Magnetite nanoparticles enhance growing rate of *Bradyrhizobium japanicum*

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ABSTRACT

The aim of this study was to investigate whether magnetite nanoparticles affect *Bradyrhizobium* growth rate (BGR) in liquid media, with a view to controlling sensitivity to high pH and, hence, survival in extreme conditions. Treatments were different concentrations of nanoparticles suspended in liquid medium (yeast mannitol broth, YMB) mixed with and adhering to the Histic strain of *Braydrhizobium japanicum*. Experimental design was a complete randomized block (CRB) with four replications. The most important variables were growth rate coefficient (GRC), mean generation time (MGT), and number of generations (NG) of bacteria before the stationary phase. The nanoparticles increased GRC and NG, and decreased MGT; the optimal quantity of nanoparticles determined. In the presence of these nanoparticles the pH of the culture remained almost constant from zero time to 96 hours of growth, but without nanoparticles the pH increased from 6.8 to 7.1 during the same interval.

Keywords: magnetite nanoparticles, *Bradyrhizobium japanicum*

1 INTRODUCTION

The feasibility of biotechnology often depends on the bacterial growth rate (BGR) and duration of exponential growth (which is also an indicator of survivability). pH (along with temperature) is one of the most important environmental parameters controlling growth. BGR is of considerable practical importance when preparing inocula of bacteria; for example in agriculture. *Bradyrhizobium japanicum* is one of the symbiotic nitrogen fixers belonging to the genus *Bradyrhizobium*, which comprises slow-growing, Gram-negative, heterotrophic bacteria, able to form root nodules on the soybean [2, 8]. During *Bradyrhizobium* growth in batch culture, medium pH typically becomes more alkaline toward the end of growth. The optimum growth pH of *Bradyrhizobium japanicum* is 6.8 ± 0.2 [7]; it is more tolerant to low pH than high pH [9]; BGR starts to decrease at pH >7, and beyond 7.5 the decrease will be significant. The main reason for the pH increase is secretion of alkaline protein degradation products during metabolism, which interm alter the ionization state of nutrient molecules, diminishing their availability to the *Bradyrhizobium* [6]. On the other hand, fermentation of carbohydrates can lead to the production of organic acids, which decreases the pH of the medium, but the decrease is typically not great [3].

Magnetic nanoparticles, especially Fe_3O_4 (magnetite), have been widely used for complexing many bioactive substances such as proteins, including oligopeptides and enzymes [5]; for example, for the purposes of decontamination. Another interesting application is to allow such nanoparticles to coat the bacteria undertaking a biotechnological process [1]; this facilitates separation of the products. The binding of nanoparticles to such substances involves

interactions, especially between the amine residues of proteins and the metal oxide [4, 5, 6], creating hybrid biomolecular entities of enhanced stability. We expect that magnetite nanoparticles are able to complex amino acids secreted by the bacteria during growth; the degree of binding will be affected by medium (broth) parameters, especially pH. Furthermore, it seems that magnetite nanoparticles can act as oxidase enzymes in the medium [5, 6]. Our aim is to determine the effect of magnetite nanoparticles on culture of *Bradyrhizobium*. We observed a significant enhancement of BGR and number of generation before senescence. Possible inclusions are discussed.

2 MATERIALS AND METHODS

2.1 Bacterial strains and culture media

The Histic strain of *Bradyrhizobium japanicum* was obtained from the Soil and Water Institute, Tehran. For preparation of the bacterial culture 50 ml of YMB [7] in a 250 ml flask was autoclaved (121 °C, 15-20 min, 103 kPa), and after cooling the Histic strain was introduced to the flask. One loop full from Histic strain was transferred to YAM broth and inoculated flask was incubated on a rotary shaker at 150 rpm at 28-30ºC for 8 days. At early stationary growth phase, 1 ml of broth culture was transferred to 250 ml flasks of YAM (50 ml) broth with varying concentrations of magnetite nanoparticles: control (without nanoparticles), 20, 40, 60, and 80 μg ml^{-1} in a statistical design of complete random blocking (CRB) with four replications.

The inoculated flasks were held on a rotary shaker at 150 rpm at 28-30 ºC for 7 days, then every day 1ml from each flask was removed separately and eight times sequentially tenfold diluted in test tubes. To count the bacteria, twice 100 μl of each of these eight tubes was removed and spread on two 9 cm Petri dishes containing YMA solid medium [7]. The Petri dishes were immediately transferred to the incubator and kept at 28-30 ºC for 6-8 days. Counting the number of colony forming unit N_f on the surface of the Petri dishes was started after 5-6 days.

Measurement of medium pH was carried out simultaneously for all treatments at 7 days by using a glass electrode. The variables were calculated during the logarithmic phase, bacterial culture mimics a first-order chemical reaction, i.e., the rate of increase of cells is proportional to the number of bacteria present at that time. GRC is determined from the following equation [5];

$$\ln N_s - \ln N_0 = \mathrm{GRC}(t_s - t_0) \qquad (1)$$

where N_0 is the number of viable cells at t_0, the time at the end of the lag phase, N_s is the number of viable cells at end of the log phase, at time t_s. The number of generation (NG) is an index of the bacterial growth for comparison of strains during the logarithmic phase growing under different conditions, and is calculated from the following equation [5]:

$$\mathrm{NG} = (\log_{10} N_s - \log_{10} N_0)/ \log_{10} 2 \qquad (2)$$

The mean generation time(MGT) is calculated from the following equation [5]:

$$\mathrm{MGT}=(t_s - t_0)/\mathrm{NG} \qquad (3)$$

2.2 Preparation of magnetite nanopaticles

A 0.5 M solution of sodium hydroxide (about 125 ml) was poured in to a three necked flask under nitrogen gas with vigorous stirring at 65 ºC. 12.5 ml of an equimolar mixture of iron (II) and iron (III) (counterion = Cl^-) (each 0.9 M) was prepared in deaerated distilled water and further purged with nitrogen gas for 30 min. Then the iron solution was added dropwise to the sodium hydroxide during 30 min while stirring vigorously. The suspensions were separated and purified by centrifuging and resuspending three times in water and then HCl at 20,000 *g*. The particles were finally dried in a vacuum oven at 70-80 ºC.

3 REZULTS AND DISCUSSION

According to the analysis of variance (Table 1), magnetite nanoparticles in the liquid media increased the growth rate constant and number of generations, and decreased the mean generation time (Figure 1). Among the treatments magnetic nanoparticles with concentration (40 μg/ml) gave the maximum GRC (about 0.25 h^{-1})and NG (about 22), whereas it had the least MGT (about 2.69 h).

Likewise, the results illustrated that the treatments of T_4 and T_5 in the stationary phase gave a maximum number viable cells per ml (about more 10^9) (Table 2). Also, measurement of the number of viable cells after 24 hours as an index of bacterial growth vigour illustrated that the control treatment had the least vigorous population, and its maximum population was about 10^8 viable cells ml^{-1}.

Table 1. Mean squares from the analysis of variation of the growth indices of coated *Bradyrhizobium japanicum* Histic with magnetite nanoparticles.

Sources of Variation	df †	MGT/ h	GRC / h^{-1}	NG
Replication	3	0.0032	3.98	0.165
Treatment	4	0.190**	0.0011**	9.56**
Replication × Treatment	15	0.0036	0.000026	0.161

† df is degree of freedom; ** F test indicated significance at $P < 0.01$

Comparison of treatments' means based on Duncan's test (least signification range test) showed that effects of concentrations of 40 and 60 μg/ml on the mean generation time and number of generation were not significantly different, while there was a difference of the GRC at $P< 0.05$ (Table 3). Moreover, differences of other comparisons were significant at $P< 0.01$ (Table 3). Measurement of pH in the growth stages of rhizobia showed that all treatments (except the control treatment T_1) could maintain the pH near the optimum for bacteria growth (Table 4). Therefore, it seems that the magnetite nanoparticles can complex OH^- ions in the medium coming from bacterial secretion.

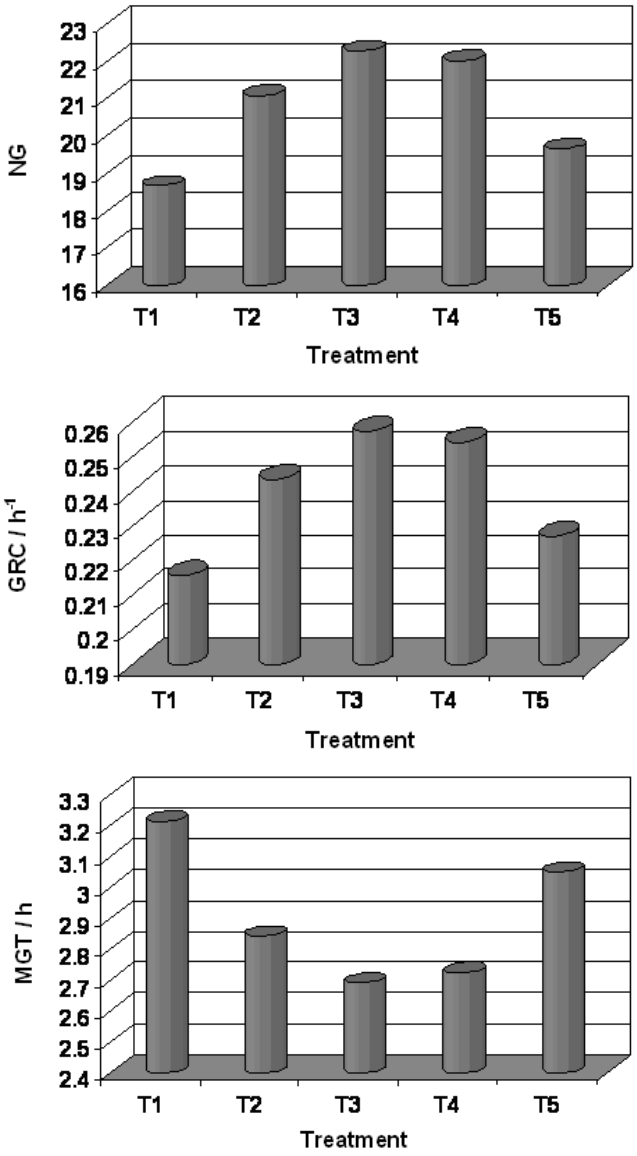

Figure 1. Effect of magnetite nanoparticles concentration on the number of generations (NG), mean generation time (MGT), and generation rate constant (GRC) of *Bradyrhizobium japanicum* Histic during the log phase.

Table 2**.** Mean of the log number of viable cells per ml between treatments.

		Time / h								
Treat[†]	NP[††]	0	12	24	48	72	96	120	144	168
T_1	0	1.6	3.1	5.1	7.5	8.7	8.7	8.8	8.7	8.4
T_2	20	1.6	2.5	6.4	7.7	8.6	8.8	8.9	8.8	8.4
T_3	40	1.8	2.2	6.2	8.3	8.1	8.9	8.7	8.7	8.5
T_4	60	1.8	2.4	6.3	7.6	8.8	9.0	9.0	9.1	8.8
T_5	80	2.1	3.1	6.2	7.9	8.8	9.0	9.1	9.0	8.8

[†]experimental treatments;[††]nanoparticles concentration / μg ml^{-1}.

Table 3**.** Comparison of treatment means based on Duncan's test.

Treatment	MGT / h	GRC / h^{-1}	NG
T_1 -T_2	0.357**	0.027**	2.4**
T_1 -T_3	0.512**	0.041**	3.7**
T_1 -T_4	0.474**	0.038**	3.3**
T_1 -T_5	0.152**	0.012**	1**
T_2 -T_3	0.155*	0.014**	1.3**
T_2 -T_4	0.117*	0.011**	0.91**
T_2 -T_5	0.205**	0.011**	1.4**
T_3 -T_4	0.038 n.s.	0.003*	0.3 n.s.
T_3 -T_5	0.360**	0.025**	2.6**
T_4 -T_5	0.322**	0.021**	2.3**

*, ** F test indicates significance at $P < 0.05$ and $P < 0.01$, respectively; n.s. indicate that there is no significant difference.

Table 4**.** Variation of pH between treatments during growth[†].

		Time / h							
Treatment	NP[††]	0	12	24	48	72	96	120	144
T_1	0	6.80	7.00	7.50	7.20	7.10	6.90	6.90	7.70
T_2	20	6.80	6.80	6.90	7.10	6.60	6.50	6.40	6.40
T_3	40	6.80	6.80	6.86	6.78	6.70	6.50	6.45	6.43
T_4	60	6.80	6.80	6.82	6.73	6.62	6.45	6.34	6.27
T_5	80	6.80	6.80	6.77	6.70	6.55	6.45	6.30	6.21

† Uncertainly of pH determination is ± 0.02,
[††] nanoparticles concentration / μg ml^{-1}.

The main question to answer is how the presence of the magnetite in the medium affect the growth. The following possible explanation seems to the merit consideration: 1. The pH results (Table 4) suggest that the nanoparticle have a pH buffering effect either by adsorbing alkaline bacterial secretion, or by reacting with them. 2. The nanoparticles catalyse reaction tending to keep pH neutral; e.g., K_2HPO complexing with $HC_2H_3O_2$. 3. The nanoparticles may complex and inactive oxygen scavenges in the medium hence increasing oxygen availability [6]. The most likely explanation is a pH- buffering effect of the nanoparticles.

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