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Synthesis of silver nanoparticles by polysaccharide bioflocculant produced from marine *Bacillus subtilis* MSBN17

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ABSTRACT

The polysaccharides are emerging as stabilizing and reducing agents for nanoparticles synthesis, however the commercial polysaccharides are not economically viable. Therefore, the exopolysaccharide from microbial origin such as bioflocculants are promising alternate for the synthesis and stabilization of nanoparticles. In this report, a bioflocculant (MSBF17) was produced from marine sponge-associated *Bacillus subtilis* MSBN17 under submerged fermentation using the economical substrates. The production was statistically optimized with most significant factors such as palm jaggery, NH_4NO_2, K_2HPO_4 and NaCl. The maximum bioflocculant production obtained with statistically optimized medium was 13.42 g/l. Based on the biochemical composition and FT-IR analysis, the flocculant compound was predicted as a polysaccharide derivative. The flocculating activity of the MSBF17 was invariably considerable at high salinity and temperature. It was found that the nano-scale silver can be synthesized in reverse micelles using the bioflocculant as stabilizer. The silver nanoparticles (AgNPs) were characterized by UV-spectroscopy, FT-IR and TEM analysis. The AgNPs were spherical shaped (60 nm) and stable for 5 months. Therefore, the bioflocculant-mediated synthesis of nanomaterials can be considered as environmental benign greener approach.

1. Introduction

Currently flocculating agents are prevalent in various industrial processes such as wastewater treatment, drinking water purification and downstream processes in food and fermentation processes [1]. The flocculants are mainly of three types such as: inorganic flocculants, synthetic flocculants and bioflocculants. The major components of bioflocculant are extracellular polymeric macromolecules such as polysaccharide, protein and nucleic acid produced by bacteria, actinomycetes and fungi during their growth [2,3]. It has been shown that bioflocculant produced by *Bacillus subtilis* IFO3335, *B. subtilis* DYU1, *Vagococcus* sp. W31, *Enterobacter aerogenes* W-23, a haloalkaliphilic *Bacillus* sp. I-471, halophilic *Halomonas* sp. V3a, *Alcaligenes cupidus* KT201 and *Pseudoalteromonas* sp. SM9913 are polysaccharides [4]. In general bioflocculants are biodegradable and nontoxic than inorganic and synthetic flocculants. However, high production cost has constrained the commercialization of bioflocculants for industrial applications. In order to reduce cost of production, research should be concentrated on isolation of hyper bioflocculant producing microbial strains capable of utilizing low-cost substrates and optimization of process conditions to increase bioflocculant yield. Response surface methodology (RSM) is a promising tool that has been successfully applied to optimize bioflocculant production and offers reliable information to perform the industrial production processes [5–8].

The sudden outbreaks due to infectious diseases caused by multi-drug resistant bacteria have created the awareness about novel chemotherapeutic agents. In the present scenario, nano-scale materials such as inorganic molecules have emerged as promising antibacterial agents owing to their high surface area to volume ratio and the unique chemical and physical properties [9–11]. The key advantages of inorganic antimicrobial agents are improved safety and stability, as compared with organic antimicrobial agents [12]. The interaction between nanoparticles and biological macromolecules are most exciting and brings out new prospects in

Abbreviations: AgNPs, silver nanoparticles; $AgNO_3$, silver nitrate; ANOVA, analysis of variance; CFU, colony forming unit; CCD, central composite design; FT-IR, Fourier transform infrared spectroscopy; K_2HPO_4, di-potassium hydrogen phosphate; MHA, Muller Hinton agar; MTCC, microbial type of culture collection; MSBF, marine sponge bacterial flocculant; NH_4 NO_2, ammonium nitrite; NaCl, sodium chloride; PB, Plackett–Burman design; RSM, response surface methodology; TEM, Transmission electron microscopy; ZMA, Zobell marine agar.

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biomedical field [13–15]. It is established that many microorganisms [16,17] can produce nano-materials on either intra or extracellular level and there is a need to develop an eco-friendly approach for nanomaterials synthesis that is devoid of using toxic chemicals in the synthesis protocol in order to meet the requirements and exponentially growing technological demand [18].

The polysaccharides purified from plants and animal sources were used as reducing and stabilizing agents for the synthesis of metal nanoparticles [11,18,19]. Polysaccharides have hydroxyl groups, a hemiacetal reducing end, and other functionalities that can play important roles in both the reduction [20] and the stabilization of metallic nanoparticles that creates vast opportunities for their utilization and potential mass production. Polysaccharide bioflocculants can be used for high-performance nanomaterials production, since they easily form a variety of liquid crystals in aqueous solutions and bioflocculant-mediated processes are highly profitable. The production of polysaccharide bioflocculants is not species specific and each strain of same species produces different kinds of polysaccharides with different biological applications. For that reason, maximum strains should be investigated to find out polysaccharide with promising biotechnological properties. Nowadays the marine sponge-associated bacteria have been recognized as rich source of biological macromolecules that are of potential interest to various industries [21]. It is presumed that extensive research on sponge-associated endosymbionts will provide to be a remarkable source of bioflocculants. Therefore, the present study aimed to isolate and identify the hyper bioflocculant producing novel strains from marine sponges. The special emphasis has been given to optimize, purify and characterize the polysaccharide bioflocculant produced by *B. subtilis* MSBN17 which is ultimately used to synthesize the silver nanoparticles (AgNPs). The obtained AgNPs were characterized by UV-spectroscopy, FT-IR and TEM analysis. To the best of our knowledge this would be first report on microbial polysaccharide bioflocculant based synthesis of metal nanoparticles.

2. Experimental details

2.1. Isolation and screening of bioflocculant producers

Marine sponge *Callyspongia diffusa* was collected from the southeast coast (10°026.51°N 79°13′45.39°E) of India by SCUBA diving at 10–15 m depth. To avoid cross contamination, only unbroken samples were used for microbiological analysis. The specimens were kept for 2 h in sterilized aged seawater (SAS) to remove loosely associated microorganisms from inner and outer sponge surfaces. For the isolation of sponge-associated bacteria, 1 cm^3 of sponge tissue was excised from the internal mesohyl area using a pair of sterile scissors. The excised portion was thoroughly washed three times with sterile SAS to remove any bacteria within current canals and then the tissue was homogenized with phosphate buffered saline using a tissue homogenizer. The resultant homogenate was serially diluted with SAS and the aliquot was placed on various isolation media including sponge agar 1, sponge agar 2 [21] and Zobell marine agar (ZMA). Amphotericin B (30 μg/μl) was added to inhibit the growth of fungi and the plates were incubated at 28 °C for 7 days in dark. The morphologically distinct colonies were reisolated and maintained on ZMA (HiMedia) at 4 °C. The composition of the bioflocculant screening medium was as follows: glucose, 10 g/l; yeast extract, 0.5 g/l; KH_2PO_4, 2 g/l; K_2HPO_4, 5 g/l; $(NH_4)_2SO_4$, 0.2 g/l; urea, 0.5 g/l and NaCl, 0.1 g/l with the initial pH 7. Each isolated strain was inoculated in 250 ml Erlenmeyer flasks containing 50 ml of screening medium and incubated in a shaker at 200 rpm for 48 h at 30 °C. The culture broth was determined for flocculating activity. Strains with high flocculating ability were selected for further studies.

2.2. Identification of bioflocculant producer

The hyper producer MSBN17 was identified morphologically and biochemically according to Bergeys's manual of determinative bacteriology [22] and the genomic DNA was obtained by the method of Enkicknap et al. [23] for molecular identification. The PCR analysis was performed with 16S rRNA eubacterial primers 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) [24]. A 25 μl reaction volume PCR was carried out using about 10 ng of genomic DNA, 1X reaction buffer (10 mM Tris–HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Triton X-100), 0.4 mM (each) deoxynucleoside triphosphates (Invitrogen) and 0.5 U of DNA polymerase (New England Labs, UK) and 1 mM each forward and reverse primers. The PCR temperature profile was used as follows: 95 °C for 3 min, then 30 cycles consisting of 95 °C for 45 s, 55 °C for 45 s, 72 °C for 1.45 min and finally an extension step at 72 °C for 10 min. The 16S amplicon was cloned by the TA cloning method using TOPO TA Cloning kit according to manufactures instructions (Invitrogen) for sequencing. The 16S rRNA gene sequence obtained from the isolate MSBN17 was compared with other bacterial sequences by using NCBI mega BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for their pair wise identities. Multiple alignments of these sequences were carried out by Clustal W 1.83 version of EBI (www.ebi.ac.uk/cgi-bin/clustalw/) with 0.5 transition weight. Phylogenetic trees were constructed in MEGA 5.0 version (www.megasoftware.net) using neighbor joining (NJ), minimum evolution (ME) and unweighted pair group method with arithmetic mean (UPGMA) algorithms.

2.3. Production and purification of bioflocculant MSBF17

Production of the bioflocculant was performed in several 500 ml Erlenmeyer flasks containing 200 ml culture medium with 200 rpm agitation at 30 °C. The composition of the modified production medium was as follows: palm jaggery, 20 g/l; yeast extract, 2.5 g/l; NH_4NO_2, 1.0 g/l; $MgCl_2$, 0.2 g/l; K_2HPO_4, 5 g/l and NaCl, 0.1 g/l. The initial pH was adjusted to 7. After incubation for 48 h, the culture was centrifuged at 10,000 rpm for 15 min. For the purification of bioflocculant, cold ethanol was added into the supernatant with stirring at the ratio of 2:1 (v/v). The precipitate was obtained by centrifugation at 10,000 rpm for 3 min. After three such ethanol precipitations, the bioflocculant was dialyzed against de-ionized water overnight and then lyophilized to obtain purified bioflocculant.

2.4. Determination of flocculating activity

The flocculating activity was predicted using a solution of kaolin clay as the test material. Briefly, 5.0 ml of a 1% (w/v) $CaCl_2$ solution and 0.2 ml of a centrifuged fermentation culture supernatant were added in turn to 95 ml of kaolin suspension (5.0 g/l, pH 8.0). The mixture was stirred for 4 min and then allowed to incubate for 5 min. The optical density (OD) of the aqueous phase was measured at 550 nm with a spectrophotometer. A control was prepared in the same way except that 0.2 ml of cell free culture filtrate replaced the culture broth. The flocculating activity was measured according to the following mathematical equation [25].

$$\text{Flocculating activity} = \frac{B - A}{A} \times 100\% \quad (1)$$

where A and B are the OD of the culture sample and the control, respectively.