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Manuscript Draft

Manuscript Number: MLBLUE-D-15-04085R1

Title: Preparing and characterizing Fe3O4@cellulose nanocomposites for effective isolation of cellulose-decomposing microorganisms

Article Type: Short Communication

Keywords: Magnetic nanoparticles; Cellulose; Uncultivable microorganisms; Cellulose-decomposing

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Abstract: This study developed Fe3O4@cellulose nanocomposites by co-precipitation synthesis for bacteria capture and isolation. By surface modification with cellulose, the Fe3O4@cellulose nanocomposites have 20 nm average particle size and 3.3-24.9 emu/g saturation magnetization. Living bacteria could be captured by the Fe3O4@cellulose nanocomposites and harvested by magnetic field, with high efficiency (95.1%) and stability (>99.99%). By metabolizing cellulose and destroying the Fe3O4@cellulose@bacteria complex, cellulose-decomposing microorganisms lost the magnetism. They were therefore able to be isolated from the inert microbial community and the separation efficiency achieved over 99.2%. This research opened a door to cultivate the uncultivable cellulose-decomposing microorganisms in situ and further characterize their ecological functions in natural environment.

Cover Letter

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19/08/2015

Dear Editor

Manuscript title: Preparing and characterizing Fe_3O_4@cellulose nanocomposites for effective isolation of cellulose-decomposing microorganisms

Magnetic nanoparticles (MNPs) have been widely applied in biomedical and biological research.Surface modified MNPs are recently used to investigate the microbial behavior and functions in a complex microbiota, but the modification method is not well established for wider range of functional bacteria. This study developed a new surface modification method and synthesized the Fe_3O_4@cellulose nanocomposites.The bacterial capture efficiency was above 95.1% and the stability is above 99.99%. More importantly, the Fe_3O_4@cellulose nanocomposites successfully isolate the cellulose-decomposing *Aeromonasveronii* from an artificial microbial community.This work broadens the applicable potential of MNPs in assessing more unknown cellulose-decomposing bacteria in natural environment and their metabolic pathways.

Novelty statement

- Fe3O4@cellulose nanocomposites can be used for the first time to isolate cellulose-decomposing bacteria from complex microbial community.
- Fe3O4@cellulose achieves high bacteria capture efficiency (>95.1%) and stability (>99.99%).
- Fe3O4@cellulose successfully isolates cellulose-decomposing *Aeromonasveronii*and the separation efficiency is 99.2%.

Conflict of Interest

No conflict of interest exits in the submission of this manuscript, and the manuscript has approved by all authors for publication. The authors would like to declare that the work described is original research that has not been published previously, and is not under consideration for publication elsewhere, in whole or in part. It has been submitted to *Materials Letters* before (MLBLUE-D-15-02845) and is re-submitted after the inquiry of Editor Prof. A.F.W. Willoughby.

Thanks for your consideration. If you have any questions, please feel free to contact with me.

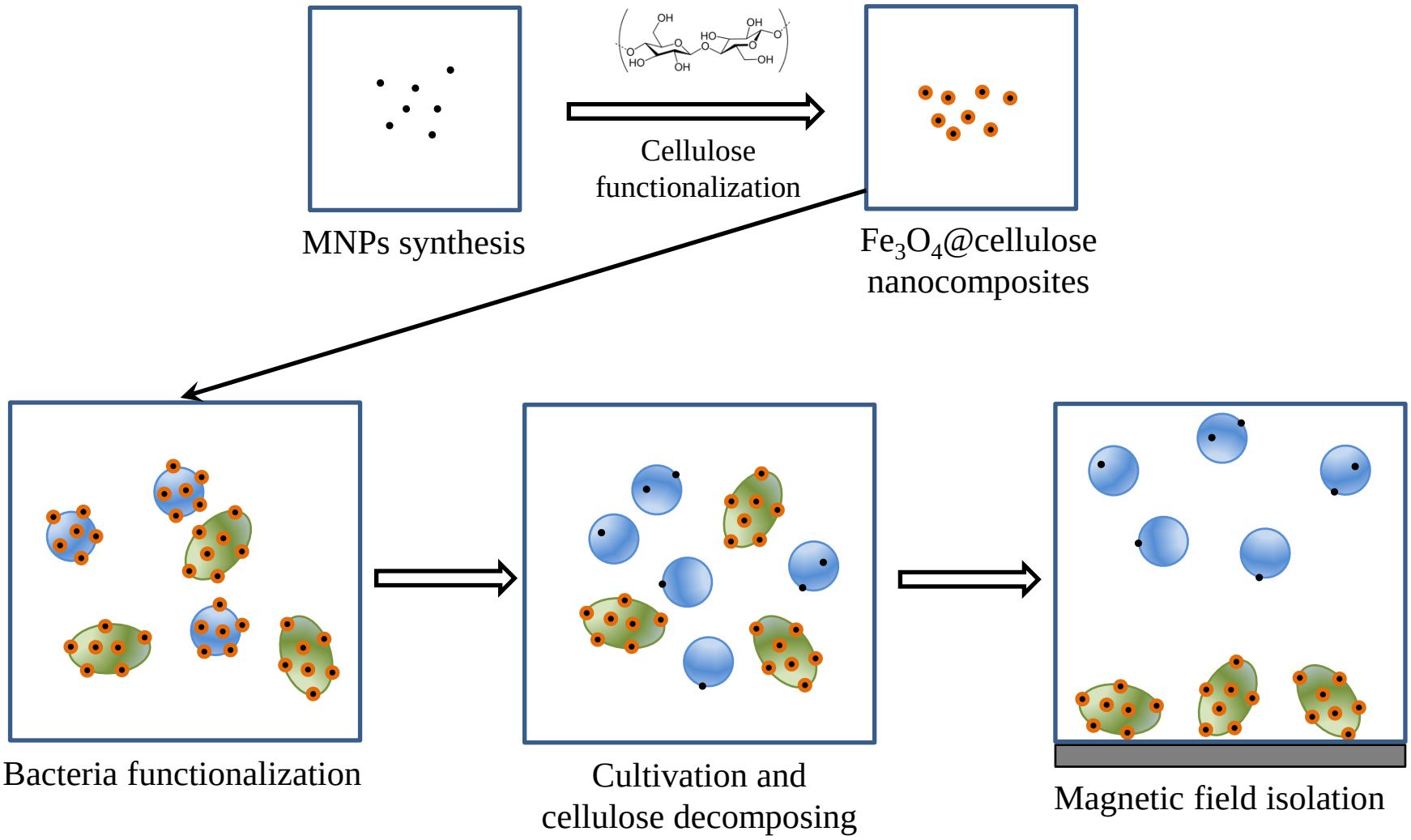
Yours sincerely

Dr Dayi Zhang

Response to Reviewers

Thanks for the reviewers' comments and editors's suggestion. We have corrected the manuscript by adding Figure S1, 2 and 3 into the main manuscript and correcting the Figure number and caption.

Graphical Abstract



Highlight

1. Fe_3O_4@cellulose nanocomposites for cellulose-decomposing bacteria isolation.
2. Bacteria capture efficiency >95.1% and stability >99.99%.
3. Cellulose-decomposing bacteria separation efficiency over 99.2%.
4. Fe_3O_4@cellulosecan identify unknown cellulose-decomposingmicrobes*in situ*.

Preparing and characterizing Fe_3O_4@cellulose nanocomposites for effective isolation of cellulose-decomposing microorganisms

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Abstract

This study developed Fe_3O_4@cellulose nanocomposites by co-precipitation synthesis for bacteria capture and isolation. By surface modification with cellulose, the Fe_3O_4@cellulose nanocomposites have 20 nm average particle size and 3.3-24.9 emu/g saturation magnetization. Living bacteria could be captured by the Fe_3O_4@cellulose nanocomposites and harvested by magnetic field, with high efficiency (95.1%) and stability (>99.99%). By metabolizing cellulose and destroying the Fe_3O_4@cellulose@bacteria complex, cellulose-decomposing microorganisms lost the magnetism. They were therefore able to be isolated from the inert microbial community and the separation efficiency achieved over 99.2%. This research opened a door to cultivate the uncultivable cellulose-decomposing microorganisms *in situ* and further characterize their ecological functions in natural environment.

Keywords:

Magnetic nanoparticles; Cellulose; Uncultivable microorganisms;
Cellulose-decomposing

1. Introduction

With the capability of remote control by magnetic field, magnetic nanoparticles (MNPs) introduce many possibilities in biochemical processes as a novel tool in microbial biotechnology [1]. MNPs surface modification is widely investigated to improve their stability and biocompatibility. Antigen functionalized MNPs achieve high throughput bacteria or cell separation by flow cytometry [2], and chitosan functionalization allows accurate and targeting gene/drug delivery via MNPs by the tagging biological entities [3]. In environmental engineering, MNPs are functionalized with poly-allylamine-hydrochloride to improve biosensor sensitivity [4, 5], and remove pathogens for drinking water purification [6, 7].

Uncultivable microorganisms account for over 99% of all the species and their functions are important for ecological system [8]. Particularly, cellulose metabolism is a key component of carbon cycle on the planet [9], but the majority of cellulose-decomposing microorganisms remain uncultivable and unknown. The recent progress to cultivate the uncultivable microorganisms with MNPs is the cutting edge for environmental ecology [10], opening a door to reveal the physiological behaviour and ecological functions of uncultivable bacteria from complex microbial community. Nevertheless, the macromolecular poly-allylamine-hydrochloride reduces the accessibility of cellulose-decomposing microorganisms to cellulose. New surface functionalization technique can broaden its applicable potential in assessing the fate of various polymers in natural environment.

We developed a novel cellulose functionalization method and prepared the biocompatible Fe_3O_4@cellulose nanocomposites. Two different bacterial strains, *Acinetobacter baylyi* and *Aeromonas veronii* (cellulose-decomposing bacterium),

were functionalized by Fe_3O_4@cellulose nanocomposites and investigated for their magnetism change after cultivation. The successful isolation of *A. veronii* from *Acinetobacter-Aeromonas* community proved the feasibility to cultivate the functional cellulose-decomposing bacteria *in situ*.

2. Experimental Section

Synthesis of Fe_3O_4@cellulose nanocomposites: All the chemicals were analytical grade from Sigma-Aldrich (UK) without specific statement. Cellulose suspension was prepared by dissolving 0.4 g cellulose in 20 mL alkaline solution (NaOH:urea:H_2O=7:12:81), mixed well and standing at 4°C overnight [11]. MNPs were synthesized by co-precipitation method [4]. The Fe_3O_4@cellulose nanocomposites was subsequently synthesized by gently mixing the MNPs and cellulose suspension (with ratios of 0.60, 0.55, 0.50, 0.45, 0.43, 0.36, 0.30, 0.24, 0.18, 0.12 and 0.06, m/m) for 5 minutes, captured by permanent magnet for 5 minutes, and finally washed by deionized water 2 to 3 times until the pH value was 7.0.

Cellulose-decomposing microorganisms isolation from microbial community: *Acinetobacter baylyi* (no cellulose-decomposing capacity) and *Aeromonas veronii* (cellulose-decomposing bacterium) were used in this study. The artificial microbial community was made by mixing *A. baylyi* and *A. veronii* in water (1:1). To isolate the cellulose-decomposing microorganism from *A. baylyi*, *A. veronii* and microbiota, a hundred microliter of each bacterial suspension (diluted to 1.0×10^8 CFU/mL) was mixed with 900 µL Fe_3O_4@cellulose suspension. After successful functionalization and cultivation for 5 days, the targeting cellulose-decomposing bacteria were harvested from the supernatant (Graphic abstract, details see Supplementary Material).

Measurements and data analysis: The morphology of MNPs and Fe_3O_4@cellulose nanocomposites were analysed by transmission electron microscopy (TEM, JEM-2100, 100 kV, Japan). Phase identification was carried out by X-ray diffraction (XRD,

D8-Advance, Bruker, UK). The magnetic properties were measured by a vibrating sample magnetometer (VSM, Lake Shore, 7304, USA) at 25°C and in a magnetic field varying from -1.7 T to +1.7 T. The nanoparticle fingerprint was obtained by InVia Raman microscopy (Renishaw, UK) with a 785-nm excitation laser and 10 second acquisition time. The number of magnetic-free bacteria was determined by quantitative polymerase chain reaction (qPCR, Supplementary Material) [12, 13].

3. Results and discussion

From the TEM morphology (Fig. 1A), raw MNPs showed a round shape and had strong self-aggregation attributing to the large surface-to-volume ratio and the expressed surface energy [14]. The XRD pattern (Fig. 1C) identified the diffraction peaks of MNPs as 2θ=30.0°, 35.4°, 43.2°, 53.6°, 57.1° and 62.7°, indexed to (220), (311), (400), (422), (511) and (440) lattice planes [15]. The mean size of MNPs was calculated as 8 nm by Scherer equation ($D=\kappa\lambda/\beta\cos\theta$). Fe_3O_4@cellulose nanocomposites had bigger size (20 nm, Fig. 1B) but with less aggregation since polymer functionalization could improve their stability by steric repulsion [4].

The Raman spectra (Fig. 1D) showed that the characteristic peaks of Fe_3O_4@cellulose nanocomposites fitted well with those of MNPs (magnetite at 678 cm^{-1}) and cellulose (υ(C-O-C) asym at 1094 cm^{-1} and 1120 cm^{-1}, υ(C-O-C) at 906 cm^{-1}, $\delta(CH_3)$ at 1380 cm^{-1} and $\delta(CH_3)$ asym at 1460 cm^{-1}) [16]. All the magnetization curves behaved S shape, and raw MNPs had the highest the saturation magnetization (43.4 emu/g, Fig. 1E). The saturation magnetization of Fe_3O_4@cellulose was positively related to the ratio of MNPs to cellulose, as 24.9 emu/g for 0.6:1 (MNPs:cellulose), 11.4 emu/g for 0.4:1 and 3.3 emu/g for 0.12:1, respectively.

Fe_3O_4@cellulose nanoparticles could effectively capture bacteria via electrostatic adsorption. The ratio of MNPs to cellulose affected the bacteria capture efficiency (Fig. 2A). When the MNPs:cellulose ratio was above 0.1, the bacteria

capture efficiency was above 90%, whereas it declined to 84.3% at the ratio of 0.06. The optimized ratio was set as 0.4 to achieve both high capture efficiency and sufficient cellulose for bacterial growth.

The capture efficiency was above 90% when the bacterial amount was less than 4.0×10^{14} CFU/g Fe_3O_4@cellulose (Fig. 2B). Langmuir isotherm equation (Equation 1) can describe the adsorption isotherm of Fe_3O_4@cellulose nanocomposites and fitted well with the experimental data (Fig. 2B).

$$Q_e = Q_{max}\frac{K_L C_e}{1+K_L C_e} \qquad (1)$$

Here, Q_e (CFU/g Fe_3O_4@cellulose) refers to the captured bacterial cells on the Fe_3O_4@cellulose surface, and C_e (CFU/mL) represents the equilibrium bacterial amount in the suspension. Q_{max} (CFU/g Fe_3O_4@cellulose) is the maximum adsorption capacity for monolayer adsorption in Langmuir isotherm model, and K_L (mL/CFU) is the Langmuir constant associated with adsorption energy. In this study, Q_{max} is 17.57×10^{14} CFU/g Fe_3O_4@cellulose, and K_L is 187.56×10^{-14} mL/CFU. The results described the monolayer adsorption equilibrium of the adsorbed bacterial cells on Fe_3O_4@cellulose nanocomposites. Given the larger particle size of Fe_3O_4@cellulose nanocomposites than raw MNPs, a significant less specific surface area was found for the bacteria captured by Fe_3O_4@cellulose than cyanobacteria harvesting by raw MNPs [17]. Nevertheless, the high K_L value indicated the high binding affinity of bacteria to Fe_3O_4@cellulose nanocomposites. Since the electrostatic mechanism is the ion exchange interaction between carboxyl (-COOH) or thiol (-SH) functional groups on bacterial membrane and the positively charged position on Fe_3O_4@cellulose nanocomposites [7], the electrostatic adsorption of living bacteria is therefore non-selective. This feature secures to magnetize all the bacteria via Fe_3O_4@cellulose nanocomposites in the environmental microbiota.

Fe_3O_4@cellulose@bacteria complex had high stability and bacteria maintained their magnetism even after long period storage. Resuspended in deionized water, the majority of both *A. baylyi* and *A. veronii* remained effective attachment on Fe_3O_4@cellulose nanocomposites (Fig. 2C), and the released magnetic-free bacteria were less than 2×10^5 CFU/mL. The short-term stability was 99.992% for *A. baylyi* and 99.987% for *A. veronii* respectively. Stored in deionized water, only less than 1.5×10^4 CFU/mL cells of *A. baylyi* or *A. veronii* were detected in the supernatant after 5 days (Fig. 2D), and the long-term Fe_3O_4@cellulose@bacteria stability was over 99.99%. Cultivated in M9 medium, *A. baylyi* could not metabolize cellulose, remaining inert and captured by the Fe_3O_4@cellulose with less than 6.0×10^4 CFU/mL in the supernatant (<0.007% of the total bacteria amount, Fig. 2E). As for cellulose-decomposing *A. veronii*, the Fe_3O_4@cellulose@bacteria complex was destroyed due to cellulose consumption and *A. veronii* were then released from the magnetic pellet into the supernatant. The enriched *A. veronii* raised from 3.4×10^4 CFU/mL (Day 1) to 4.4×10^6 CFU/mL (Day 5).

After 5 days cultivation of the artificial microbial community of *A. veronii* and *A. baylyi*, Fe_3O_4@cellulose nanocomposites successfully isolated the cellulose-decomposing *A. veronii* from the complex microbiota (Fig. 3). Without Fe_3O_4@cellulose functionalization (direct cultivation), inert *A. veronii* ranged from 32.8% to 61.7% of the whole microbial community after 5 days cultivation, not significantly dominant and hard to be isolated (Fig. 3). In Fe_3O_4@cellulose treatment, the targeting *A. veronii* became dominant on Day 4 (65.3%) and Day 5 (99.2%). Thus, the cellulose-decomposing *A. veronii* was enriched and isolated in the supernatant from the artificial microbiota. The results prove that Fe_3O_4@cellulose nanocomposite is an effective tool to identify and isolate living cellulose-decomposing bacteria from

environmental microbiota and the magnetic-free fraction is suitable for further analysis of cellulose degradation genes and pathways.

4. Conclusions

By co-precipitation synthesis and surface functionalization with cellulose, living bacteria were successfully captured and harvested by Fe_3O_4@cellulose nanocomposites with high efficiency (95.1%) and stability (>99.99%). The Fe_3O_4@cellulose nanocomposites achieved 99.2% isolation efficiency of cellulose-decomposing bacteria from artificial microbial community. With the novel Fe_3O_4@cellulose nanocomposites functionalization method, uncultivable and unknown cellulose-decomposing bacteria are able to be effectively isolated from complex microbiota to investigate the potential new cellulose-decomposing functional genes and reveal their ecological roles in natural environment.

5. Acknowledgement

The authors would like to thank National Natural Science Foundation of China (No. 41301331) for financial support.

6. Reference

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Figure caption

Fig. 1. TEM images of MNPs (A) and Fe_3O_4@cellulose composites (B). The XRD pattern of Fe_3O_4@cellulose composites (C). Raman microscopy of cellulose, MNPs and Fe3O4@cellulose (D). The magnetization curve of synthesized MNPs and Fe_3O_4@cellulose (E).

Fig. 2. (A) Bacteria capture efficiency against the ratio of MNPs to cellulose during synthesis process. (B) Bacteria capture efficiency against the ratio of bacteria amount to MNPs weight. (C) Bacteria functionalization stability of *A. baylyi* and *A. veronii*. (D) Long-term (5 days) stability of Fe_3O_4@cellulose@bacteria complex in water. (E) The isolation efficiency of Fe_3O_4@cellulose@bacteria complex after 5 days cultivation in M9 medium.

Fig. 3. Cellulose-decomposing bacteria isolation from artificial *Acinetobacter-Aeromonas* microbial community by cultivation and Fe_3O_4@cellulose cultivation.

Figure

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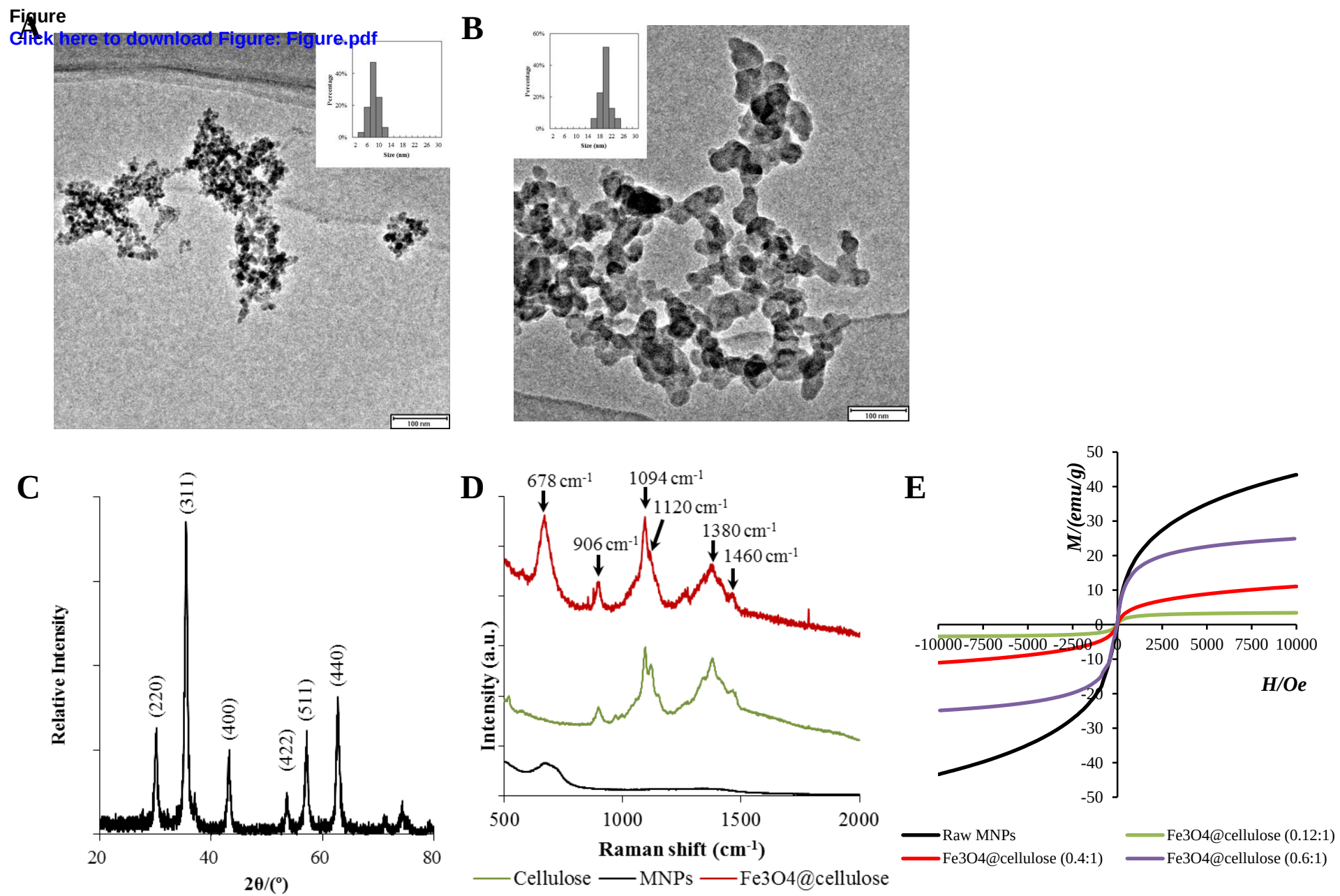

Fig. 1

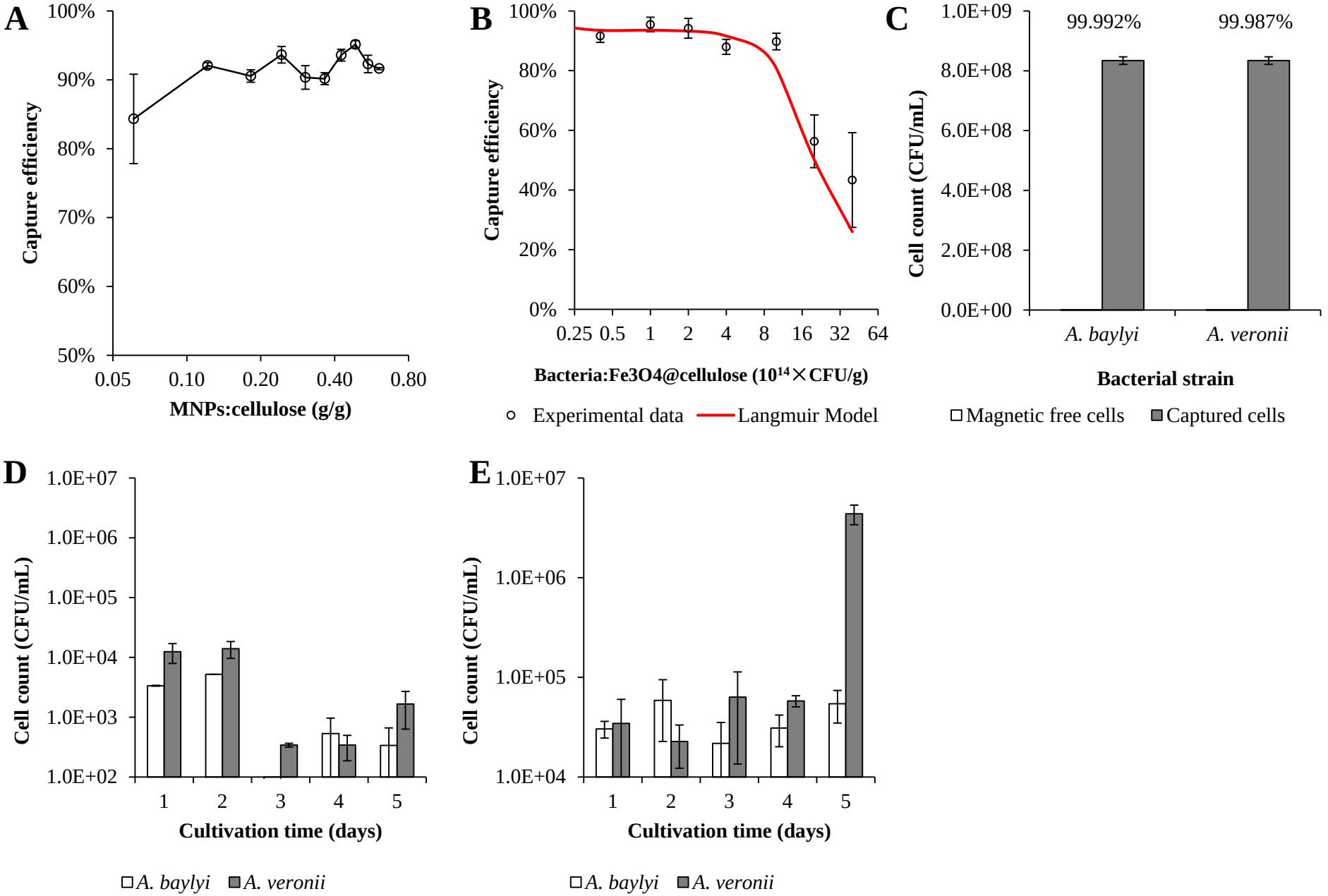
A
100%
90%
80%
70%
60%
50%
Capture efficiency
0.05
0.10
0.20
0.40
0.80
MNPs:cellulose (g/g)
B
100%
80%
60%
40%
20%
0%
Capture efficiency
0.25
0.5
1
2
4
8
16
32
64
Bacteria:Fe3O4@cellulose (10¹⁴×CFU/g)
Experimental data
Langmuir Model
C
1.0E+09
8.0E+08
6.0E+08
4.0E+08
2.0E+08
0.0E+00
Cell count (CFU/mL)
99.992%
99.987%
A. baylyi
A. veronii
Bacterial strain
Magnetic free cells
Captured cells
D
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
Cell count (CFU/mL)
1
2
3
4
5
Cultivation time (days)
A. baylyi
A. veronii
E
1.0E+07
1.0E+06
1.0E+05
1.0E+04
Cell count (CFU/mL)
1
2
3
4
5
Cultivation time (days)
A. baylyi
A. veronii

Fig. 2

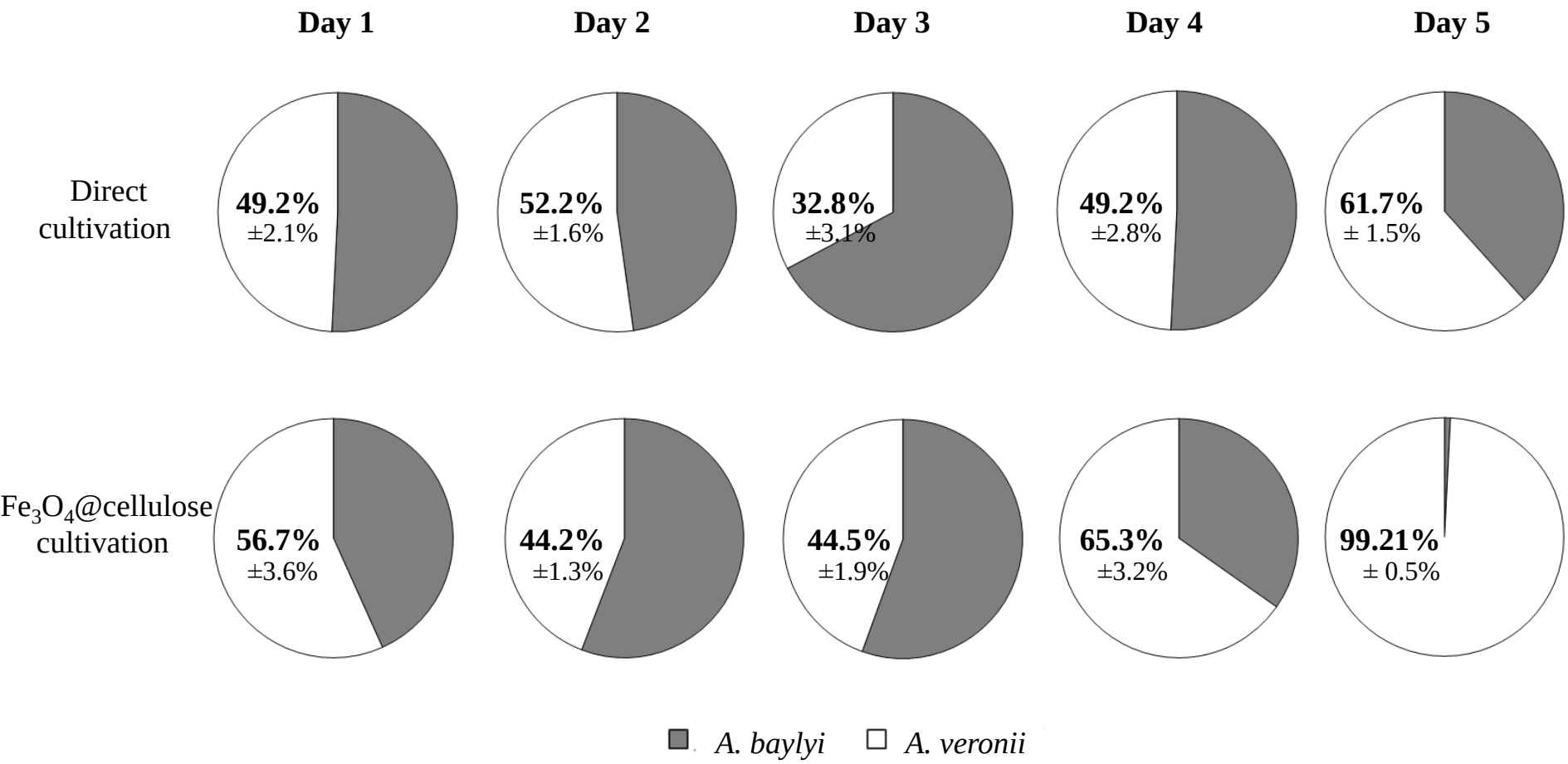
Day 1
Day 2
Day 3
Day 4
Day 5
Direct cultivation
49.2% ±2.1%
52.2% ±1.6%
32.8% ±3.1%
49.2% ±2.8%
61.7% ± 1.5%
Fe_3O_4@cellulose cultivation
56.7% ±3.6%
44.2% ±1.3%
44.5% ±1.9%
65.3% ±3.2%
99.21% ± 0.5%
A. baylyi
A. veronii

Fig. 3

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