nitrate Consumption. Batch culture of AMB-1 was carried out in a flask with a working volume of 4 liter. Fig.1 shows the relationship between BMP production and growth of AMB-1 in batch culture. AMB-1 can only produce BMPs in the logarithmic growth phase. When the growth of AMB-1 reached stationary phase, BMPs were not produced. Therefore, to achieve maximum BMP production, it is necessary to keep AMB-1 cells in the logarithmic growth phase. Fig.2 shows the consumption of nitrate in correlation with growth of AMB-1. Consumption of nitrate began at the start of logarithmic growth. When the initial nitrate (1.4 mM) was consumed up after 70 hrs, the growth of AMB-1 stopped. The final cell concentration was $1.5x10^8$ cells/ml. This result indicates that nitrate limited the growth of AMB-1 under these conditions.

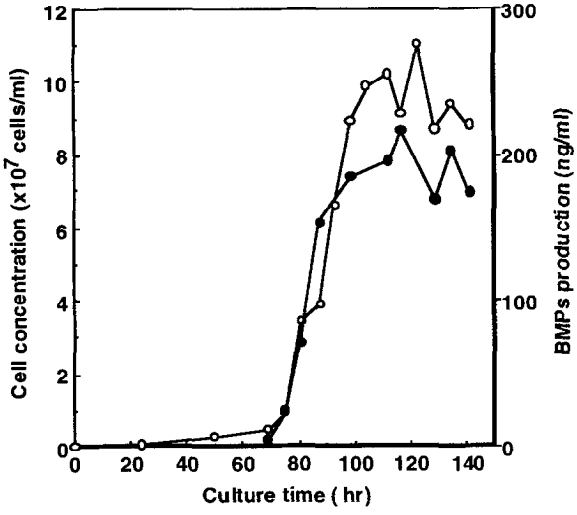

Figure 1 Growth curve of magnetic bacterium AMB-1 (o) and BMP production (●).

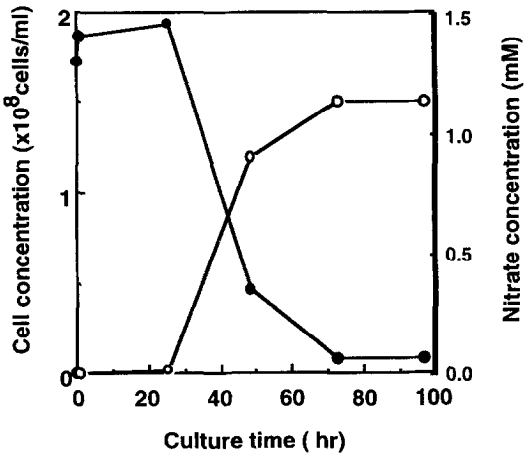

Figure 2 Growth of AMB-1 (o) and the concentration of nitrate in medium (●). The initial nitrate concentration was 1.4 mM.

Effect of Initial Nitrate Concentration on the Growth of AMB-1. AMB-1 is a denitrifying bacterium and the growth of the bacteria depends on the nitrate concentration in the medium (Mahne and Tiedje, 1995). The growth of *Magnetospirillum magnetotacticum* MS-1 was enhanced by addition of nitrate to the medium (Bazylinski and Blakemore, 1983). To investigate the effect of nitrate concentration on the growth of AMB-1, batch culture at various initial nitrate concentrations was carried out in a flask containing 40 ml modified MSGM. When the initial nitrate concentration was 2.8 mM, AMB-1 seemed to reach greater cell densities than when the initial nitrate concentration was 1.4 mM (Fig.3). From this data, it can be concluded that an adequate nitrate

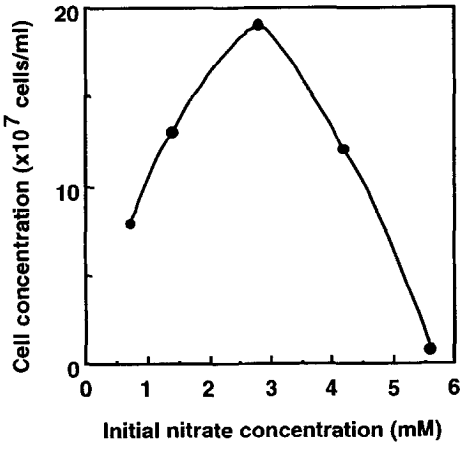

Figure 3 Effect of initial nitrate concentration on the cell concentration of AMB-1 after 96 hours of incubation.

supply is necessary for the growth of AMB-1, and that the optimum initial nitrate concentration is 2.8 mM. However when the initial concentration of nitrate is above 4.2 mM, growth of AMB-1 is inhibited. At high nitrate concentrations, intermediates such as nitrite, nitric monoxide and dinitrogen monoxide accumulated during consumption of nitrate as the nitrogen source (Bazylinski and Blakemore, 1983; Blackmer and Bremner, 1978).

Nitric Acid and Succinate Fed-batch Culture. Fed-batch culture of AMB-1 was carried out in a jar fermentor. When nitrate was used as a nitrogen source, the pH of the medium gradually increased. Therefore nitric acid was used as a supply of nitrogen and to control pH. As a result, the final cell concentration reached 1.2×10^9 cells/ml (Fig.4). However, when HCl was used to control pH, growth of AMB-1 was not enhanced. Thus it appears that supplying additional nitrogen enhances growth. Succinate solution was used as a carbon source. When succinate concentration decreased, 1 M succinate solution was added to the 4 liter culture in order to adjust the succinate concentration at 6.3 mM. In this case the final cell concentration reached 2.2×10^9 cells/ml (Fig.5). At this cell concentration the dry cell weight and

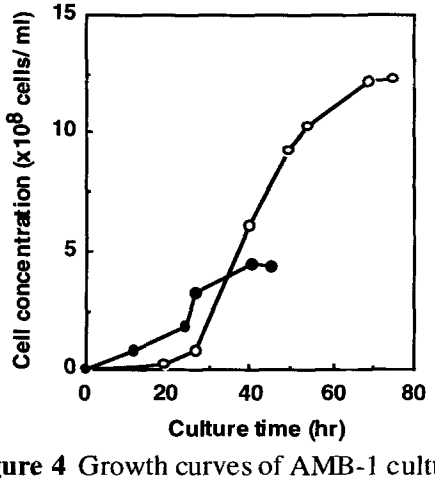

Figure 4 Growth curves of AMB-1 cultured in a fermentor when the pH of medium was controlled using nitric acid (○) or HCl (●).

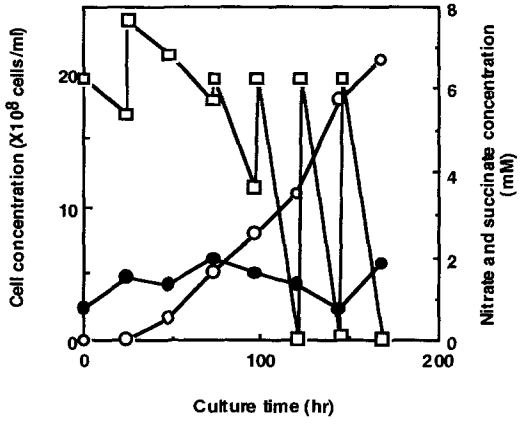

Figure 5 Growth of AMB-1 (○) with intermittent succinate addition, and concentrations of nitrate (●) and succinate (□) in medium. The pH was controlled using nitric acid.

BMP production were 0.34 g/l and 4.5 mg/l, respectively. These results indicate that sufficiently high nitrate concentrations can be maintained in the culture of AMB-1, by adding nitric acid throughout growth.

Acknowledgments. We are grateful to Dr. J.G. Burgess, Department of Biological Sciences, Heriot-Watt University, for reading the manuscript.

References

Bazylinski DA, Blakemore RP (1983). Appl Environ Microbiol 46, 1118-1124.

Blackmer AM, Bremner JM (1978). Soil Biol Biochem 10, 187-191.

Blakemore RP, Maratea D, Wolfe RS (1979). J Bacteriol 140, 720-729.

Mahne I, Tiedje J (1995). Appl Environ Microbiol 61, 1110-1115.

Matsunaga T (1991). Trend Biotech 9, 91-95.

Matsunaga T, Hashimoto K, Nakamura N, Nakamura K, Hashimoto S (1989). Appl Microbiol Biotechnol 31, 401-405.

Matsunaga T, Kamiya S (1987a). Biomagnetism 410-413.

Matsunaga T, Kamiya S (1987b). Appl Microbiol Biotechnol 26, 328-332.

Matsunaga T, Sakaguchi T, Tadokoro F (1991). Appl Microbiol Biotechnol 35, 651-655.

Matsunaga T, Tadokoro F, Nakamura N (1990). IEEE Trans Magnet 26, 1557-1559.

Matsunaga T, Takano H, Burgess JG (1993) Biogenic magnetite and inorganic ultrafine particles and their applications. In: Havorson HO and Hunter-Cevera J (eds) Short Communications of the 1991 International Marine Biotechnology Conference.Wm. C. Brown Publishers, Dubuque, Iowa, 256-264.

Nakamura C, Burgess JG, Sode K, Matsunaga T (1995). J Biol Chem 270, 28392-28396.

Sakaguchi T, Burgess JG, Matsunaga T (1993). Nature (London) 365, 47-49.

Takeyama H, Yamazawa A, Nakamura C, Matsunaga T (1995). Biotechnol Techniques 9, 355-360.