SUPPLEMENTARY MATERIAL

Role of silica coated magnetic nanoparticle on cell flocculation, lipid extraction and linoleic acid production from *Chlorella pyrenoidosa*

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Abstract

In the present work, it has been observed that magnetic (Fe₃O₄) - silica core- shell nanoparticles helps in flocculation of *Chlorella pyrenoidosa* cells with simultaneous production of linoleic acid. The mean particle size in Dynamic light scattering (DLS) of the silica coated magnetic nanoparticle was estimated 444.7 nm. The characterization of nanoparticles was also performed by X-ray diffraction technique (XRD). Apart from flocculation, it has been observed that in presence of magnetic silica core- shell nanoparticles the amount of lipid obtained was four times than that of control. On the contrary, in presence of these nanoparticles, linoleic acid (18:2) has been produced in *Chlorella pyrenoidosa* cells almost by 80% whereas, it has been noticed only 8.73% in control. This is the first report where the linoleic acid has been obtained as major component of microalgal fatty acid methyl esters (FAME) having important application in neutraceutical and pharmaceutical sectors.

**Keywords:** Microalgae, silica coated magnetic nanoparticle, magnetic flocculation, lipid extraction, FAME, linoleic acid
4. Experimental

4.1. Synthesis of Nanoparticles

4.1.1 Synthesis of silica nanoparticles

Silica nanoparticles were synthesized from tetraethoxy orthosilicate (TEOS) by sol gel method. The overall scheme for the synthesis and microalgae flocculation is represented in Figure S 7.

4.1.2 Synthesis of modified magnetic nanoparticles

To prepare the magnetic nanoparticles, ferric chloride (2.6 g) and ferrous chloride (1g) were dissolved in 120 ml of distilled water at room temperature (25°C). Then 10 ml of liquor ammonium was added under vigorous stirring at 60°C for 1 h. The magnetic nanoparticles were separated from the suspension by centrifugation at 8000 rpm and re-dispersed in 200 ml of trisodium citrate (0.3M) solution. The suspension was stirred again for 1 hour at 80°C, centrifuged and washed with acetone. The dark black precipitate of modified magnetic nanoparticles were obtained (Alizadeh, et al. 2012)

4.1.3 Synthesis of silica coated magnetic nanoparticles (Fe₃O₄@SiO₂)

For the synthesis of silica coated magnetic nanoparticles, 1g of magnetic nanoparticle was taken and dispersed in 50 ml of distilled water and sonicated for 10 min. To the above suspension, 3 ml of liquor ammonia and 50 ml of ethanol was added. The resultant mixture was stirred continuously at 40°C. Separately, 1ml of TEOS was added in 20 ml of ethanol and this ethanolic solution of TEOS was added to previously prepared ammonical solution of magnetic nanoparticles. Light brown colour precipitate was obtained after 14 h of continuous stirring, which indicated that the silica coating over the core black magnetic nanoparticles. The precipitate was separated by centrifugation at 4000 rpm, washed with water and dried at 100°C to get Fe₃O₄@SiO₂ nanoparticles (Alizadeh et al. 2012). The process and its application in microalgae flocculation is summarised in Fig.1.

4.2. Characterization

The particle size of the formed nanoparticles was characterized by Dynamic light scattering (DLS) using model Malvern S-90, UK. The crystallinity of the nanoparticles was checked by X-Ray diffraction (XRD) studies in the range of 10-80° using Miniflex 600 Diffractometer.

4.3. Cell Culture and Flocculation

4.3.1 Microalgae and growth conditions

Chlorella pyrenoidosa NCIM 2738, obtained from National Collection of Industrial Microorganism, National Chemical laboratory, Pune, India was grown photoautotrophically in 5L Erlenmeyer flask carrying 3L sterilised Fogg’s media (Rai et al. 2013). The cultures were executed in temperature controlled culture room at 28±1°C providing 24 hour continuous illumination with light intensity of 40.5 μmol m⁻² s⁻¹. Inoculation was carried out
using exponentially growing cells such that initial optical density of the culture is 0.1. The set up was maintained for 15 days in photoautotrophic culture conditions.

4.3.2 Cell harvesting using Nanoparticles
In 500 ml microalgae culture 1.25 g of magnetic silica core shell and silica nanoparticles were added in two flasks separately and one was kept as control. To utilize the magnetic property of Fe$_3$O$_4$ silica core shell nanoparticles, common house magnet was placed at the bottom of the flask. The Optical density of the supernatant was observed after every hour upto three hours using UV-Vis spectrophotometer (Shimadzu UV-1800). Cells were collected in pre-weighed petri dishes and total biomass was measured. Flocculation efficiency was calculated by using following equation:

Flocculation efficiency (%) = (OD$_i$ - OD$_f$ / OD$_i$) × 100

Where, OD$_i$ and OD$_f$ are optical density before and after the flocculation.

4.3.3. SEM analysis
The C. pyrenoidosa cells have been studied by scanning electron microscopy (Carl Zeiss microscope, SUPRA 40 model, Germany) with and without incorporating nanoparticles. The cell samples were taken during the process of lipid extraction in presence of nanoparticles and compared with control cells.

4.4. Lipid extraction and estimation
Fenton’s reagent was prepared according to the standard protocol for lipid estimation (Stretti et al. 2014). The reagent was poured dropwise to 1g of wet algal biomass harvested from each mentioned culture set up. The mixture was left for constant stirring for 18 hours using magnetic stirrer. Cells were separated by centrifugation at 5000 rpm for 15min and the lipid was recovered using hexane: water mixture with 2:3 ratio. The upper layer was taken and dried to estimate lipid content in each set to obtain the effect of nanoparticles on lipid extraction compared to control.

4.5. Conversion of microalgae lipid to FAME by direct transesterification
The process of in-situ transesterification was performed in acidic condition using wet microalgae biomass (Kim et al 2015). The wet C. pyrenoidosa microalgae biomass was taken equivalent to 100 mg dry weight, allowed to saturate using hydrochloric acid and deionised water and kept overnight at ambient temperature. The magnetic core shell silica nanoparticles are kept associated with algae cells. Chloroform and methanol were added and mixed homogeneously in a round bottom flask connected to reflux unit. Reaction was maintained at 120°C for 2 h. After the reaction 2 ml deionized water was added and centrifuged for 10min at 5000rpm for the phase separation. After centrifugation, two phases appeared water phase containing cell with methanol and chloroform layer (upper layer) with FAME. The FAME containing chloroform phase was collected for GC-MS analysis.

4.6. FAME analysis:
Fatty acid composition was determined using GC-MS. Fatty acid methyl esters (FAMEs) were identified by comparison of the retention times with those of the standard (supelco TM 37 component FAME mix, sigma-aldrich co.).

Figure S 1. DLS of (a) silica, (b) magnetic (Fe₃O₄) nanoparticles, (c) silica coated magnetic (Fe₃O₄@SiO₂) nanoparticles
Figure S 2. XRD of (a) silica, (b) magnetic (Fe$_3$O$_4$) nanoparticles, (c) silica coated magnetic (Fe$_3$O$_4$@SiO$_2$) nanoparticles
Fig S 3: (a) Flocculation and (b) lipid extraction from *C. pyrenoidosa* in presence of silica and magnetic silica nanoparticles compared to control.

Figure S 4. Scanning electron micrograph (a) *C. pyrenoidosa* control cells without nanoparticles (scale bar: 2µm), (b) *C. pyrenoidosa* cells with silica coated magnetic nanoparticles (scale bar: 1µm)
Figure S 5 Chromatogram showing the FAME profile of *C. pyrenoidosa* in presence of magnetic-silica core-shell nanoparticle.

![Chromatogram](image)

Fig S 6. Comparative percentage of compounds present in FAME extract of *C. pyrenoidosa* shows highest quantity of linoleic acid.

![Bar chart](image)
Fig S 7 Synthesis and application of Fe₃O₄@SiO₂ nanoparticles in microalgae flocculation

Table S1: Effect of silica and magnetic silica nanoparticles on C. pyrenoidosa cell harvesting in terms of flocculation efficiency

| Samples          | Initial O.D. (660 nm) | Final O.D. (660 nm) | Flocculation Efficiency |
|------------------|------------------------|---------------------|-------------------------|
| Control          | 0.541                  | 0.492               | 9.05%                   |
| Silica           | 0.541                  | 0.422               | 22.0%                   |
| Magnetic Silica  | 0.541                  | 0.088               | 83.7%                   |

Table S2: Major compounds present in microalgal FAME with their retention time and percentage area obtained from chromatogram

| Name                                      | Molecular Weight | Retention Time | Area % |
|-------------------------------------------|------------------|----------------|--------|
| n-hexadecanoic acid methyl ester          | 284              | 19.340         | 1.2    |
| 9,12-octadecadienoic acid (Z,Z)           | 238              | 23.609         | 80.78  |
| 2- methyltetradecanoic acid               | 352              | 25.82          | 1.02   |
| 9-octadecenoic acid (Z)                   | 282              | 28.310         | 1.29   |
| 2- methyl nervonate                       | 352              | 30.122         | 1.51   |
| 4A,8A-Naphthalenediol, octahydro-, cis-   | 170              | 30.480         | 1.19   |
| Methyl docosahexaenoate                    | 342              | 34.20          | 2.80   |
| 2H-1-Benzopyran-6-ol                      | 148              | 41.252         | 1.84   |
| Stigmast-5-en-3-ol, (3.BETA)              | 678              | 43.924         | 1.38   |
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