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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

quadratic effect, *bij* = coefficient of interaction effect when $i < j$ and k which are the involved variables (media components).

2.6. Bioflocculant purification

The fermentation broth was centrifuged (3000 rpm, 30 min, 15 °C) and cell pellets separated from the supernatant by decantation. The supernatant was mixed with ice cold ethanol (95%), at volume to volume ratio of 1:4 and kept at 4 °C in a cold cabinet for 16 h. The ethanol and cell free broth mixture was centrifuged (10,000 rpm, 30 min, 15 °C) and the residue re-dissolved in distilled water at ratio 1:4 (v/v). The procedure was successively repeated twice and the purified bioflocculant was lyophilized and vacuum dried [23,24]. The lyophilized fraction was used for further studies.

2.7. SEM imaging and FTIR spectroscopy of the purified bioflocculant

Purified bioflocculant was placed on carbon coated stub and gold coated in a gold coating chamber, using Eiko IB.3 ION coater. Scanning electron microscopic (SEM) image of the gold coated bioflocculant was obtained using JEOL JSM-6390LV FEI XL30 (JEOL; USA) scan electron microscope. Similar procedures were carried out in obtaining the micrograph images of flocculated and the unflocculated Kaolin clay samples. Functional groups present in the bioflocculant were determined using a Fourier transform infrared (FTIR) spectrophotometer (2000 FTIRS Spectrometer; Perkin Elmer System) over a wave number range of 4000–500 cm^{-1}.

2.8. Molecular weight and compositional analyses of purified bioflocculant

The molecular weight of the purified bioflocculant was determined using the LS-MS (AB SCIEX TripleTOF 5600 LS-MS/MS System, MA, USA). About 10 mg of the purified bioflocculant was solubilized in 200 mL of HPLC grade water; further dilution of the solution was made at a ratio of 1:10. Injection of the solubilized sample was made into a high performance liquid chromatography system (Shimadzu XR) which uses Phenomenex Kinetex C18 column (1.7 μm, 50 mm × 2.1 mm). Gradient elution using water and acetonitrile, containing 0.1% formic acid (HPLC grade) was used at a flow rate of 0.5 mL/min over 30 min. A TripleTOF 5600 + system with a DuoSpray source (electrospray ionization) was used for data acquisition in positive mode, over a mass range of 100–1000 m/z. An automated calibration was performed using an external calibrant delivery system (CDS) which infused calibration solution prior to sample introduction. TOF MS survey scan experimentation was performed with collision energy of 45 eV and a spread of ±15 eV. Data were processed using PeakView Software.

Total sugar and protein contents of purified bioflocculant were determined by the phenol–sulfuric acid and folin–phenol methods using glucose and bovine serum albumen (BSA) as standards [25,26]. Neutral sugars, amino sugars and uronic acids were determined following anthrone reaction, Elson–Morgan and Morgan–Elson assay and the carbazole–sulfuric acid technique [27–29].

3. Results

3.1. The effect of nutritional sources on bioflocculant production

The carbon sources evaluated showed sucrose to optimally support bioflocculant production by yielding flocculation activity of 91% and 4.01 ± 0.22 (g/L) on quantification of bioflocculant yield. Conversely, lower flocculation activities as well as lower bioflocculant yields were obtained with other carbon sources (Table 1). However, peptone and magnesium chloride served as the preferred nitrogen and cation sources; optimum flocculation activities of 820% and 78% were respectively obtained with corresponding bioflocculant yields of 3.21 ± 0.13 g/L and 3.29 ± 0.42 g/L. Other nitrogen and cation sources with significant influence in bioflocculant production were $(NH_4)_2SO_4$ (79% flocculation activity; 3.18 ± 0.24 g/L bioflocculant yield), $(NH_4)_2Cl_4$ (71% flocculation activity; 2.92 ± 0.51 g/L bioflocculant yield), $MnCl{\cdot}4H_2O$ (62.0% flocculation activity; 2.81 ± 0.19 g/L bioflocculant yield) and $CaSO_4{\cdot}H_2O$ (57.0% flocculation activity; 2.39 ± 0.33 g/L bioflocculant yield) respectively (Table 1).

3.2. Variables influencing the production of bioflocculant

The Plackett–Burman design matrix showing the experimental results for the screening of media components (sucrose, peptone, $MgCl_2$, K_2HPO_4 and KH_2PO_4) critical for the production of bioflocculants are presented in Table 2. The flocculation activities observed (measured from experimental trials) and predicted (generated through regression analysis) were in close accord at an alpha level of 0.05. Similarly, optimum flocculation activity of 95.0% was recorded at runs no. 6 with media component concentrations of (g/L); 12.5 (sucrose), 1.0 (peptone), 0.3 ($MgCl_2$), 5.0 (K_2HPO_4) and 2.5 (KH_2PO_4). The explanation for optimum performance at runs No. 6 is not clear as PB design is not equipped to address such. However, regression analysis indicated that sucrose, peptone and $MgCl_2$ had positive effect on bioflocculant production while K_2HPO_4 and KH_2PO_4, respectively, did not. The level of significance of the variables with positive effects were; $p \geq 0.0114$ (sucrose), $p \geq 0.0071$ (peptone) and $p \geq 0.0866$ ($MgCl_2$). This is shown in Table 3 respectively.

Table 1
The effects of nutritional factors on bioflocculant production by *Streptomyces* sp. and *Cellulomonas* sp. in mixed culture fermentation.

Carbon source	Glucose	Lactose	Fructose	Sucrose	Maltose	Starch
Max. flocculation activity (%)	88	79	84	91	68	77
Bioflocculant yield (g/L)	$3.91^a \pm 0.55$	$3.08^b \pm 0.27$	$4.08^a \pm 0.71$	$4.01^a \pm 0.22$	$3.65^c \pm 0.51$	$3.54^c \pm 0.82$

Nitrogen source	Urea	$(NH_4)_2SO_4$	$(NH_4)_2NO_3$	$(NH_4)_2Cl_4$	Peptone
Max. flocculation activity (%)	58	79	68	71	82
Bioflocculant yield (g/L)	$2.13^b \pm 0.11$	$3.18^a \pm 0.24$	$2.04^b \pm 0.71$	$2.92^a \pm 0.51$	$3.21^a \pm 0.13$

Cation source	KCl	NaCl	$MgCl_2$	$CaSO_4{\cdot}H_2O$	$MnCl{\cdot}4H_2O$	$FeSO_4$	$FeCl_3$
Max. flocculation activity (%)	41	33	78	57	62	21	18
Bioflocculant yield (g/L)	$1.77^c \pm 0.13$	$1.26^c \pm 0.66$	$3.29^a \pm 0.42$	$2.39^b \pm 0.33$	$2.81^{ab} \pm 0.19$	$1.31^c \pm 0.23$	$1.01^c \pm 0.18$

The mean values and deviations as denoted by different lowercase letter (superscript) are significantly different ($p < 0.05$).

Table 2
PB design for the screening of critical media components involved in bioflocculant production.

Runs	Coded levels/concentrations (g/L)					Flocculation activity (%)	
	Sucrose	Peptone	$MgCl_2$	K_2HPO_4	KH_2PO_4	Observed	Predicted
1	1 (12.5)	1 (1.5)	−1 (0.3)	1 (6.5)	1 (2.5)	79	84.5
2	1(12.5)	−1(1.0)	1 (0.5)	1 (6.5)	1 (2.5)	93	92.2
3	−1(10.0)	1 (1.5)	1 (0.5)	1 (6.5)	−1 (2.0)	74	74.2
4	1 (12.5)	1 (1.5)	1 (0.5)	−1 (5.0)	−1 (2.0)	82	80.5
5	1 (12.5)	1 (1.5)	−1 (0.3)	−1 (5.0)	−1 (2.0)	83	81.2
6	1 (12.5)	−1 (1.0)	−1 (0.3)	−1 (5.0)	1 (2.5)	95	95.8
7	−1 (10.0)	−1 (1.0)	−1 (0.3)	1 (6.5)	−1 (2.0)	76	83.2
8	−1 (10.0)	−1 (1.0)	1 (0.5)	−1 (5.0)	1 (2.5)	94	91.8
9	−1 (10.0)	1 (1.5)	−1 (0.3)	1 (6.5)	1 (2.5)	91	81.2
10	1 (12.5)	−1 (1.0)	1 (0.5)	1 (6.5)	−1 (2.0)	88	85.8
11	−1 (10.0)	1 (1.5)	1 (0.5)	−1 (5.0)	1 (2.5)	76	83.5
12	−1 (10.0)	−1 (1.0)	−1 (0.3)	−1 (5.0)	−1 (2.0)	88	86.2

Table 3
Regression analysis showing critical media components in bioflocculant production.

No.	Media components	Estimate	*t*-Value	*p*-Value
*x*1	Sucrose	0.826	10.878	0.0114
*x*2	Peptone	0.566	6.194	0.0071
*x*3	$MgCl_2$	0.245	3.176	0.0866
*x*4	K_2HPO_4	−0.104	−1.190	0.460
*x*5	KH_2PO_4	−0.230	−1.667	0.746

3.3. Critical media component optimization with RSD for bioflocculant production

The identification of sucrose, peptone and $MgCl_2$ as critical media components, consequent to the PB design, resulted in the application of a 3-factor-5-level central composite design for the optimization of media components. Subsequently, enhanced bioflocculant yield was obtained and the data shown in Table 4. The proportion of the independent variables which yielded optimal flocculation activity (98.9%) was 16 g/L (sucrose), 1.5 g/L (peptone) and 1.6 g/L (magnesium chloride). The second order response surface model fitting following analysis of variance is depicted in Table 5 and the adequacy of the model was indicated by the high regression coefficient value ($R^2 = 0.938$) obtained, thus an account for 93.8% variability with respect to enhanced flocculation activity obtained. The coefficient of adjusted R^2 was similarly high hence, an indication of the significance of the model. Further validation of this result is shown in the *F*-test which yielded a low probability value (0.00006), and a lack-of-fit value ($R^2 = 0.035$) which was not statistically significant ($p \leq 0.38$). This is an indication of the adequacy of the model for predicting enhanced flocculation activity within the limits of the assay conditions. The works of Karthikeyan et al. [30] and He et al. [10] is in corroboration with the above observation as the same model similarly optimized product yield. In the same vein, high regression coefficient obtained for the quantified bioflocculants yields, when subjected to analysis of variance, similarly showed adequacy ($R^2 = 0.833$) to the model with 83.3% adaptability. Likewise the low probability value (0.0066) of the *F*-test and the lack-of-fit value ($R^2 = 0.149$) which showed no statistical significance ($P \leq 0.018$) as is shown in Table 5.

The modeled nutritional sources (sucrose, peptone and $MgCl_2$) were all significant in the production of bioflocculant at above 96% probability level however; peptone appeared to have the most significant contribution at about 99.9% probability level (Table 6). The quadratic model yielded regression coefficients (estimates) with negative values which are indicative of low significance in bioflocculant production as it negatively impacted on yield. Conversely, the interaction within the three variables showed only sucrose and $MgCl_2$ as well as sucrose and peptone to have influenced bioflocculant production positively (Table 6).

On the other hand, graphical representation of the regression equation yielded a three dimension surface response plot (Fig. 1). These plots show the relationship between concentrations of nutritional sources and flocculation activity which was an indirect

Table 4
Central composite design matrix for critical media components showing observed and predicted values for flocculation activity and bioflocculant yield.

Runs	Sucrose	Peptone	$MgCl_2$	Flocculation activity (%)		Bioflocculant yield (g/L)	
				Observed	Predicted	Observed	Predicted
1	12.0 (−1)	0.5 (−1)	1.2 (−1)	93.4	93.5	3.55	3.44
2	12.0 (−1)	0.5 (−1)	1.6 (+1)	95.8	95.4	3.94	3.86
3	12.0 (−1)	1.5 (+1)	1.2 (−1)	96.6	96.7	4.01	3.96
4	12.0 (−1)	1.5 (+1)	1.6 (+1)	98.4	97.9	4.39	4.12
5	16.0 (+1)	0.5 (−1)	1.2 (−1)	96.1	96.4	3.37	3.52
6	16.0 (+1)	0.5 (−1)	1.6 (+1)	98.7	98.4	4.24	4.17
7	16.0 (+1)	1.5 (+1)	1.2 (−1)	97.2	97.4	4.13	4.09
8	16.0 (+1)	1.5 (+1)	1.6 (+1)	98.9	98.7	4.42	4.45
9	10.64 (−1.73)	1.0 (0)	1.4 (0)	95.1	95.5	3.61	3.88
10	17.36 (+1.73)	1.0 (0)	1.4 (0)	98.7	98.6	4.36	4.26
11	14.0 (0)	0.36 (−1.73)	1.4 (0)	95.5	95.6	3.59	3.65
12	14.0 (0)	1.74 (+1.73)	1.4 (0)	97.4	97.6	3.98	4.15
13	14.0 (0)	1.0 (0)	1.06 (−1.73)	96.8	96.2	3.74	3.71
14	14.0 (0)	1.0 (0)	1.84 (+1.73)	98.8	99.3	4.31	4.43
15	14.0 (0)	1.0 (0)	1.4 (0)	97.7	97.8	4.21	4.16
16	14.0 (0)	1.0 (0)	1.4 (0)	97.4	97.8	4.19	4.16
17	14.0 (0)	1.0 (0)	1.4 (0)	98.6	97.8	4.28	4.16
18	14.0 (0)	1.0 (0)	1.4 (0)	97.8	97.8	4.12	4.16
19	14.0 (0)	1.0 (0)	1.4 (0)	97.3	97.8	4.06	4.16
20	14.0 (0)	1.0 (0)	1.4 (0)	97.8	97.8	4.11	4.16

Table 5
Analysis of variance showing fitted quadratic polynomial model for optimization of flocculation activity.

Source	DF	SS	MS	*F*-ratio	*p*-Value	R^2
Flocculation activity						
Regression model	9	37.37691	4.152991	16.86	0.000064	0.938176
Linear	3	29.92558	9.975192	40.50	0.000007	0.751144
Quadratic	3	4.63759	1.545864	6.28	0.011457	0.116405
Lin × Lin	3	2.81375	0.9379167	3.81	0.046822	0.070626
Total error	10	2.463084	0.2463084			0.061824
Lack of fit	5	1.409751	0.2819501	1.34	0.378454	0.035385
Pure error	5	1.053333	0.2106667			0.026439
Bioflocculant yield						
Regression model	9	1.491207	0.1656897	5.54	0.006586	0.833033
Linear	3	1.222067	0.4073556	13.63	0.000725	0.682683
Quadratic	3	0.2065032	0.0688344	2.30	0.139029	0.115359
Lin × Lin	3	0.0626375	0.020879	0.70	0.573965	0.034991
Total error	10	0.2988875	0.029889			0.166967
Lack of fit	5	0.2670042	0.053401	8.37	0.017931	0.149156
Pure error	5	0.0031883	0.006377			0.017811

DF = degree of freedom; SS = Sum of square; MS = mean square.

measure of bioflocculant production. Hence, the pair wise interaction between sucrose, peptone and $MgCl_2$ is visualized in Fig. 1: A (sucrose and $MgCl_2$), B (peptone and sucrose) and C (peptone and $MgCl_2$). Interaction was observed at all combination however, only the interaction in Fig. 1A (sucrose and $MgCl_2$) and Fig. 1B (sucrose and peptone) was positive toward enhanced bioflocculant yield at a probability level of 90.1% and 99.9% respectively. The optimal concentrations of sucrose, peptone and $MgCl_2$ for the production of bioflocculant by mixed culture fermentation of *Streptomyces* sp. Gansen and *Cellulomonas* sp. Okoh were 16 g/L, 1.5 g/L and 1.6 g/L respectively. Consequently, maximum flocculation activity of 98.9% and bioflocculant yield of 4.42 g/L was achieved, while the estimates following the regression model were 98.7% flocculation activity and 4.45 g/L bioflocculant yield, respectively.

3.4. SEM images of bioflocculant and flocculation behavior

Scan electron micrographic imaging of the kaolin clay particles (Fig. 2a), kaolin clay suspension treated with bioflocculant free broth which served as control (Fig. 2b), kaolin clay suspension treated with bioflocculant (Fig. 2c) and the purified bioflocculant (Fig. 2d) revealed a loose particulate materials for the kaolin clay and some forms of loose clumps for the suspension treated without bioflocculant. However, the reverse was the case for the kaolin clay treated with bioflocculant as it appeared as a tightly knit continuous stretch with no noticeable interstice at 1 μm spacing. Additionally, the bioflocculant micrograph revealed membranous sheets, stacked, in a close manner that resembles a maze like structure (Fig. 2d). The interstices between horizontal pleated sheets were between 1 μm and 50 μm which were sporadically distributed on the sheet stacking as is shown in the micrograph. Holistically, the micrograph may equally be seen as showing a clumped structure of sheets with undulations as the only regular pattern. The compact nature of the bioflocculant may have contributed to the high flocculation activity demonstrated nonetheless, no other physical distinguishing attribute was apparent when compared to those produced by axenic cultures, besides the clump-like nature.

3.5. Compositional characteristics and molecular weight of the purified bioflocculant

Broad stretching peaks of 3437.63–3299.14 cm^{-1} characteristic of hydroxyl groups from polymeric and dimeric OH stretch were obtained, other peaks characteristics of different functional groups includes 2960.37–2853.93 cm^{-1} showing weak C—H stretching band from methylene groups, 1657.62–1545.14 cm^{-1} indicative of aromatic ring presences [15,31]. Furthermore, wave numbers 1452.03–1402.85 cm^{-1} and 1242.09–1080.89 cm^{-1} shown were typical of phenol and tertiary alcohol OH bend indicative of the presence of carboxylic groups, carboxylate ions, aromatic ring stretch and C—O and C—O—C from polysaccharides [31].

The LC–MS chromatogram of the purified bioflocculant obtained over 30 min time interval showed a wave of low and high molecular weight constituents of the bioflocculant (Fig. 3). The molecular weights ranged from 494.81 Da to 18,300.26 Da obtained over 30 min interval; the highest fraction was around 494.81 Da. There was not distinct pattern of molecular weight distribution in the segmented chromatogram as designated by a, b and c respectively in Fig. 3 following LC–MS retention time.

Chemical analyses of the purified bioflocculant revealed polysaccharides (34.4%) and proteins (18.56%) to account for about 52.96% of the bioflocculant composition. Further analyses of the

Table 6
Second order polynomial model following regression analysis of flocculation activity optimization.

Parameter	Estimate	Standard error	*t*-Value	*p*-Value
Intercept	54.79618			
Sucrose	8.908607	1.119815	2.60	0.026610
Peptone	17.49924	3.75848	4.66	0.000900
$MgCl_2$	9.884886	1.586189	1.03	0.326761
Sucrose^2	−6.866854E−02	3.276237E−02	−2.10	0.062500
Peptone^2	−2.636729	0.6876642	−3.83	0.003295
$MgCl_2{}^2$	−1.568268	2.484065	−0.63	0.541985
Sucrose × peptone	0.5625	0.1754667	3.21	0.009400
Sucrose × $MgCl_2$	0.03125	0.4386667	0.07	0.944612
Peptone × $MgCl_2$	−1.875	1.754667	−1.07	0.310375

polysaccharide constituent showed the presence of neutral sugars (5.7 mg), amino sugars (9.3 mg) and uronic acids (17.8 mg) out of 100 mg of the purified bioflocculant.

4. Discussion

The reason for this preference of sucrose and peptone is not quite clear as respective axenic cultures (*Streptomyces* sp. Gansen and *Cellulomonas* sp. Okoh) in the consortium preferred glucose as carbon source and NH_4SO_4 and $(NH_4)_2NO_3$ respectively as nitrogen sources for optimal bioflocculant production. Various accounts of the utilization of different carbon sources have been documented on axenic culture in the optimum production of bioflocculant [10,32]. Although, Zhang et al. [17] reported the ability of the consortium of *Staphylococcus* sp. and *Pseudomonas* sp. to optimally utilize brewery wastewater as carbon source however, a dearth of information exists on the suitability of nutritional sources in mixed culture fermentation for the production of bioflocculant.

The application of the PB model has been used effectively in the screening of critical media components necessary for production of poly-γ-glutamic acid and bioflocculants among other secondary metabolites [10,33] from axenic cultures. Nonetheless, its significance was demonstrated; as critical media components were identified in the enhanced production of bioflocculant via mixed culture fermentation with *Streptomyces* sp. Gansen and *Cellulomonas* sp. Okoh. The emergent of carbon and nitrogen sources as the critical media components may be attributed to the requirement of these components for cell growth while magnesium chloride may, similarly, have served as essential ion for cell physiological functions including enzyme activities. Similar observations have been made on *Porphyridium* sp., *Chlorella sorokiniana* and *Clostridium pasteurianum* respectively [10,34–36].

Bioflocculant yield optimization was effectively achieved through the application of central composite design model, higher yield and flocculation activities were achieved. Consequently, the higher flocculation activities observed may be attributed to the presence of more bioflocculants (higher concentrations) in the cell free broth or better still, the production of bioflocculants with high density of surface charges as compared to those produced by axenic cultures hence, the account for the high flocculation activity. The later most probably holds true as the molecular weight distribution of the bioflocculants showed a heterogeneous composition between low molecular weight and high molecular weight constituents. Consequently, more surface charges were available for the flocculation of dispersed particles (Kaolin clay) in the media as has been shown with the high flocculation activity. In the vein, the chemical analyses revealed that a myriad of constituents may have constituted the bioflocculant thus, the plausible reason for the high flocculation activity observed.

The high regression coefficients obtained in the model proves adequacy of the models. Additionally, the model has been successfully applied in the optimization of dextran and bioflocculant production respectively by axenic cultures [10,30]. The contributions of the carbon, nitrogen and cation sources were clearly

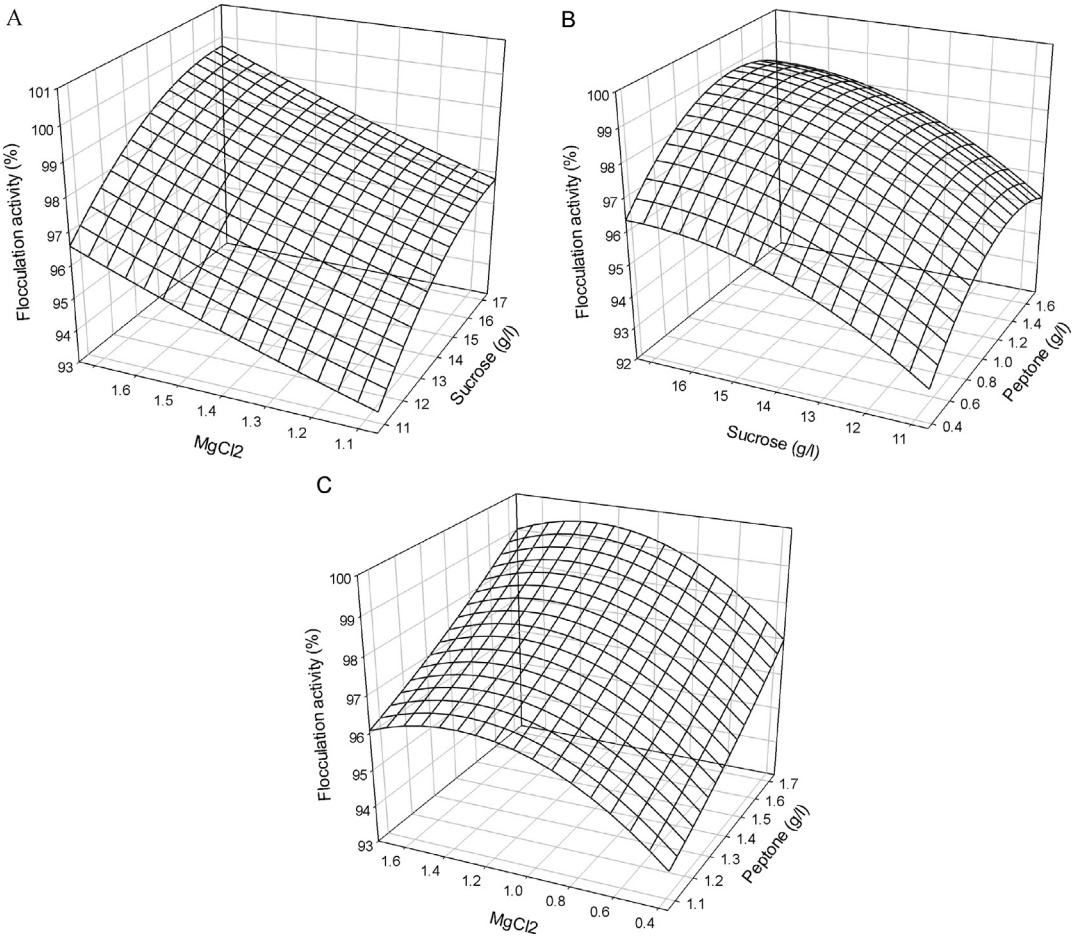

Fig. 1. Response surface plots representative of critical media components in flocculation activity. Interactions are respectively represented as A (sucrose and $MgCl_2$), B (Peptone and sucrose) and C (peptone and $MgCl_2$). *Description of the illustration*: it describes the relationship between concentrations of critical media components and flocculation activity which was an indirect measure of bioflocculant production. Hence, the visualization of the pair wise interaction between sucrose, peptone and $MgCl_2$ thus revealing concentration for optimal bioflocculant production.

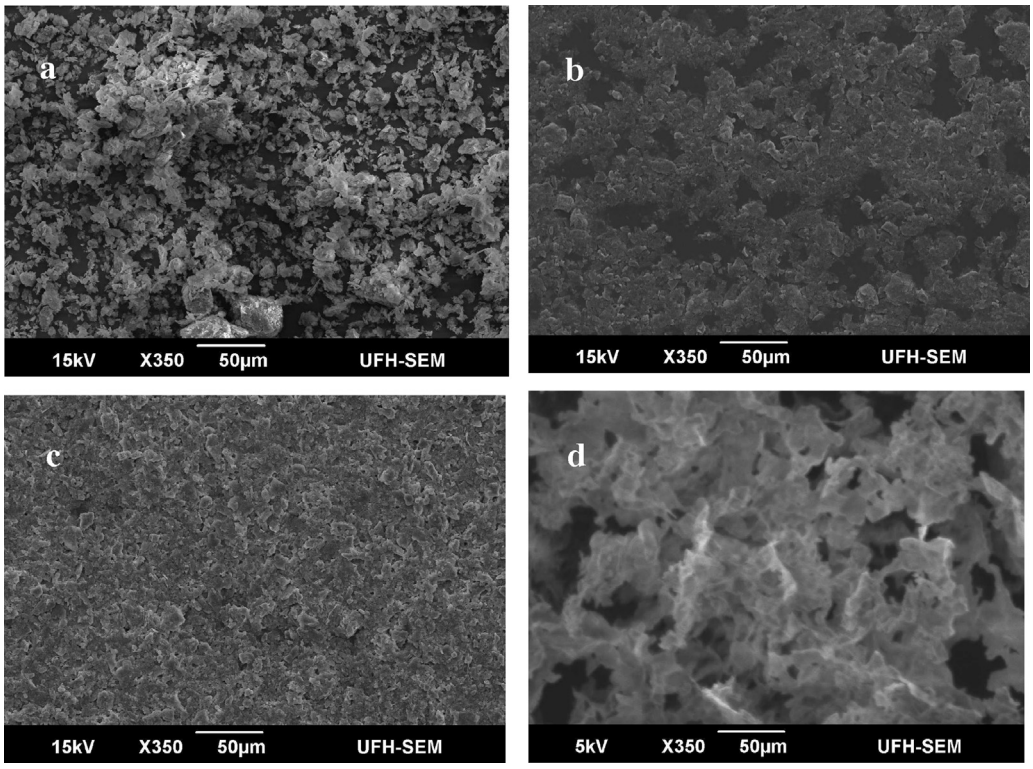

Fig. 2. SEM images of the purified bioflocculant from mixed culture fermentation of *Streptomyces* sp. Gansen and *Cellulomonas* sp. Okoh and the flocculated kaolin clay suspension. a = kaolin clay; b = kaolin clay suspension treated with bioflocculant free broth (control); c = kaolin clay suspension treated with bioflocculant; d = bioflocculant from the mixed culture. *Description of the illustration*: it describes the morphological state of the bioflocculant produced by the mixed cultures of *Streptomyces* sp. Gansen and *Cellulomonas* sp. Okoh after purification and lyophilization. It further depicts the morphology of the Kaolin clay before and after treatment with bioflocculant showing how closely knit the particles become following flocculation by the bioflocculant.

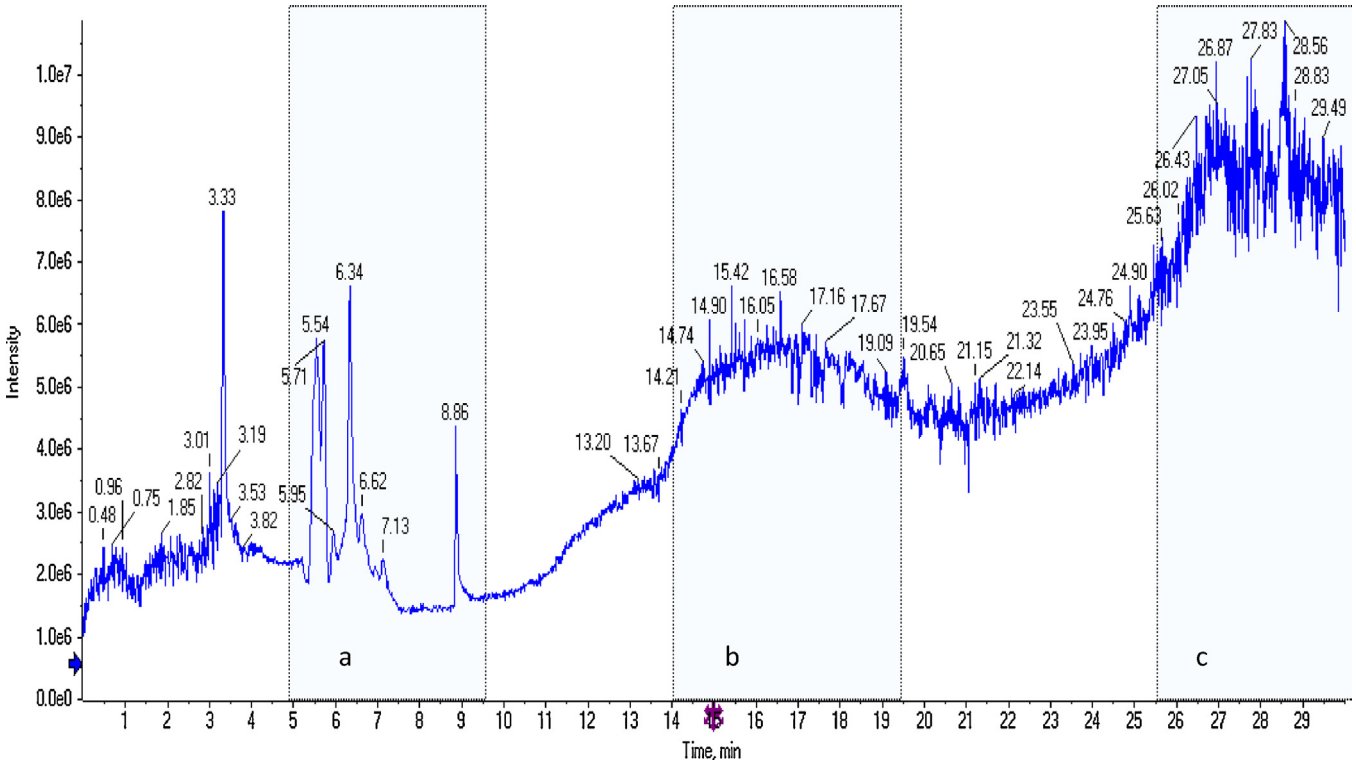

Fig. 3. LC–MS of the purified bioflocculant produced by the mixed cultures of *Streptomyces* sp. Gansen and *Cellulomonas* sp. Okoh. a–c = molecular weights of compounds obtained between 5 and 10 min, 15 and 20 min and 25 and 30 min respectively. *Description of the illustration*: it describes the molecular weight distribution of the constituents of the bioflocculant following liquid chromatography mass spectrometric analysis.

shown and this is as expected as these nutritional sources are essential in the formation of cellular structures and metabolites. Nonetheless, the revelation of the roles of carbon to nitrogen ratio and the ratios of other nutritional sources toward the production of bioflocculants is novel. Hence, the need for medium optimization in mixed culture fermentation.

5. Conclusion

The mixed culture fermentation of *Streptomyces* sp. Gansen and *Cellulomonas* sp. Okoh produced bioflocculant with high yield and flocculation activity. Sucrose, peptone and magnesium chloride respectively served as preferred carbon, nitrogen and cation sources. Central composite design showed the significance of respective critical media components; peptone made the highest contribution. The pair wise interactions of the critical variables revealed that carbon and nitrogen ratio as well as carbon and cation ratio were crucial for the consortium to produce bioflocculant. Functional groups characteristic of aromatic, allylic, hydroxyl, carboxyl, esters and amino moieties were obtained from the bioflocculant, suggestive of an amalgam of polymers including uronic acids and carbohydrates. Furthermore, the untargeted analysis of the molecular weight of the bioflocculants revealed the diversity of the constituents as depicted by the range of molecular weights obtained following LC–MS analysis hence, an additional accord to the poly-diversity of the bioflocculant besides the diversity shown with the results of the chemical analyses. The high bioflocculant yield of the consortium and high flocculation activity of the bioflocculant portend industrial applicability.

Author's contributions

Nwodo UU executed the experiment, extracted the data and wrote the initial draft of the manuscript. Green E co-supervised the research. Mabinya LV, Rumbold K and Obi CL advised on the models appropriate for the experimentation, analysis of data and interpreted the results obtained from the models. Okaiyeto K was of technical assistance in the lab while Okoh AI designed and supervised the research as well as proof read the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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