



Production of Microbial Exopolysaccharide by Cost-effective Medium Optimization Method

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Authors' contributions

This work was carried out in collaboration among all authors. Author GOA designed the study. Author IGN performed the experiment, statistic analysis, wrote the protocol and wrote the first draft of the manuscript. Authors GOA and OKA supervised the study. All authors read and approved the final manuscript.

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ABSTRACT

Microbial exopolysaccharide (EPS) emerged as a fast and high yielding sustainable polymeric substance which can be used as an alternative to synthetic polymer in industry. In this study, the influence of various nutritional and environmental factors of fermentation medium on bacterial growth and EPS production was evaluated by one factor at a time optimization. Efficient production medium was chosen from four different basal media and its carbon and nitrogen substrates were varied among organic and inorganic sources. Feasibility of bacterial utilization of some agricultural wastes as carbon and nitrogen sources to synthesize exopolysaccharide was compared. Carbon source of the fermentation medium was replaced with hydrolysates of sugarcane bagasse (SCB), sweet potato peels (SPP) or ripe plantain peels (RPP) at various concentrations while the nitrogen substrates were replaced with extracts of poultry droppings (PP), groundnut pod (GP) or beans bran (BB). Response results observed from single factor optimization were explored as center points to

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design a model for Response Surface Methodology study. Cell growth was determined from the biomass population of the fermentation broth after 5 days of incubation in a rotary shaker at 120 rpm at 30°C. EPS was precipitated with pre-chilled ethanol (at 4°C) from cell-free broth and overnight incubation at 4°C. Total carbohydrate content was estimated by phenol-sulphuric acid method. Result obtained showed that 2% concentration Hydrolysate of SPP containing medium gave maximum yield of 2.26 g EPS/l of the medium as compared to sucrose containing medium with yield of 1.25 g EPS/l of the medium while highest production yield of 9.46 gEPS/l of the medium was obtained from 10 g/l BB extract medium as compared to yeast extract medium (5.41 gEPS/l). Results indicated that agricultural wastes such as sweet potato peel hydrolysate and bean bran extract could be developed as inexpensive alternative route to synthesize EPS from bacteria than inorganic substrates.

Keywords: *Bean bran; bacteria; exopolysaccharide; RSM; sweet potato peel.*

1. INTRODUCTION

In complex biodiversity of marine habitat, most microorganisms synthesize biopolymers as secondary metabolites for adherence and thriving which enable their adaptation and survival in adverse environmental conditions. The biological roles of these biopolymers include cell defense, cell interactions and adherence to surfaces, energy reserves and virulence expression [1]. Such polymeric substances comprise of polysaccharides, lipids, protein, nucleic acid and humic acid substances [2]. Microbial polysaccharide is the most important biopolymer secreted either by bacteria, fungi or yeast as natural, non-toxic, biodegradable and renewable sugar monomers [3]. However, a variety of microorganisms identified as exopolysaccharide producing bacteria was isolated from extreme environments. Extremophiles adapt to and tolerate harsh environmental conditions by synthesizing extracellular polysaccharides to withstand the external stress. Such microorganisms include *Alcaligenes*, *Streptococcus*, *Halomonas*, *Pseudomonas* and *Shewanella* [4].

Considerable emerging interest of researchers in exploring new microbial exopolysaccharides in the past few decades is owing to the unique diverse and modifiable functionalities attributed by their physiochemical and rheological properties. They serve as additives for thickening, stabilizing, gelling and emulsifying, bioflocculators and viscosifiers in various fields and industrial sectors such as drilling, medicines, pharmaceutical, cosmetics, food and agriculture [5,6,7].

Although, higher plants polysaccharides are extracted from their biomass, microbial polysaccharides are very much preferable as

they are recovered from their fermentation broth cultures. Besides, microbial polysaccharides are more advantageous and economical at industrial scale for the production of bioactive materials due to their fast and higher production yield under well-controlled fermentation process within limited time, uninterrupted geographical and seasonal variations and non-interference with land for agricultural purposes. Moreover, various organic sources can be utilized for microbial production unlike plants that solely depend on solar energy [8].

Nevertheless, the global biopolymer market is highly dominated by plants polymers superseding smaller fraction of microbial polysaccharides such as Xanthan and Gellan with commercialized industrial values because of cost intensive steps in production process and other downstream process [9,10]. The rising interests to overcome the economic hindrance of microbial EPS in industrial purposes call elucidation of several approaches. Approaches adopted to minimize production cost of EPS biosynthesis in industrial scale involve using cheaper fermentation feedstock, optimizing production conditions to improve yield or genetic manipulation to develop strains and optimizing downstream processing [11,12].

Numerous studies on EPS production have been reported, however, only a few were directed toward production using low cost medium [13]. Fermentation medium represent 30% cost of production of bioactive molecules from microorganisms. The costly synthetic complex media commonly employed for EPS production resulted from high amount of individual nutrients such as sugars (carbon source), yeast extract and peptone (nitrogen source) and other salts hence are economically not feasible. Bacterial cells efficiently utilize carbon and nitrogen

sources available in the medium under certain critical environmental factors to release polysaccharide into the surroundings [13]. Almost 70% of the energy require for biosynthesis of exopolysaccharide are from carbon source [2]. It is crucial to develop environmentally friendly and cost effective fermentation medium in order to increase production yield at reasonable cost by designing and employing cheaper alternative fermentation substrates such as agro-industrial by-products and other waste materials [6].

This study aimed to optimize EPS production of bacterial strain by Response Surface Methodology using different agricultural wastes to develop an economic culture medium, the effect of environmental factors on its production and characterization of produced EPS.

2. MATERIALS AND METHODS

2.1 Isolation of Bacteria

Water sample was aseptically collected from Bonny Island using a sterile container and immediately transported to the laboratory. It was serially diluted from 10^{-1} to 10^{-7} in normal saline and 0.1 ml of each dilution was inoculated in nutrient agar plate supplemented with 4% sucrose in duplicate by spread plate technique [14]. Bacteria producing EPS characterized by thick slime or mucoid colonies were selected and subcultured.

2.2 Screening and Selection by EPS Quantification

Pure cultures of selected isolates were inoculated in a freshly prepared nutrient broth supplemented with 4% sucrose and incubated for 24 hrs [14]. The cell-free broths after centrifuging at 10,000 rpm for 10 minutes were mixed with two volumes of cold ethanol and stored at 4°C overnight. The precipitates were collected centrifuging at 10,000 rpm for 20 minutes, dried, weighed and dissolved in distilled water [15]. They were quantified by phenol sulphuric acid method using glucose as standard [16].

2.3 Morphology and Biochemical Identification

Most potent EPS producing bacterial isolate selected was identified by morphological characterization, examined by Grams staining reaction and further confirm by biochemical test

based on key to Bergey's Manual of Determinative Bacteriology.

2.4 Preparation of Extracts of Organic Carbon Sources

Ripe plantain peel (RPP), sugarcane baggasse (SCB) and sweet potato peel (SPP) were diluted individually with distilled water containing 1% concentrated sulfuric acid. The mixtures were autoclaved at 121°C for 40 minutes, cooled and filtered. The pretreated filtrates were separately used as source of carbon.

2.5 Preparation of Extracts of Organic Nitrogen Sources

Bean bran (BB), groundnut pod (GP) and poultry droppings (PD) were collected, dried and processed. They were diluted in 0.5M K_2SO_4 solutions to extract the nitrate [17]. The solutions were autoclaved at 121°C for 40 minutes, cooled and filtered for clear extracts. The extracts were used as economic media for nitrogen source.

2.6 Optimization by Classical Method

One-factor-at-a-time was used to investigate the EPS production by the selected strain.

2.7 Effect of Different Synthetic Media on EPS Production

Influence of different media composition was investigated on growth and production of EPS by the bacterial strain. The media include A (g/l): peptone 10, yeast extract 3, sodium chloride 5 and sucrose 20 at pH 6.5 [18]; B (g/l): yeast extract 1.0, $MgSO_4$ 0.5, KH_2PO_4 1.0 and sucrose 30 at pH 7.0 [19]; C (g/l): KH_2PO_4 0.2, K_2HPO_4 1.5, $MgSO_4 \cdot 7H_2O$ 0.2, $CaSO_4 \cdot 2H_2O$ 0.1, $FeCl_3$ 0.002, yeast extract 0.5 and sucrose 20 [7]; D (g/l): peptone 10, glucose 15, NaCl 75 and $MgSO_4$ 1.5 at pH 10 [5] and E: nutrient broth supplemented with sucrose 20 g/l [14].

Aliquot of 24 hrs old actively growing culture of the isolate was used at constant inoculum size for each medium. The best medium that produced the highest growth and EPS yield was selected as the production media for further study.

2.8 Effect of Different Carbon Sources

Different inorganic carbon substrates (glucose, fructose and sucrose) and different organic

carbon substrates (RPP, SPP and SCB extracts) were altered in the medium while other factors remaining constant. 100 ml of each medium in 250 ml conical flask was inoculated with 5% of 24 hrs old active culture of the isolate and incubated for 96 hrs at 200 rpm in orbital shaker incubator at 30°C.

2.9 Effect of Different Nitrogen Sources

Varying synthetic nitrogen sources (yeast extracts, ammonium sulfate and urea) and organic nitrogen sources (BB, GP and PD extracts) were altered in the medium while other factors remained constant. 100 ml of each medium was inoculated with actively growing culture at constant inoculum size and incubated at 30°C in orbital shaker incubator at 200 rpm for 96 hrs.

2.10 Effect of Different pH

EPS production was carried out in the medium at varying initial pH range from 5, 6, 7 to 8 to monitor the influence on bacterial growth and EPS yield. 100 ml of the medium in 250 ml conical flask were adjusted with 1M HCl and 1M NaOH at different pH range before sterilization. About 5% of 24 hrs old active culture of the isolate and incubated for 96 hrs at 200 rpm in orbital shaker incubator at 30°C.

2.11 Effect of Different Incubation Period

Varying period of incubation (3, 6 and 9 days) were investigated for their effect on growth and production of EPS when cultured at constant inoculum size.

2.12 Optimization of Culture Medium by Response Surface Methodology

The tested variables were selected from one-factor-time and employed to determine the optimum conditions and concentration for production of EPS by bacterial strain through RSM. The four independent variables included in the model were organic carbon source SPPE (10-50 g/l), organic nitrogen source BBE (3.75-8.75 g/l), pH (4.5-6.5) and incubation time (4.5-10.5 days).

2.13 Characterization of EPS

The crude EPS was characterized by Fourier Transform Infrared Spectrometer.

2.14 FTIR

The functional groups of the EPS from strain code WAS 11 was analyzed with FT IR spectroscopy [20]. About 2 mg of the crude EPS was mixed with 200 mg of dry potassium bromide pellets and compressed into mould in a disc. The FT IR spectrum was recorded in the frequency range of 4000-400 cm^{-1} by using FTIR system.

3. RESULTS

Bacterial isolates were chosen based on their ability to form mucoid on nutrient agar plates. Mucoid-forming isolate with the highest potential of producing exopolysaccharide was selected quantitatively by EPS dry weight and phenol sulfuric acid test after cold ethanol precipitation. Bacterial strain WAS11 which has the highest potential of producing EPS was selected, identified as Gram-negative, catalase and oxidase positive, hydrogen sulphide utilizing rod and used for further study in this research. WAS11 was identified as *Shewanella chilikensis* WAS11 (Accession number: MN511702).

Result showed that all the shortlisted culture media favored the growth and production of EPS by the bacterial strain inoculated. Optimum cell growth of 1.62 (OD) with the highest EPS yield of 1.29 g/l was obtained from medium C. The least EPS yield of 0.78 g/l along with highest cell growth of 1.84(OD) was observed in medium B as shown in Fig. 1.

When the carbon source of the medium C was varied with three inorganic carbon substrates; fructose, glucose and sucrose, result obtained showed that highest EPS yield of 0.18 g/l was obtained from sucrose culture medium at 20 g/l along with highest bacterial cell growth of 1.16 (OD) after 24 hrs. Least EPS yield of 0.11g/l was observed from glucose containing medium while lowest biomass of 0.48 (OD) was observed from fructose medium as shown in Fig. 2. The carbon substrate of the medium was supplemented with pretreated extracts of SPP, RPP and SCB separately. Result signified that SPP containing medium was the best carbon source for EPS production at 1.23 g/l yield with optimum growth rate of 0.6 (OD) while the least yield of .897 g/l with highest bacterial biomass of 0.9 (OD) was found from SCB containing medium followed by 0.92 g/l EPS yield of RPP containing medium as shown in Fig. 2.

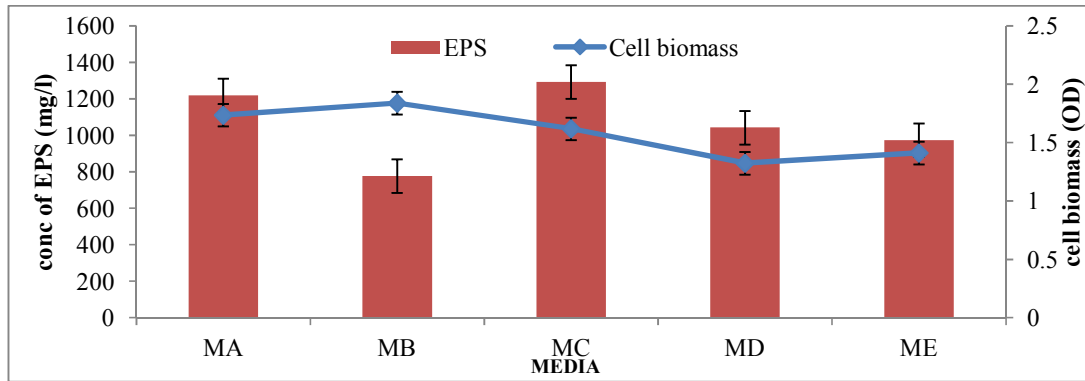


Fig. 1. Effect of different media on cell growth and EPS production

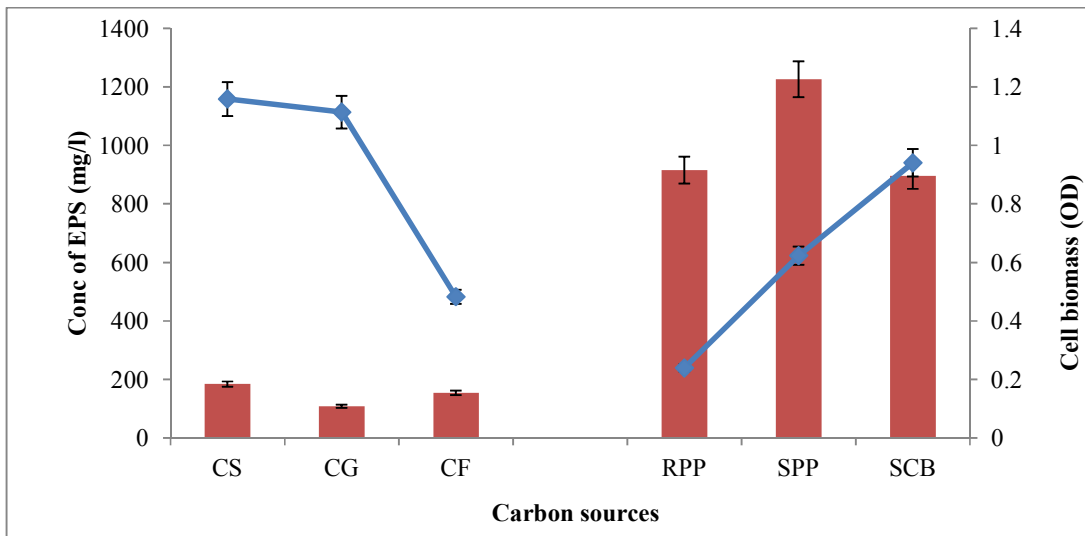


Fig. 2. Effect of organic and inorganic carbon substrates on EPS and cell biomass

Out of the three synthetic nitrogen sources used for this optimization study, 0.5% yeast extract containing medium produced maximum EPS yield of 0.54 g/l and optimum growth of 0.71 (OD) after 24 hrs cultivation. Under the same condition, maximum cell growth of the strain (1.94(OD)) was noted in urea containing medium as shown in Fig. 3. The synthetic nitrogen source of the medium composition was replaced by three different organic nitrogen extracts individually (PD, GC and BB). Result obtained indicates that bacterial isolate was able to grow in the three tested nitrogen substrate media and BB containing medium produced the highest yield of 0.95 g/l at optimum cell growth of 0.97(OD) as shown in Fig. 3.

Result of the influence of initial pH on bacterial growth and production of EPS illustrated that

there was correlation between bacterial growth and exopolysaccharide production as the initial pH of the culture media was varied from range of 5 to 8. The maximum yield of 1.98 g/l EPS and uppermost biomass yield of 2.02(OD) was from lowest initial pH 5 while smallest amount yield of 1.15 g/l EPS with least cell biomass of 0.83(OD) was observed from highest initial pH 8 as shown in Fig. 4.

As the time of incubation increased, biomass yield of the bacterial isolate multiplied indicating exponential growth of bacteria. But maximum EPS yield of 1.16 g/l was observed on day 6 while peak of the cell density, 1.06(OD) was observed on day 9 as shown in Fig. 5.

RSM result showed that trial 17 produced the highest EPS yield of 38.14 g/l with initial

pH of 5 for the culture medium containing SPP 20 g/l as carbon source and BB 5 g/l as limiting nitrogen source incubated for the period of 6 days as shown in Table 1 and Fig. 6 showing the interaction among initial pH, incubation time, carbon and nitrogen sources.

4. DISCUSSION

According to several researchers, the main criteria for selection of the most potential bacteria

exopolysaccharide producers were based on highest EPS yield of the isolates [21]. Liu and others reported isolate with the maximum EPS yield capability as the most potent producer bacteria (2010). Numerous authors suggested that media composition is essential parameters in determining EPS biosynthesis of bacteria strain [22,23]. The results showed that the most potent isolate code WAS11 used for this study was able to grow and produce EPS in all the selected media but the maximum EPS yield occurred in media C.

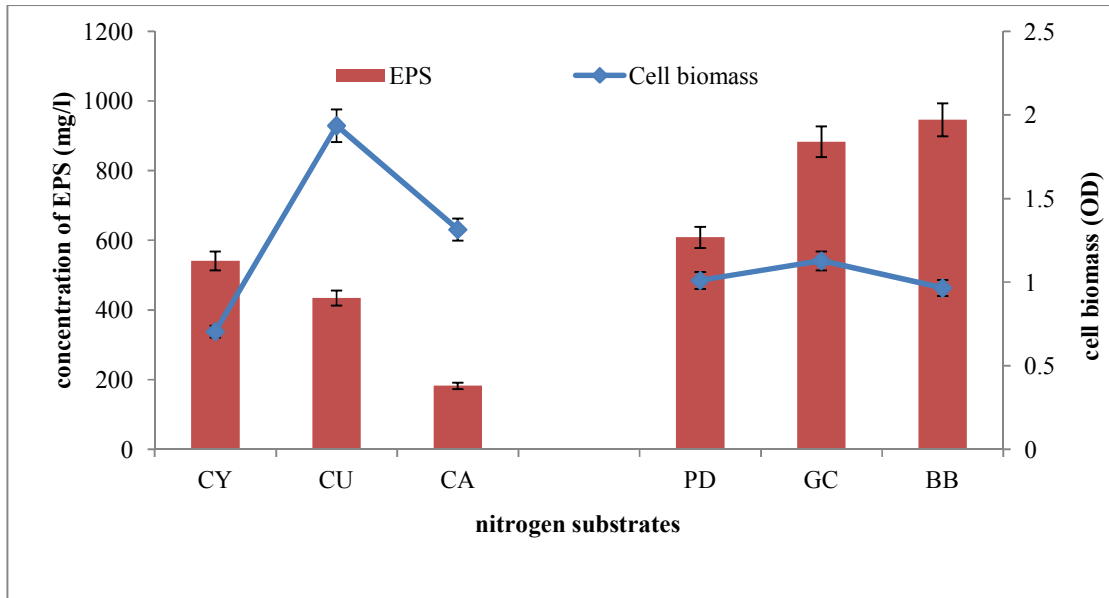


Fig. 3. Effect of synthetic and organic nitrogen substrates

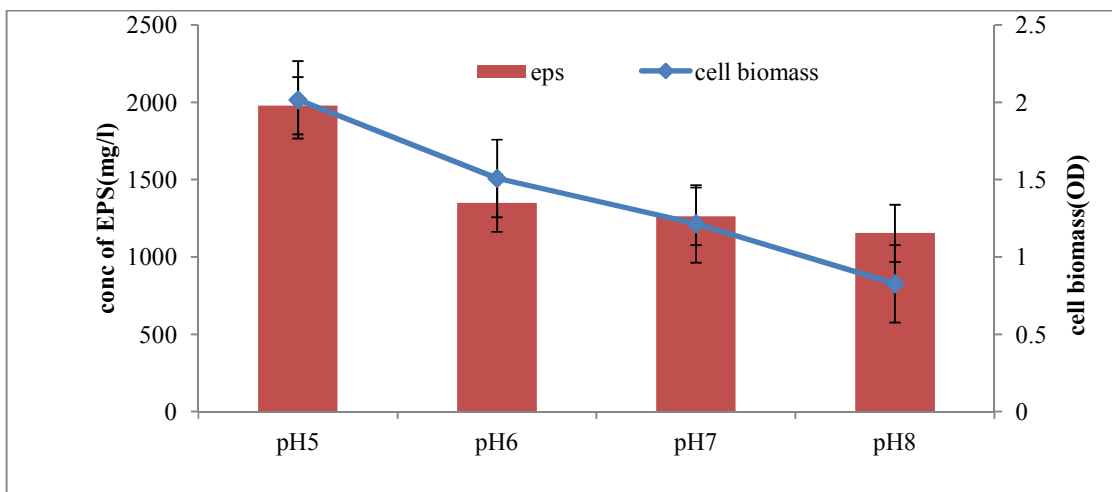


Fig. 4. Effect of different initial pH

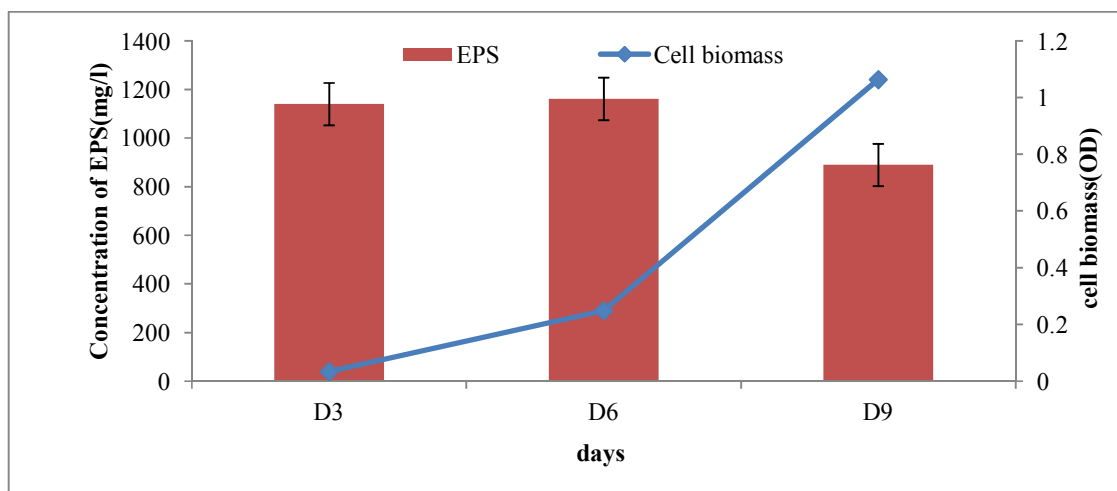


Fig. 5. Effects of incubation days

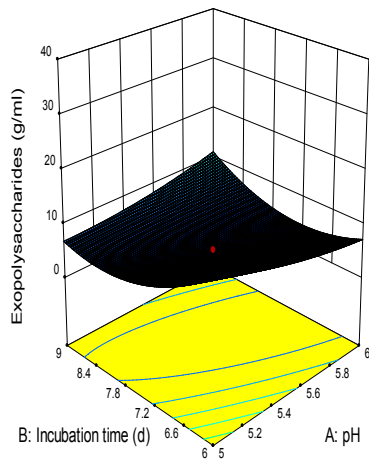
Table 1. Response surface experimental design for culture conditions influencing EPS production

	Factor 1	Factor 2	Factor 3	Factor 4	Actual response	Predicted response	
Std	Run	A:pH	B:Incubation time	C:Carbon source	EPS	EPS	
			Days	g/l	g/l	g/l	
20	1	5.5	10.5	30	6.25	18.34	20.82
19	2	5.5	4.5	30	6.25	24.08	24.74
4	3	6	9	20	5	15.81	7.60
21	4	5.5	7.5	10	6.25	18.60	13.57
16	5	6	9	40	7.5	26.50	25.86
3	6	5	9	20	5	10.54	17.94
11	7	5	9	20	7.5	15.38	8.33
14	8	6	6	40	7.5	19.49	13.66
22	9	5.5	7.5	50	6.25	2.13	10.30
5	10	5	6	40	5	26.71	17.89
30	11	5.5	7.5	30	6.25	5.20	4.89
17	12	4.5	7.5	30	6.25	4.99	10.76
7	13	5	9	40	5	14.10	6.44
23	14	5.5	7.5	30	3.75	4.68	11.28
26	15	5.5	7.5	30	6.25	5.20	4.89
6	16	6	6	40	5	8.07	10.40
1	17	5	6	20	5	38.14	34.06
27	18	5.5	7.5	30	5	5.20	7.28
2	19	6	6	20	5	9.14	11.67
10	20	6	6	20	7.5	2.48	5.42
15	21	5	9	40	7.5	7.29	6.34
12	22	6	9	20	7.5	2.55	12.95
9	23	5	6	20	7.5	9.89	12.86
13	24	5	6	40	7.5	2.70	6.19
28	25	5.5	7.5	30	6.25	5.20	4.89
8	26	6	9	40	5	12.41	11.01
24	27	5.5	7.5	30	8.75	8.91	4.93
18	28	6.5	7.5	30	6.25	10.52	7.89
25	29	5.5	7.5	30	6.25	5.20	4.89
29	30	5.5	7.5	30	6.25	5.20	4.89

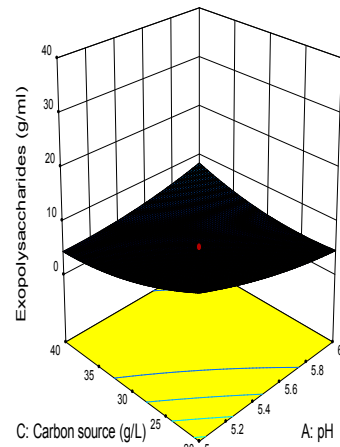
Table 2. Analysis of variance

Analysis of variance table [Partial sum of squares - Type III]						
Source	Sum of squares	df	Mean square	F value	p-value Prob> F	
Model	17.88	14	1.28	8.42	< 0.0001	significant
A-pH	0.13	1	0.13	0.84	0.3734	
B-Incubation time	0.051	1	0.051	0.34	0.5693	
C-Carbon source	0.27	1	0.27	1.76	0.2046	
D-Nitrogen source	2.464E-003	1	2.464E-003	0.016	0.9003	

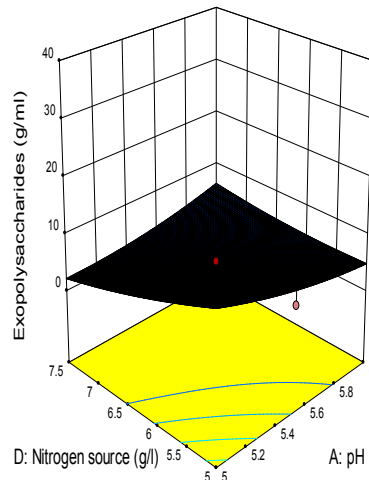
Design-Expert® Software
 Factor Coding: Actual
 Exopolysaccharides (g/ml)
 • Design points above predicted value
 38.14
 2.13
 X1 = A: pH
 X2 = B: Incubation time
 Actual Factors
 C: Carbon source = 30
 D: Nitrogen source = 6.25



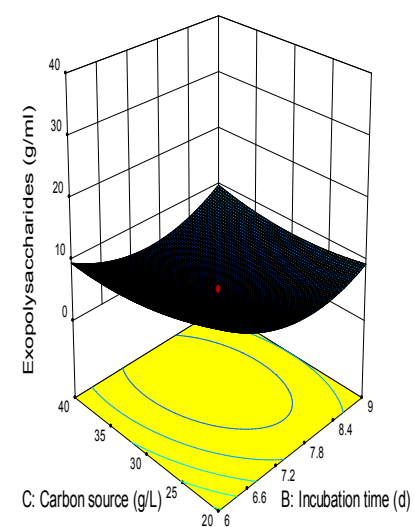
Design-Expert® Software
 Factor Coding: Actual
 Exopolysaccharides (g/ml)
 • Design points above predicted value
 38.14
 2.13
 X1 = A: pH
 X2 = C: Carbon source
 Actual Factors
 B: Incubation time = 7.5
 D: Nitrogen source = 6.25



Design-Expert® Software
 Factor Coding: Actual
 Exopolysaccharides (g/ml)
 • Design points above predicted value
 • Design points below predicted value
 38.14
 2.13
 X1 = A: pH
 X2 = D: Nitrogen source
 Actual Factors
 B: Incubation time = 7.5
 C: Carbon source = 30



Design-Expert® Software
 Factor Coding: Actual
 Exopolysaccharides (g/ml)
 • Design points above predicted value
 38.14
 2.13
 X1 = B: Incubation time
 X2 = C: Carbon source
 Actual Factors
 A: pH = 5.5
 D: Nitrogen source = 6.25



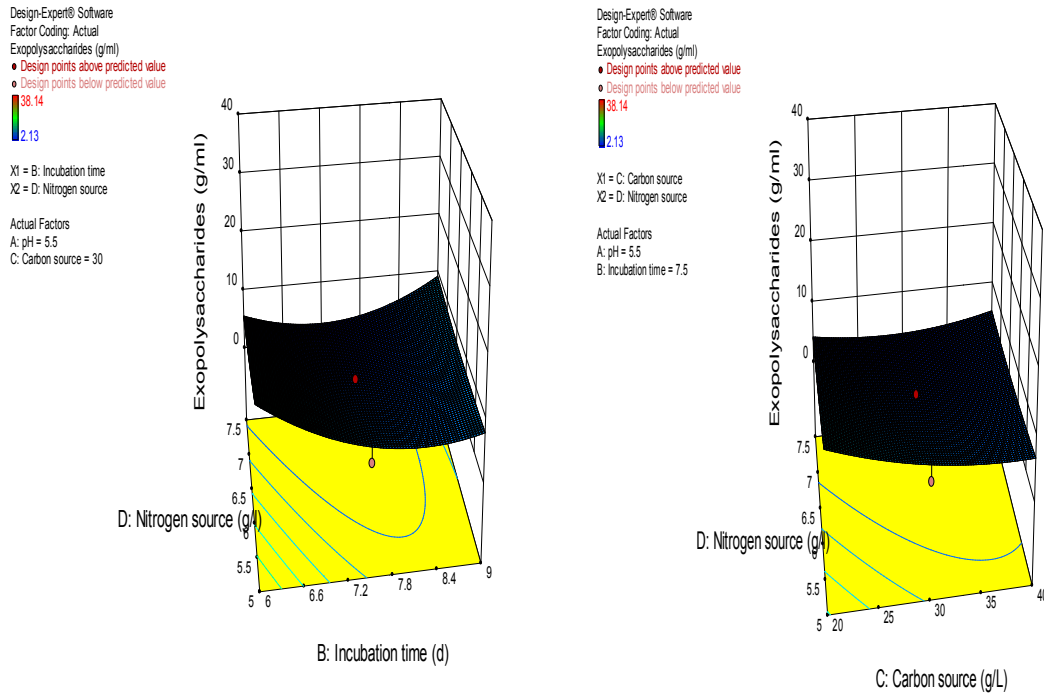


Fig. 6. Response surface design of interaction among initial pH, incubation time, carbon source and nitrogen source

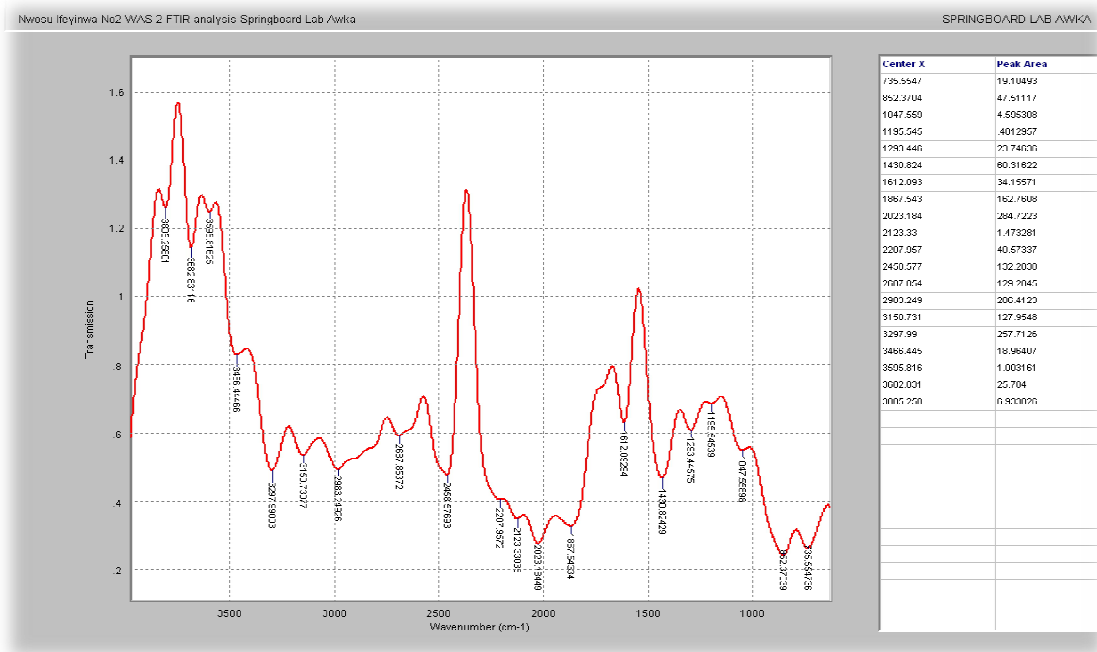


Fig. 7. FT-IR spectrum of strain code WAS11 exopolysaccharide

Carbohydrates are the key nutritional requirement for the growth and cell development. Influence of carbohydrates on bacteria growth and EPS biosynthesis was reported to depend on strain type, specific sugar and cell carbohydrate characteristics [24]. This research studied the effect of both organic and inorganic carbon sources. Result of different inorganic carbon sources showed that sucrose substrate produced highest EPS yield than glucose and fructose substrates after 96h incubation. This was corresponding to the result obtained from the study of different carbon optimization by Shukla and others [14]. Supplementing the carbon source of medium by utilizing organic carbon extracts; SPP, RPP and SCB, results indicated that irrespective of the organic sources selected as carbon substrate, syntheses of EPS were higher than in inorganic carbon media. SPPE stimulated EPS production at least twice more than sucrose which was most suitable sugar used for this study. There study is synonymous with the study of Vaishnav and others [19]. Although cell growths were not stimulated suggesting that most sugars released from the organic extracts were employed for EPS production with little or no energy for growth [25].

Moghannem and others recommended that EPS production and yield were highly affected by the type and concentration of carbon sources in the medium (2018). Result obtained from the optimization study of the concentration of agricultural waste media indicated that SPPE was most effective and economical carbon source for EPS production among other carbon sources used. Bacterial growth and EPS yield maintained higher levels at different concentrations of SPPE comparable to skim milk medium supplemented with whey protein concentrate [26].

Cerning [27] stated that carbon source along with nitrogen source and other ion sources are important composition of media that enhance EPS production. Yeast extract was reported to greatly influence EPS yield by *L. plantarum* MTCC9510 and *Paeniactillus polymyca* EJS-3 [28,29]. Likewise study by Shankar and others showed that yeast extract was more efficient nitrogen substrate than other nitrogen sources for growth and production of EPS by *S. phocae*. The nitrogen substrates in this study were compared, though the maximum cell growth cell biomass was observed by urea containing medium but highest EPS yield was formed by yeast extract medium. This may be due to

presence of large quantities of free amino acids, short peptides and more growth factors on yeast extracts [30]. The production yield in BBE has a great economic interest as nitrogen source. Data obtained from single factor optimization indicated that the use of SPPE as carbon source and BBE as nitrogen source were significantly more economical nutritional requirement for production of EPS by isolate code WAS11 at industrial scale.

Zisu and Shah [31] described correlation exist between pH, EPS production yield and bacterial growth. The report of the influence of initial pH on production of EPS showed that as the pH reduce from 8 to 5, the EPS yield and the cell growth increase. Patel and others investigated the effect of pH on EPS production from pH range of 6 to 9 and results showed that lower pH gave higher EPS yield as compared to other pH (2018). Result confirmed that the highest bacterial growth and EPS yield was obtained at pH 5.

Throughout the optimization study of the influence of incubation days, EPS reached its maximum production yield at post stationary phase of the growth. Report illustrated that bacterial growth were retained at higher level while there was resulting decline of EPS yield after day 6 probably due to the action of glycohydrolase enzyme possibly released in the culture medium which catalyzed the polysaccharide degradation [32].

RSM depends on data obtained from single factor optimization and data analysis indicated that the maximum EPS yield was estimated to be 34.06 g/l and the actual yield obtained was 38.14 g/l which was in close accordance with the model prediction.

The FT-IR spectrum of the crude EPS code WAS11 strain revealed some characteristic functional groups according to data in literature. The broad stretching absorption peaks exhibited at 3595, 3466 and 3297 cm^{-1} were assigned to the hydroxyl (O-H) group of polysaccharide (3600-3200 cm^{-1}) [33]. The absorption at 2983 cm^{-1} was attributed to the symmetrical stretching vibration of aliphatic (CH_2) group which revealed the presence of sugar content [34]. The weak absorption peak noticed at 2687 and 2458 cm^{-1} correspond to amide stretching vibration bands. An asymmetrical stretching peak was observed at 1612 cm^{-1} which corresponds to the ring stretching of galactose and mannose (1662-1593 cm^{-1}) [35]. A peak at 1430 cm^{-1}

represents asymmetrical bending of carboxylic acid group [35]. The stretching of C-O-C and C-O at region of 1195 cm^{-1} was as result of the glycosidic linkage corresponds to the presence of carbohydrates [36]. Furthermore, the peak at 1047 cm^{-1} ($1000\text{-}1125\text{ cm}^{-1}$) range confirmed the presence of uronic acid, O-acetyl ester linkage bonds [37]. Moreover, the small absorption at approximately 852 cm^{-1} band was attributed to sugar monomers in polysaccharide [38,39]. The absorption at approximately 735 cm^{-1} ($910\text{-}665\text{ cm}^{-1}$) peaks corresponds to the N-H wag of primary amines [36].

5. CONCLUSION

This study concluded that bacterial strain code WAS11 posses the ability to grow and produce exopolysaccharide in defined medium whose carbon and nitrogen sources were replaced with hydrolyzed extracts of sweet potato peel and bean bran as inexpensive resources. Furthermore, the optimization study shows that 20 g/l of the sweet potato peels and 5 g/l of bean bran in the medium at pH 5 after 6 days produced highest yield (38.14 g/l) of the exopolysaccharide.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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