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Bioflocculant production by a consortium of *Streptomyces* and *Cellulomonas* species and media optimization via surface response model☆

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ABSTRACT

Species of actinobacteria previously isolated from Tyume River in the Eastern Cape Province of South Africa and identified by 16S rDNA sequence as *Cellulomonas* and *Streptomyces* species were evaluated as a consortium for the production of bioflocculant. Sucrose, peptone and magnesium chloride were the nutritional sources which supported optimal production of bioflocculant resulting in flocculation activities of 91%, 82% and 78% respectively. Response surface design revealed sucrose, peptone and magnesium chloride as critical media components following Plackett–Burman design, while the central composite design showed optimum concentration of the critical nutritional source as 16.0 g/L (sucrose), 1.5 g/L (peptone) and 1.6 g/L (magnesium chloride) yielding optimal flocculation activity of 98.9% and bioflocculant yield of 4.45 g/L. FTIR spectrometry of the bioflocculant indicated the presence of carboxyl, hydroxyl and amino groups, typical for heteropolysaccharide, while SEM imaging revealed an interwoven clump-like structure. The molecular weight distribution of the constituents of the bioflocculants ranged 494.81–18,300.26 Da thus, an indication of heterogeneity in composition. Additionally, the chemical analyses of the purified bioflocculant revealed the presence of polysaccharides and proteins with neutral sugar, amino sugar and uronic acids in the following concentration: 5.7 mg, 9.3 mg and 17.8 mg per 100 mg. The high flocculation activity of the bioflocculant suggests commercial potential.

1. Introduction

Flocculants are used to mediate flocculation, which is an imperative step, in wastewater and municipal water treatment process. The conventionally used flocculants includes salts of aluminum (aluminum sulfate and poly-aluminum chloride), derivatives of polyacrylamide and polyethylene imines [1–3]. These conventional flocculants are cost effective and efficient in flocculation process; however, they have been linked to detrimental health effects including dementia (Alzheimer's disease), cancer and neurotoxicity [4–7]. These colossal demerits militate against their continual usage in water treatment amongst other processes.

Conversely, flocculants of microbial origin referred to as bioflocculants are innocuous, environmentally friendly and have been variously documented to show flocculation efficiency comparable to those of conventionally used flocculants; aluminum salts, polymers of acrylamide and ethylene [8,9]. However, high production cost and low yield has limited the application of bioflocculant in industrial processes such as water treatment [10]. Axenic cultures including *Bacillus firmus* [11], *Arthrobacter* sp. Raats [3], *Enterobacter cloacae* WD7 [12], *Bacillus* sp. Gilbert [13] and *Pseudoalteromonas* sp. SM9913 [14] of the extreme deep sea psychrophilic milieu have been respectively shown to produce bioflocculants. Maneuvering of microbial fermentation conditions and nutritional requirements are areas of research which has proven useful toward bioflocculant yield enhancement [15]. Other useful techniques for the maximization of metabolites of interest in microbial fermentation include microbial mutational analyses to obtain more efficient

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strains, utilization of cheap nutritional sources and fermentation using microbes in consortia [16,17]. Additionally, factorial experiment and surface response design (SRD) are statistical models with high efficiency toward yield optimization. Nonetheless, SRD has the advantage of been inexpensive as less experimental trials are required, time saving and able to identify the contributions of input variables (independent variable) respectively [18–20].

In our previous studies, axenic cultures of *Streptomyces* sp. Gansen [15] and *Cellulomonas* sp. Okoh [21] produced bioflocculants characterized as proteoglycan and glycosaminoglycan polysaccharide bioflocculants respectively. These bioflocculants were stable to extremes of pH and high temperature. Accordingly, these actinobacterial species were evaluated in consortium for bioflocculant yield optimization. Furthermore, to ascertain media components with significant contribution to bioflocculant production, Plackett–Burman (PB) experimental design was used to screen media constituents and the central composite design (CCD) applied to optimize critical media components determined with PB design. Application of PB and CCD was necessitated by the paucity of information on media optimization for consortia culture fermentation; likewise media optimization is pivotal for cost reduction in fermentation processes. The bioflocculant produced was purified and characterized.

2. Materials and methods

2.1. Actinobacterial strains

The bacteria strains were reactivated from glycerol stock stored at −80 °C as part of the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, South Africa. They were previously isolated from Tyume River of the Eastern Cape of South Africa and identified by 16S rDNA sequence. BLAST analyses of the nucleotide sequences revealed one of them to have 99% similarity to three *Cellulomonas* species (strain 794, *Cellulomonas flavigena* DSM 20109 and *Cellulomonas flavigena* NCIMB 8073) and the sequences was deposited in GenBank as *Cellulomonas* sp. Okoh (accession number HQ537132). The other bacterial strain also had 99% similarities to *Streptomyces* sp. MEC01 and *Streptomyces cavourensis* subsp. cavourensis strain NRRL 2740 and the nucleotide sequence was deposited in GenBank as *Streptomyces* sp. Gansen (accession number HQ537129). The reactivation of the bacteria were achieved by inoculating 20 μl of the glycerol stock into a sterile 5 mL sterile broth composed of 3 g beef extract, 10 g tryptone and 5 g NaCl (per liter) respectively and incubated overnight at 28 °C.

2.2. Screening of carbon, nitrogen and cation sources for bioflocculant production

Aliquot of 2 mL, each, of the activated cultures of *Streptomyces* sp. Gansen and *Cellulomonas* sp. Okoh adjusted to cell density of about 1.5×10^8 cfu/mL were inoculated into 200 mL of sterile basal salt media composed of the following (g/L): glucose, 10; tryptone, 1; K_2HPO_4, 5; KH_2PO_4, 2 and $MgSO_4{\cdot}7H_2O$, 0.3. The fermentation medium was adjusted to pH 7 and incubated at a temperature of 30 °C and agitation speed of 160 rpm for a period of 72 h. Afterwards, the broth was centrifuged at 3000 rpm for 30 min at 15 °C and the cell-free supernatant was assessed for flocculation activity. Fructose, sucrose, lactose, maltose and starch respectively served as sole carbon source. Sole nitrogen and cation sources respectively evaluated included; urea, ammonium sulfate, ammonium nitrate, ammonium chloride, peptone, monovalent salts (KCl and NaCl), divalent salt ($MgSO_4$, $CaSO_4{\cdot}H_2O$, $MnCl{\cdot}4H_2O$, and $FeSO_4$) and trivalent salt ($FeCl_3$).

2.3. Determination of flocculation activity

About 0.3 mL of 1% $CaCl_2$ and 0.2 mL of cell free broth (bioflocculant rich broth) were added to 10 mL of Kaolin suspension (4.0 g/L) in a test tube. The mixture was vortexed using a vortex mixer (VM-1000, Digisystem) for 30 s and kept still for 5 min, after which 2 mL of the upper layer was carefully withdrawn and its optical density (OD) read spectrophotometrically (Helios Epsilon, USA) at 550 nm wavelength. Control included repeating same process however, the bioflocculant broth was replaced with sterile (un-inoculated) fermentation medium [15,17]. All assays were in triplicates and flocculation activity calculated using the following equations:

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

A and B are OD_{550} (optical density: 550 nm) of the control and sample, respectively.

2.4. Plackett–Burman design for the screening of media components

Plackett–Burman (PB) design used for n variable screening in n + 1 experiment was employed [10]. The carbon, nitrogen and cation sources yielding optimal flocculation activity were evaluated with other media components to ascertain respective influence in bioflocculant production. Five independent variables (media components); Sucrose, $MgCl_2$, peptone, K_2HPO_4 and KH_2PO_4 were investigated, two levels (concentrations) of each variable, "high" and "low", were used and was designated as +1 and −1 respectively (Table 2). All runs were carried out in triplicate and the average flocculation activity was used as the response variable. Regression analysis revealed the media components with significant ($p < 0.05$) effect on flocculation activity and these components were evaluated in further optimization experiments. NCSS 2007 (Statistical analysis and graphics software, Kaysville, UT), was used to design and developed the PB experimental design based on the following first-order model:

$$Y = bo + \sum_{i=1}^{k} bixi \quad (2)$$

Y = the response (flocculation activity), bo = model intercept, bi = linear coefficient, xi = level (concentrations) of the independent variable, and k = number of involved variables (media components).

2.5. Optimization of critical media components by central composite design

Media components with significant input in bioflocculant production as identified by PB design, were optimized through the response surface design (RSD). Thus, a central composite design (CCD) model was generated and this model was applied to the independent variables; Sucrose, $MgCl_2$ and peptone using 3-factor-5-level CCD [22]. Experimental trials were all carried out in triplicate and the average of both flocculation activity and bioflocculant yield at each run were used as the response variable. The linear relationship between the response variables (flocculation activity and bioflocculant yield, respectively) and the independent variables were respectively fit to the second order polynomial model as shown below:

$$Y = bo\sum_{i=1}^{k} bixi + \sum_{i=1}^{k} biix^2 + \sum_{i=1}^{k} \sum_{i=1}^{k} bijxixj, \quad i \neq j \quad (3)$$

Y = response variable (flocculation activity), bo = coefficient of interception, bi = coefficient of linear effect, bii = coefficient of the