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Biosynthesis of silver nanoparticle and its application in cell wall disruption to release carbohydrate and lipid from *C. vulgaris* for biofuel production

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ABSTRACT

Microalgae are the fledging feedstocks yielding raw materials for the production of third generation biofuel. Assorted and conventional cell wall disruption techniques were helpful in extracting lipids and carbohydrates, nevertheless the disadvantages have led the biotechnologists to explore new process to lyse cell wall in a faster and an economical manner. Silver nanoparticles have the ability to break the cell wall of microalgae and release biomolecules effectively. Green synthesis of silver nanoparticles was performed using a novel bacterial isolate of *Bacillus subtilis*. Characterisation of nanosilver and its effect on cell wall lysis of microalgae were extensively analysed. Cell wall damage was confirmed by lactate dehydrogenase assay and visually by SEM analysis. This first piece of research work on direct use of nanoparticles for cell wall lysis would potentially be advantageous over its conventional approaches and a greener, cost effective and non laborious method for the production of biodiesel.

1. Introduction

Alternate transport fuel is an emerging research worldwide due to rapidly decreasing fossil fuel resources and raising environmental concern on global warming. Fossil fuels are not only dominant in energy sectors and global economy but also responsible for life threatening infection by the accumulation of toxic gases while burning mainly from transport division which contributes to 21% CO_2 emissions [1–4]. Hence, promising alternate to petrodiesel are considered to be biodiesel and bioethanol, termed as "green" fuels, as they are ecofriendly, non-toxic, renewable and biodegradable in nature [5–7].

Biofuel is generally produced from vegetable oil and agricultural crops [8]. However rise in food prices, consumption of more arable land and water for cultivation create serious problems of utilizing them [9]. Moreover, high lignin content in plant biomass makes saccharification difficult during bioethanol production [9,10]. Nowadays microalgae have been recognised as the third generation biofuel feedstock by many scientists and advantageous to plant based feedstock due to their simple structure, fast growth rate, can be grown in non agricultural land and waste water, accumulate large amount of lipids and carbohydrates [11,12]. However, biorefinery process of microalgae has several steps including species selection, mode of cultivation, harvesting, and cell disruption. Among all these steps cell disruption is the principal step because algal cell wall generally consist of multilayered rigid cell wall [13–15], which restrict the complete extraction of compound of interest from microalgae. Therefore, to extract lipid and carbohydrate from algal cells, the cell wall must be disrupted to smallest pieces using pretreatment process using organic solvents [16]. Methods of cell wall disruption and extracting solvents decide the efficiency of oil and carbohydrate extraction from microalgae [13].

Many pretreatment methods can be used for microalgal cell disruption such as physical treatment including ultrasonication [14,17], thermal [13,18], microwave [13,14,19], bead milling [14,20] and cryogrinding [13,21], chemical methods including acid [22,23], alkaline [24], solvent soaking [21] and osmotic shock [13] and enzymatic treatments [10,25–28]. Nevertheless, these methods have some limitations; physical or mechanical methods are not effective and not suitable for large-scale operations [27], chemical methods cause pollution [29] and also destruct other cellular components [27], and enzymatic treatment is cost intensive and very slow process [17].

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The disadvantages can be overcome by an efficient alternate method and it has to be developed for better microalgae cell disruption. One such potential alternate is the use of nanotechnology in biotechnological applications. Nanoparticles have carved their own niche in renewable energy sector having been implemented in various processes. Metal nanoparticles are recognized to be effective catalytic agents in chemical and biological sciences. Due to nanoparticles' tiny structure, they penetrate and easily interact with the biomolecules acting upon them efficiently [30]. To the best of our knowledge and from extensive literature survey, no report on cell wall lysis of microalgae using silver nanoparticles for biofuel synthesis had been published. The main objectives of this study are the extracellular synthesis of silver nanoparticle (AgNP) from a novel bacterial isolate *B.subtilis* (KF681508) and evaluate its effects on cell wall rupture of fresh water microalga to extract lipid and carbohydrate from *Chlorella vulgaris* for biofuel production.

2. Materials and methods

2.1. Microalgal culture

Fresh water microalga *Chlorella vulgaris* was obtained from CAS in Botany, University of Madras (Guindy Campus), Chennai, Tamilnadu, India. It was grown in 14 L PBR using sterile Bold's Basal Medium (BBM) consisting of (g/L) $NaNO_3$ (0.25), K_2HPO_4 (0.075), KH_2PO_4 (0.175), NaCl (0.025), $CaCl_2.2H_2O$ (0.025), $MgSO_4{\cdot}7H_2O$ (0.075), EDTA.2Na (0.05), KOH (0.031), $FeSO_4{\cdot}7H_2O$ (0.005), H_3BO_3 (0.008), $ZnSO_4{\cdot}7H_2O$ (0.0015), $MnCl_2.4H_2O$ (0.0003), MoO_3 (0.00025), $CuSO_4{\cdot}5H_2O$ (0.0003), $Co(NO_3)_2{\cdot}6H_2O$ (0.0001) and mixing was provided by sparging air (0.3vvm) from the bottom of the PBR. The lighting was supplied by cool-white fluorescent light with an intensity of 5000 lux under 12:12 light/dark cycle.

2.2. Isolation and molecular identification of bacterium for AgNP synthesis

Silver nanoparticle producing bacteria was isolated from soil sample that was collected from the Annamalai University campus, Tamilnadu, India, using serial dilution technique and 0.1 ml sample was spreaded on Nutrient Agar plates (Hi Media Laboratories Ltd., Mumbai, India) and incubated at 37 °C for 24 h. After incubation, the plates were checked for white, swarmed mucoid colonies and then biochemical characterisation was performed to tentatively identify the isolate as *Bacillus* sp. The culture was finally subjected to strain identification and was confirmed using 16SrRNA primers: 27 F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492 R (5′- TAC GGT TAC CTT GTT ACG ACT T-3′). The gene sequence obtained from the organism was compared with other Bacillus strains for pairwise identification using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and multiple sequence alignments of the sequences were performed using Clustal Omega version of EBI (www.ebi.ac.uk/Tools/msa/clustalo). Finally Phylogenetic tree was constructed by Clustal Omega of EBI (www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny) using neighbor joining method.

2.3. Biosynthesis of silver nanoparticles

The bacterial isolate was cultivated in LB broth for 24 h at 37 °C and applied for silver nanoparticle synthesis. After incubation, the cell free supernatant was collected by centrifugation at 10,000 rpm for 10 min. In a 250 ml conical flask, 50 ml of 1 mM $AgNO_3$ solution was added to 100 ml of culture supernatant. The control was the culture supernatant without $AgNO_3$ solution. The flasks were then left for 48 h at room temperature in brightness. The colour change from pale yellow to brown was visually checked, which indicated the extracellular synthesis of AgNPs. The AgNPs were obtained by centrifugation at 12000 rpm for 10 min, washing the pellet with double distilled water and repeating the protocol thrice. The obtained AgNP pellet was then lyophilized for characterisation study.

2.4. Characterisation of AgNPs

Primary characterization of AgNP was performed by UV–vis Spectrophotometer (SL-159, Elico, India) with wavelengths ranging between 200 and 600 nm. FT-IR spectra of the nanoparticle using KBr pellet was obtained by FTIR Spectroscope (Bruker Optics, GmBH, Germany). Morphology of AgNP was observed through TEM (Jeol, Japan) and the XRD pattern was measured using diffractometer (Xpert-Pro, England) at room temperature with current of 30 mA, voltage of 40 kV and the peaks were recorded at 2θ.

2.5. Pretreatment of microalga using AgNPs

C.vulgaris culture was harvested when it reached stationary phase by centrifugation at 5000 rpm for 10 min. Then the microalgal paste was dried using Lyophilization and the dried biomass was subjected to pretreatment process. Various concentrations of AgNPs such as 50,75, 100, 125, 150 and 200 μg were added to the test tube contain 1 g of microalgal biomass with sterile distilled water. This mixture was then incubated at various time intervals of 10, 20, 30, 40 and 50 min in an orbital shaker at 100 rpm.

After the pretreatment process, the biomass slurry was subjected to drying at 60 °C for 8 h using hot air oven and the intracellular oil was extracted by Bligh and Dyer method [31] and oil extraction yield (%w/w) was calculated as,

$$\text{oil extraction yield}(\%) = \frac{\text{Weight of extracted oil (g)}}{\text{Weight of algal biomass (g)}} \times 100$$

The extracted oil from untreated microalgal biomass was considered as control and yield was compared with test sample.

The total carbohydrate content was determined, after extracting oil, using phenol sulfuric acid method [32]. A volume of 5 ml sample was used for the procedure and untreated sample was used as control.

2.6. Lactate dehydrogenase (LDH) assay for cell wall damage

LDH experiment was carried out according to Pakrashi et al., [33]. About 1 mL of the interacted cell suspensions was centrifuged at 8000 rpm for 10 min. A 100 μL of the supernatant was then collected and 100 μL of 30 Mm sodium pyruvate was added followed by 2.8 mL of 0.2 M Tris–HCl. Finally, a 100 μL of 6.6 mM NaDH was added just before measuring the decrease in absorbance at 340 nm as a function of 10 readings using UV–vis spectrophotometer (SL-159, ELICO Ltd, India).

2.7. Scanning Electron Microscope (SEM) Analysis

The effects of AgNP pretreated microalgal cells were subjected to morphological analysis for cell wall damage. After the pretreatment process, a small amount of sample was taken from the test tube, dried and observed with Scanning Electron Microscope (JSM 5610, Jeol, Japan).