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Heteroexpression of Bacteriophage T7 Lysozyme in *Magnetospirillum gryphiswaldense* Facilitates Large-Scale Extraction of Magnetosomes

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Abstract: To improve the efficiency of magnetosome extraction, a lysozyme expressing strain of *Magnetospirillum gryphiswaldense* MSR-1, named MLE was constructed. Compared to wild type, MLE showed no significant differences in cell growth and magnetosome formation. Colorimetric monitoring and transmission electron microscopy confirmed that MLE cells could be disrupted more effectively following freeze thaw treatment. Importantly, extraction of magnetosomes from the engineered strain could be easily performed on a large scale.

Key words: Extraction, lysozyme, magnetosomes, *Magnetospirillum gryphiswaldense*, China

INTRODUCTION

Magnetotactic bacteria synthesize magnetosomes which comprise a magnetic mineral crystal surrounded by a lipid bilayer membrane (Bazylinski and Frankel, 2004). Bacterial magnetosomes possess several unique features superior to those of synthetic magnetic nano particles. These features include paramagnetism, narrow size distribution on a nano scale and membrane-bound. Although, magnetosomes have been experimentally used as carriers for antibodies (Nakamura *et al*., 1991), nucleic acids (Takeyama *et al*., 1995) and chemotherapeutic drugs (Sun *et al*., 2007), their commercial applications were hindered by the lack of magnetosome production on a large scale (Heyen and Schuler, 2003). This includes the problems of mass cultivation and the efficiency of magnetosome extraction.

The conventional laboratory process for magnetosome extraction employs mechanical disruption of magnetotactic cells, usually by French press and sonication, followed by repeated cycles of enzymatic and sonic treatment interspersed with washing (Gorby *et al*., 1988; Nakamura *et al*., 1991; Sun *et al*., 2007). This process is both time-consuming and labour-intensive and is difficult to economically scale. The procedure is often simplified by reducing the rigour of disruption treatment, leading to inefficiencies in subsequent downstream processing operations. To improve the efficiency of magnetosome extraction, effective techniques for cell disruption are firstly required. Many methods have been used for process-scale disruption of microorganisms (Middelberg, 1995), for example, intracellular T7 lysozyme can effectively lyse cells even in small amounts and has been used in an in-fermenter chemical extraction procedure that degrades the cell wall of *Escherichia coli* and releases the inclusion bodies into the medium (Lee *et al*., 2004). However, the in-fermenter chemical extraction procedure is not appropriate for magnetotactic bacteria because Triton X-100 dissolves both cell membrane and magnetosome lipid bilayers (Gorby *et al*., 1988) and Benzonase is costlier due to lower magnetotactic cell density than that of *E. coli*. Freeze and thaw in the presence of lysozyme results in gentle lysis of *E. coli* cells (Ron *et al*., 1966). The commercially available *E. coli* strains like BL21 (DE3) plysS or Rosetta (DE3) plysS (Invitrogen) are also designed for easier cell disruption after expression, as they produce low amounts of T7 lysozyme. Unfortunately, there is no such option available for magnetotactic cells.

In this research, researchers firstly report the construction of a lysozyme-expressing strain of *Magnetospirillum gryphiswaldense*, MLE. The bacteriophage lysozyme was expressed from the promoter of *M. gryphiswaldense* glnBA operon and exhibited moderate expression when excess ammonia is present. Similar to the wild type, MLE cells harboring lysozyme expression vector grew and produced magnetosomes normally. Researchers also extend

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previous study by substituting chemical extraction with freeze and thaw followed by improved sonication for the preparation of clean magnetosomes. Importantly, researchers perform nonsolubilising extraction directly in fermentation media, coupled with enzymatic attack of the cell wall using constitutively expressed intracellular T7 lysozyme encoded by lys (Accession V01146 Region: 10706..11161) integreted in *Magnetospirillum* genome which provided a simple and effective method for large-scale extraction of magnetosome from magnetotactic cells.

MATERIALS AND METHODS

Bacterial strains, culture media and growth conditions: *E. coli* DH5a and S17-1 (Simon *et al*., 1983) strains were grown at 37°C in Luria-Bertani (LB) medium. *M. gryphiswaldense* MSR-1 (DSM6361) was grown at 30°C in Sodium Lactate and Ammonia (SLA) medium which contains (per L): 2.25 g Na-lactate, 0.1 g yeast extract, 0.41 g NH_4Cl, 0.5 g K_2HPO_4, 0.1 g $MgSO_4 7H_2O$, 0.05 g sodium-thioglycolate, 20 mg ferric citrate and 5 mL mineral mixture (Jiang *et al*., 2002). For the mating experiment, Sodium Lactate-Glutamate (SLG) medium (similar to SLA but with 4 g monosodium glutamate substituting for the 0.1 g yeast extract and 0.41 g NH_4Cl) was used.

For large-scale growth, an MLE strain was 1st grown in SLA medium with 5 μg chloramphenicol/mL to maintain the selection of an integrated plasmid. The fed-batch cultures were carried out in a 42 L bioreactor (Biostat C, B. Braun, Germany) which was loaded with SLA lacking K_2HPO_4 (about 60% of the fermentor volume), autoclaved (121°C, 30 min) and sparged with sterile air during continuous agitation. When the temperature of the medium fell to 30°C, sterilized K_2HPO_4 was added to 0.5 g/L. The concentration of dissolved oxygen was set to 0.5% (v/v) saturation (dissolved oxygen was defined as 100% when it reached saturation) and the agitating speed was automatically adjusted. The pH was controlled at 7.0 with HCl. A stock solution containing 900 mM sodium lactate, 684 mM ammonium choloride and 3.6 mM ferric citrate was added when lactate concentration was lower than 0.4 g/L.

Construction of the lysozyme expression vector: PCR amplification, DNA isolation, transformation and DNA manipulations essentially followed standard methods. As described in Fig. 1, pSUP202-glnA-lys was constructed by the ligation of PCR-generated MSR-1 glnA and bacteriophage T7 lysozyme gene (Dunn and Studier, 1983) into appropriate sites of plasmid pSUP202 (Simon *et al*., 1983). First, a 1.4 kb glnA is PCR-amplified with primers of p1e (5'-TTGGATCCATGGCCGACAAC-3', BamHI underlined) and p2e (5'-TTAAGCTTAGACCGA GTAGTAC-3', HindIII underlined) and cloned into the pSUP202 at BamHI and HindIII sites, yielding pSUP202-glnA. Next, a 610 bp T7 lysozyme gene was PCR-amplified with primers of p3 (5'-CCCAAGCTTTGATAGA TTAAAAAGG-3', HindIII underlined) and p4 (5'-CCATCGATA CACTTGGATATGC CTC-3', ClaI underlined) and inserted into pSUP202-glnA at HindIII and ClaI sites, yielding pSUP202-glnA-lys.

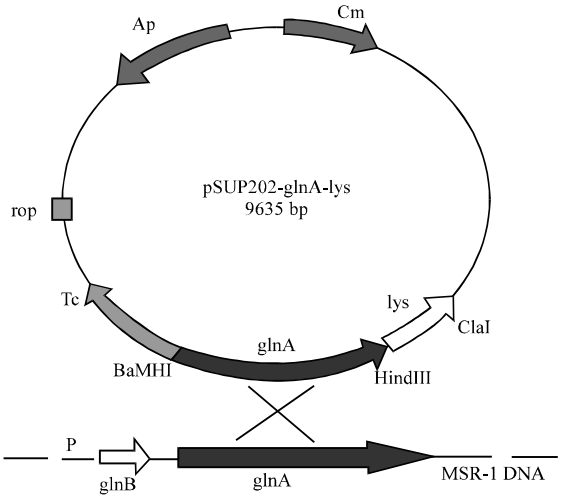

Fig. 1: Schematic diagram of a suicide vector pSUP202-glnA-lys integrated into the chromosome of *M. gryphiswaldense* MSR-1. P, glnB promoter

Mating experiments: The biparental conjugation was carried out as described with a minor modification (Li *et al*., 2005). The donor strain, an *E. coli* S17-1 derivative harboring pSUP202-glnA-lys was cultivated over night at 37°C in LB liquid medium, then diluted 5 times with LC liquid medium (similar to LB but with 5 g NaCl/L) and incubated at 30°C for another 3 h. The recipient strain *M. gryphiswaldense* MSR-1 was grown in SLA for 24-36 h until the cell density was approximate 1×10^9 cells/mL. The donor and recipient cultures were mixed in a ratio of 1-3 and then centrifuged and resuspended with 50 μL SLA medium. This bacterial suspension was spread evenly over the surface of a 0.22 μm pore size 25 mm membrane filter and incubated on SLG plate for 8 h. Cells were resuspended in 1 mL SLG medium and 0.1 mL aliquots were spread on selection plates with 5 μg nalidixic acid/mL and 5 μg chloramphenicol/mL and incubated at 30°C for 7-10 days. Isolated colonies were stabbed into a fresh broth containing chloramphenicol.

Enzymatic activity assay: The cell pellet was washed with 50 mM Tris/HCl (pH 7.0) and then suspended in 2 mL lys is buffer (50 mM sodium phosphate, pH 7.0, 0.5 M NaCl and 20 mM imidazole). The cell suspension was sonicated for 2 min by a Fisher Scientific Model 500 ultrasonic crusher (amplitude of 35%). After centrifugation, the supernatant was used for both protein and enzymatic activity assays. The protein concentration of cell extracts was determined by the Bradford Method, using crystalline BSA as the standard.

Glutamine Synthetase (GS) activity was determined, as described previously (Fisher and Sonenshein, 1984) using crude extracts in the transferase reaction. Lysozyme activity was determined with an A050-1 assay kit from Nanjing Jiancheng Bioengineering Institute (China) by following the decrease in absorbance at 450 nm of a *Micrococcus lysodeikticus* suspension in 0.1 M potassium phosphate buffer, pH 6.2 at 25°C which was adapted from a method of Colombie.

Iron content assay: Pellets from 2 mL fermentation broth were washed 3 times with 10 mM HEPES (pH 7.0), nitrified in 1 mL ultrapure nitric acid for 3 h and then diluted to 2 mL with ddH_2O. Iron content was assayed as previously described (Heyen and Schuler, 2003), using an atomic absorption spectrometer (Model Z8000, Hitachi, Japan).

Cell disruption and extraction of magnetosomes: Disruption by freeze-thaw treatment was carried out by freezing at 20 or 70°C for 2 h and thawing at room temperature for 1-2 h. It was done with 1-3 cycles.

For upscale extraction, fermentation broth was directly divided into 2 L aliquots, the cells were centrifuged at 4,000 g for 10 min and pellets were resuspended in 200 mL 0.1 M Phosphate Buffered Saline (PBS) (pH 7.4) and frozen. After the freeze-thaw treatment, suspensions were dispersed by a Fisher Scientific Model 500 ultrasonic crusher (amplitude of 20% for 15 min). Magnetosomes were absorbed using Neodymium Iron Boron magnets (100×100×25 mm, magnetic field is 0.5 T), re suspended and dispersed by low-level sonication (amplitude of 5% for 15 min, repeated for 30 cycles) and finally lyophilized using a freeze drier (Free Zone, Labconco, USA) as described previously (Sun *et al*., 2007).

Transmission electron microscopy: The suspension of cells or magnetosomes was added to a copper grid. After being air-dried, the grid was observed by transmission electron microscopy (JEM1230, Japan), as described previously (Sun *et al*., 2007).

RESULTS AND DISCUSSION

Construction of the MLE strain: As shown in Fig. 1, pSUP202-glnA-lys was constructed and integrated into the chromosome of *M. gryphiswaldense* MSR-1 by homologous recombination. In the lysozyme-expressing strain, designated MLE, the T7 lysozyme gene was cotranscribed with the glnBA operon from a glnB promoter.

Considering glnA encoded glutamine synthetase, a key enzyme of nitrogen metabolism, researchers put the *lysozyme* gene right after the *glnA* gene to avoid a possible polar effect. The correct construction was confirmed by PCR amplification and sequencing. The resulting MLE strain was stable in SLA medium without chloramphenicol.

Characterization of the MLE strain: The expression of the lysozyme and its effect on growth and magnetosome synthesis in MLE were 1st verified using a 250 mL serum bottle and a 2 L flask. Afterwards, scale-up inoculations were carried out in a 42 L bioreactor to monitor the expression of lysozyme and its effect on cell growth and magnetosome synthesis.

As shown in Fig. 2, lysozyme was expressed at moderate level (1.27±0.19 U/mg protein) in MLE during culture (Fig. 2a). The growth rate of MLE showed no significant difference compared to wild type for both wild type and MLE grew to 2.48±0.05 g DCW/l for approximately 60 h (Fig. 2b). Moreover, the activity of glutamine synthetase was comparable between MLE and wild type, indicating that the integration of T7 phage *lysozyme* gene into *M. gryphiswaldense* did not affect the expression of glnA (Fig. 2c). The intracellular iron content, a prerequisite for magnetosome formation was also measured and no significant difference was found (Fig. 2d). Since, magnetosome-bound iron was assumed to constitute >99% of total cellular iron in highly magnetic cells (Heyen and Schuler, 2003), the yields of magnetosomes from MLE and wild type were calculated as 30.81±1.04 and 32.86±1.28 mg/g DCW, respectively. All the results suggested that MLE cells could tolerate intracellular lysozyme and synthesize magnetosomes to a comparable level of wild type.

Effect of freeze-thaw treatment on disruption of MLE cells: As described in materials and methods, the freeze-thaw treatment was used to disrupt cells. The A_{260} and A_{280} were measured to initially evaluate the effect of freeze-thaw treatment on cell disruption, As shown in Fig. 3, MLE cells released larger amounts of nucleic acids and proteins than wild-type cells after freeze-thaw

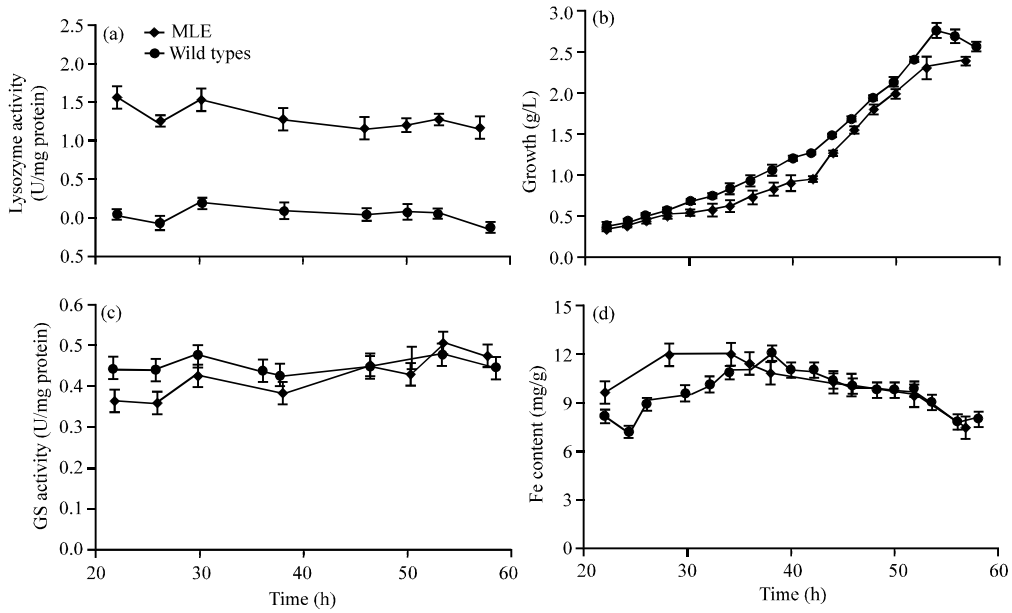

Fig. 2: Comparison of the level of: a) Lysozyme; b) Growth rate; c) Glutamine synthetase activity; d) Fe content in MLE and wild type during growth in a 42 liter bioreactor. Error bars represent standard deviations for 3 experiments

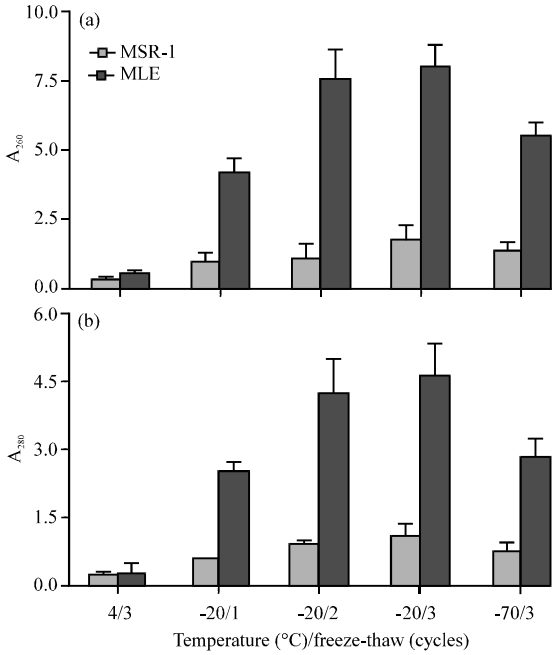

Fig. 3: Effects of the freezing temperature and the freeze-thaw cycles on the disruption of MLE and wild-type cells. Broth samples were diluted to OD_{565} of 1.00 and subjected to freeze-thaw treatments. After centrifugation, the supernatants were monitored by optical density at 260 and 280 nm on a Beckman DU 640 spectrophotometer with untreated supernatant as blank. Error bars represent standard deviations for three experiments

treatment indicating that this treatment was effective to facilitate intracellular lysozyme to disrupt MLE cells. Temperature and freeze-thaw cycles affected the efficiency of cell disruption. Freezing at 20°C showed higher A_{260} or A_{280} values when compared to the freezing at 70°C, indicating that a mild freeze treatment was more effective for cell disruption, probably due to the larger crystal formed at 20°C than at 70°C. Repeated freeze-thaw cycles at 20°C resulted in increased cell breakage, so that, 3 freeze-thaw cycles were adopted in the following work.

The pH value and the components of the cell suspensions affected the function of the lysozyme, with the optimal pH at 6.24; however, direct freeze-thaw treatment of the broth is probably the best choice for an upscale sample (Fig. 4). Both the extraction and filtration methods exerted their efficacy directly on a crude fermentation broth, eliminating the need for cell recovery and resuspension in buffer.

The effect of freeze-thaw treatment on disruption of cells was further confirmed by transmission electron microscopy. Electron micrographs clearly showed that MLE cells were disrupted with chains of magnetosomes being dispersed against a background of cell inclusion and debris after 3 freeze-thaw cycles as compared with the cell before treatment (Fig. 5ab). However, wild-type cells remained almost identically intact before and after 3 freeze-thaw cycles (Fig. 5cd). These results indicated that freeze-thaw treatment could efficiently disrupt MLE cells.

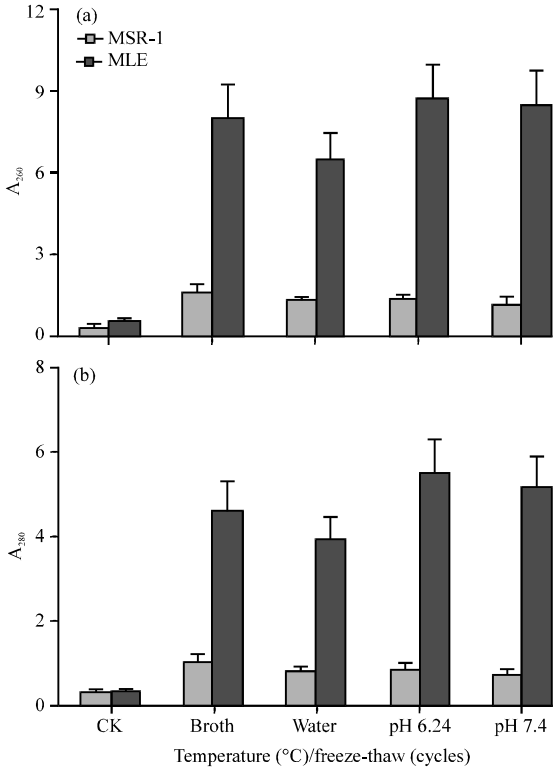

Fig. 4: Effects of freeze/thaw treatments on MLE and wild-type cells in different solutions. The 57 h sample was resuspended in fermentation broth supernatant, pure water, 100 mM KH_2PO_4/K_2HPO_4 buffer (pH 6.24), 100 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.4) and carried out 3 freeze/thaw cycles and monitored by optical density at 260 and 280 nm

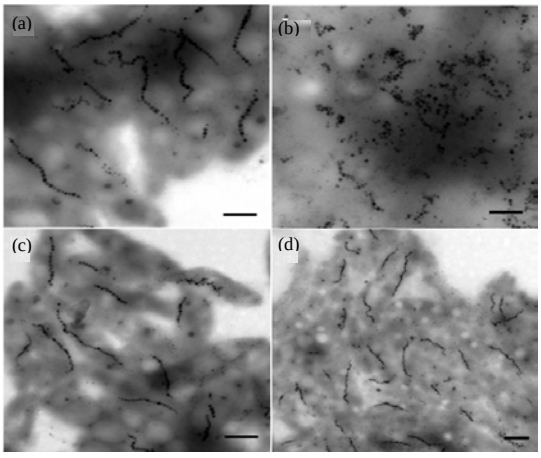

Fig. 5: Transmission electron micrographs of *M. gryphiswaldense*: a) MLE before; b) After 3 freeze-thaw cycles; c) Of wild type before; d) After 3 freeze-thaw cycles. The bar indicates 500 nm

Table 1: Effect of sonication on yields of magnetosomes from *M gryphiswaldense*

	Yields of magnetosomes (mg/g DCW)[b]	
Sonication cycle(s)[a]	Wild type	MLE
0	<0.2 (0.6)[c]	2.6±0.5 (8.4)
1	0.4±0.1 (1.2)	15.3±2.3 (49.7)
6	3.0±0.4 (9.1)	15.9±2.6 (51.6)
13	15.7±2.8 (47.8)	ND[d]

[a]After 3 freeze-thaw cycles, suspensions of wild type and MLE were further subjected to relevant cycles of sonication (One cycle was set, as amplitude of 20% and 4, 6 sec pause for 15 min); [b]DCW = Dry Cell Weight which was obtained by drying the washed cell pellet to a constant weight at 60°C (Heyen and Schuler, 2003). A culture density of OD_{565} at 1.0 corresponds to 0.30 g DCW/L; [c]Extraction yield (%) = Yields of magnetosomes/calculated yields of magnetosomes based on iron content; [d]ND = Not Determined; the data shown are the means±SD (n = 3)

Upscale extraction of magnetosomes from MLE cells: To scale up the extraction of magnetosomes, broth aliquots underwent freeze-thaw treatment simultaneously, just as in an in-fermenter way (Lee *et al*., 2004). After freeze-thaw treatment, large magnets were used and sonication conditions were explored to disperse conglomerate granules of inclusion bodies and debris.

As shown in Table 1, one sonication cycle for MLE could increase the extraction yield by 4 fold, i.e., from 2.6±0.5-15.3±2.3 mg/g DCW. More treatments did not increase the overall yield significantly, indicating that magnetosomes bound to debris were ultimately released by one ultrasonic cycle (amplitude of 20% for 15 min). However, one sonication cycle only slightly affected wild type as compared with MLE, although an approximate value of 15.7±2.8 mg/g DCW was obtained by using 13 sonication cycles. With the in-fermenter like disruption and the optimal sonication, extraction of magnetosomes from MLE cells was carried out with a recovery yield of 51.60%, comparable to that of 47.78% from wild-type cells. Moreover, magnetosomes extracted from MLE were intact and qualified for subsequent applications (Fig. 6) (Sun *et al*., 2007).

Concerns have arisen in the mass production of magnetosomes (Jiang *et al*., 2002; Heyen and Schuler, 2003) and about the effectiveness of magnetosome extraction from the cell. Researchers report here the construction of MLE, a lysozyme expressing *M. gryphiswaldense* strain which could facilitate the large-scale magnetosome extraction.

The MLE strain can tolerate intracellular lysozyme and normally synthesize magnetosomes, probably due to the controlled transcription of the *lysozyme* gene from a glnB promoter (Johansson and Nordlund, 1996; Kim *et al*., 2004). Under the current conditions with excess ammonia, mass cultivation of MLE can be easily performed in parallel with the wild type without changing any of the parameters.

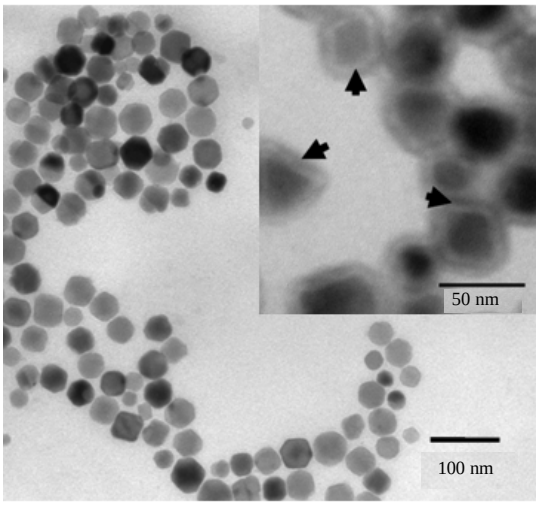

Fig. 6: Transmission electron micrographs of purified magnetosomes from *M. gryphiswaldense* MLE. Inset shows magnification of narrow size distribution (35-55 nm) of magnetosomes and magnetosome membrane (arrows)

The in-fermenter chemical extraction procedure for *E. coli* was not appropriate for magnetotactic bacteria because of the problems associated with mass cultivation and the protection of the unique functional magneto some membrane. Although, freeze-thaw treatment can efficiently disrupt MLE cells, researchers could not avoid the common issues affecting separation and purification of magnetosomes, such as in removing cell inclusion bodies and debris. Following the disruption of cells, the immediate viscosity increase resulted in partial aggregation. Because repetitive sonication cycles were used in the present method, a higher power output was easily adopted to disperse the conglomerate granules, although other methods such as nuclease treatment (Gorby *et al*., 1988) and shearing or precipitating nucleic acids may also be effective (Burgess and Guthrie, 1993). Because of the effectiveness of disruption, the overall time for extraction was greatly diminished.

CONCLUSION

The results presented here show a method for the extraction of magnetosomes using an engineered *M. gryphiswaldense* strain that expresses lysozyme and the use of repeated freeze-thaw cycles. The combination of these 2 factors showed an increase in cell disruption and magnetosomes separation. This demonstrates that the MLE strain has the potential for large-scale magnetosome production and applications.

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