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Isolation, characterization and performance of extra cellular polymer substances (EPS) producing bacteria from biofloc culture water of Nile tilapia using distillery spentwash as carbon source

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ABSTRACT

The present study aimed to isolate and characterize the Extracellular polymeric substance (EPS) producing bacteria from biofloc reared Nile tilapia (Chitralada) ponds. Distillery spentwash was used as a carbon source to maintain the C: N ratio at 10: 1 in the fish culture ponds and screening of bacteria were done fortnightly in 180 days culture. Out of 38 bacterial isolates, 7 isolates were found to produce EPS. Based on 16s rRNA sequence analysis the isolates were identified as *Bacillus subtilis, B. megaterium, B. infantis, B. cereus, Pseudomonas balearica, P. mendocina* and *P. alcaligenes.* The highest production of EPS was recorded in *B. cereus* (1.25 g/L). EPS extracted from *Bacillus cereus* was reported to have higher protein (89 µg/ml) and *B. subtilis* possessed higher carbohydrate (753.75 µg/ml). Maximum flocculating ability of 40.18% in *B. cereus* and higher emulsifying activity of 63.53% was observed in *B. megaterium*. The EPS extracted from *B. infantis* showed lower sludge volume index on its treatment with aquaculture sludge (15.38 ml/g). Absorption band in the range of 4,000/cm to 450/cm using FTIR analysis confirmed the presence of characteristic functional bands arising from polysaccharides, nucleic acids and proteins. The results indicated the presence of EPS producing bacteria in biofloc based Nile tilapia aquaculture systems.

Keywords: Biofloc, Extracellular polymeric substances, Flocculation, FTIR

Tilapia is one of the widely cultured fish globally in various aquaculture systems (FAO, 2017). However, tilapia production was hugely affected by emerging disease outbreaks thereby hindering the development of aquaculture industry (Telli *et al.* 2014, Menaga *et al.* 2019). Also, to ensure long-term sustainability, environmental impacts must be minimized and alternative ways such as flocculation process need to be applied. Flocculation helps to overcome the problem of aquaculture effluent by the addition of synthetic polymers. As sludge from aquaculture ponds is negatively charged, they can interact with these synthetic polymers in combination with cations to neutralize the surface charges thereby aiding the flocculation and settling (Higgins and Novack, 1997b). However, these synthetic polymers not only enhance the flocculation and sludge dewatering but also when discharged into the environment affects the soil microorganism as a major disadvantage. To overcome these hindrances, green technology metabolites known as bio flocculants produced by microorganisms can act similar function as synthetic flocculants (Zaki *et al.* 2011). Many microorganisms isolated from sludge were reported to secrete extracellular polymeric substances. They are mainly composed of functional proteins, polysaccharides, nucleic acid and cellulose (Kumar *et al.* 2004, Feng and Xu 2008). The bacterial extracellular polymeric substances also improve the formation of bioflocs in activated sludge and contribute to its structural, surface charge and settling properties (Houghton *et al.* 2001). Consequently, better understanding on the characteristics of EPS in biofloc provides advantages in undergoing waste water treatment process. Biofloc technology, a zero-water exchange system, can be used to overcome the problems such as sludge settling by stocking at a higher density with the enhanced fish production (Hargreaves 2013, Green *et al.* 2019). Therefore, the ultimate aim of this study was to characterize the potential flocculant-producing bacteria particularly for aquaculture wastewater treatment. In the present study 7 bacterial species isolated from Biofloc culture water were identified as EPS producing bacteria. The EPS extracted from the bacteria has been studied for various properties for its application in flocculation and sludge settling.

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MATERIALS AND METHODS

Isolation and characterization of EPS producing bacteria from Biofloc: Nile tilapia juveniles (5.5±0.21 g) were stocked at a density of 10/m^3 in 300 m^2 ponds for 180 days in triplicates. Biofloc development and maintenance

in the freshwater culture ponds were adopted as suggested by Taw (2006). Distillery spent wash obtained from M/s Rajshree Biosolutions, Coimbatore has been used as a carbon source to maintain the C : N ratio at 10 : 1. The biofloc water sample from Nile tilapia cultured ponds was collected, serially diluted and spread plated on nutrient agar. The plates were incubated at 37°C. Morphologically different colonies were picked and stained using crystal violet and 20% aqueous $CuSO_4$ solution for initial screening of EPS producing bacteria (Cain *et al.* 2009). Morphological characterization was carried out for the selected bacterial isolates according to Bergey's manual (Brown, 1939).

Genotypic identification and phylogenetic tree construction of EPS producing bacteria: The genomic DNA was isolated using Phenol-Chloroform method from EPS producing bacteria. The isolated DNA was amplified for its 16s rRNA region using the Forward primer-AGAGTTTGATCCTGGCTCAG and Reverse primer-CGGTTACCTTGTTACGACTT. The amplified DNA was sequenced and subjected to BLAST analysis. The aligned sequences were submitted in Genbank database to obtain the accession number. The phylogenetic tree was constructed to find the relationship between the EPS producing bacterial isolates.

Extraction of EPS from bacterial isolates: The bacterial isolates were incubated in liquid medium specific for EPS production containing 0.2 g KH_2PO_4, 0.8 g K_2HPO_4, 0.2 g $MgSO_4.H_2O$, 0.1 g $CaSO_4.2H_2O$, 2 mg $FeCl_3$, $Na_2MoO_4.2H_2O$ (trace), 0.5 g yeast extract, 20 g Sucrose per litre and after 48 h of incubation the cell pellet were harvested by centrifugation at 5,000 rpm for 15 minutes. The cell pellet is resuspended by adding 500 µl of 1 mM EDTA and centrifuged at 9,000 rpm for 10 minutes. To the cell free supernatant, cold acetone solution (1 : 3) was added and centrifuged to obtain the pellet. The final pellet was dried at 60ºC for 24 h to obtain the capsular EPS (Mu'minah and Subair, 2015).

Characterization of EPS

Protein and carbohydrate estimation: EPS solution were prepared by harvesting the cell pellets from the specific medium. The pellets were resuspended in twice the volume of 0.2 M cold sulphuric acid solution followed by stirring at 4°C for 3 h. The resulting suspension was centrifuged at 15,000 rpm for 20 min to obtain the supernatant (EPS solution) which can be used for further analysis. The amount of protein and carbohydrate in EPS solution was quantified using Lowry's method (1951) and phenol sulphuric method (Dubois *et al.* 1956).

Lipid emulsifying test: Emulsifying activity of EPS producing bacterial isolates were tested using the method of Yasumatsu *et al.* (1972). An equal volume of olive oil and EPS solution were incubated on a rotary shaker for 11 days. The lipid emulsifying activity was expressed as percentage of the height of emulsified layer to the height of whole layer.

Flocculation ability: To 2 ml of EPS solution, 1 ml of 6.8 mM $CaCl_2$ and 10 ml of 5 g/l activated charcoal were added and mixed well. Control was prepared without adding EPS solution. The mixture is kept incubated at room temperature and the absorbance was measured at 550 nm. Flocculation activity was calculated according to following formula (Aziz *et al.* 2012).

$$\text{Flocculating activity (\%)} = A–B/A \times 100$$

where A and B are the optical density values of control and samples respectively.

Sludge volume index: Sludge volume index (SVI) of the EPS producing bacterial isolates was estimated according to the method of Subramanian *et al.* (2010). The sludge sample was collected and 2% of extracted EPS (v/v) was added to this and mixed well. Sludge sample without EPS was considered as control. The settled sludge volume and suspended solids was measured after 30 min. SVI was calculated using the formula:

$$\text{Sludge volume index (SVI) (ml/g)} = \frac{\text{Settled sludge volume}}{\text{Suspended solids}}$$

Fourier Transform Infra-red Analysis (*FTIR*): The EPS extracted from bacterial isolates was subjected to Fourier Transform Infra-red Analysis and spectra were recorded in 4,000/cm to 450/cm range for confirmation of the functional groups in EPS arising from polysaccharides, nucleic acids and proteins.

RESULTS AND DISCUSSION

Isolation and biochemical characterization of EPS producing bacteria: Out of 38 morphologically different colonies only 7 were confirmed for EPS production using crystal violet and 20% aqueous $CuSO_4$ solution stained for initial screening of EPS producing bacteria. The morphological and biochemical characterization of 7 bacterial isolates were listed in the Table 1.

Phylogenetic tree for EPS producing bacteria: The accession numbers obtained from genbank database are *Bacillus infantis* (577 bp) -MH424755, *Bacillus subtilis* (568 bp)-MH424900, *Bacillus megaterium* (577bp)-MH424904, *Pseudomonas alcaligenes* (518 bp)-MH424895, *Bacillus cereus* (492 bp)-MH997476, *Pseudomonas balearica* (736 bp)-MH997474, *Pseudomonas mendocina* (669 bp)-MH997472. It is inferred from the phylogenetic tree that there exists a close relationship between the four *Bacillus* species: *Bacillus infantis, Bacillus cereus, Bacillus megaterium, Bacillus subtilis* and three *Pseudomonas species*: *Pseudomonas mendocina, Pseudomonas alcaligenes, Pseudomonas balearica.* Bacteria in general tend to produce EPS under unfavourable environmental conditions to prevent them from desiccation, toxic compounds, low temperatures or high osmotic pressures and during oxygen and nitrogen level fluctuations (Hirst *et al.* 2003). The EPS obtained in our study was capsular EPS and its tightly bound with the cell wall by non-covalent linkages that helps in the biofloc or biofilm formation by interacting with the negatively

Table 1. Morphological and biochemical characterization of bacterial isolates

Biochemical characteristic	*Pseudomonas mendocina*	*P. balearica*	*P. alcaligenes*	*Bacillus cereus*	*B. subtilis*	*B. megaterium*	*B. infantis*
Colony morphology	-ve rods	-ve rods	-ve rods	+ve rods	+ve rods	+ve rods	+ve rods
Indole	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Methyl red	-ve	-ve	+ve	+ve	-ve	-ve	-ve
Voges Proskauer's	-ve	-ve	-ve	+ve	+ve	-ve	+ve
Citrate utilization	+ve	-ve	-ve	-ve	+ve	+ve	+ve
Glucose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Adonitol	+ve	+ve	+ve	+ve	+ve	-ve	-ve
Arabinose	+ve	+ve	+ve	+ve	+ve	-ve	-ve
Lactose	+ve	+ve	+ve	+ve	+ve	-ve	+ve
Sorbitol	+ve	+ve	+ve	+ve	+ve	-ve	-ve
Mannitol	+ve	+ve	+ve	+ve	+ve	-ve	+ve
Sucrose	+ve	+ve	+ve	+ve	+ve	-ve	+ve

charged sludge solids (Higgins and Novack, 1997b). The results from our study reveals the fact that both gram positive and gram negative bacteria can produce EPS as most of the microorganisms in the environment have the ability to live within these biofilms. Biofloc helps the bacteria to form the microbial aggregates as an essential step for the survival (Flemming and Wingender, 2001b) in a huge bacterial population. The anionic nature of EPS can also be augmented by the presence of uronic acids or ketal-linked pyruvates thereby enhancing the interactions of divalent cations such as Calcium and Magnesium which helps in increasing the binding force in the biofilm (Donlan, 2002, Sutherland 2001). The identified isolates were dominantly *Bacillus* sp. which agrees with the findings of Hashim *et al.* (2018). Previous studies have also reported on the presence of bioflocculating bacteria in biofloc culture ponds of *P. vannamei* (Kasan *et al.* 2016).

Extraction of EPS from bacterial isolates: The EPS extracted from seven bacterial isolates were listed in Table 2. The EPS extracted from bacterial isolates of biofloc culture water varies from 0.33–1.25 g/L with the maximum production in *Bacillus cereus* (1.25 g/L). The maximum EPS production (3.2 g/L) from *Bacillus cereus* isolated from sludge water (Subramaniam *et al.* 2007) was also previously reported. The variable production of EPS by different isolates was may be due to the bacterial metabolic activity (Subramanian *et al.* 2010), genetic organization of gene clusters, biosynthesis of sugar precursors and regulatory elements (Nouha *et al.* 2016).

Table 2. EPS extracted, protein, carbohydrate content and sludge volume index of bacterial isolates

Isolates	EPS in grams/L	Protein (µg/ml)	Carbohydrate (µg/ml)	Sludge volume index ml/g
Bacillus subtilis	0.92	40	753.75	27.78
B. megaterium	0.98	26	303.80	21.43
B. infantis	0.33	19	188.75	15.38
B. cereus	1.25	89	377.50	20.10
Pseudomonas balearica	0.6	59	476.25	16.67
P. mendocina	0.483	18	667.50	30.77
P. alcaligenes	0.53	29	71.25	23.08

Carbohydrate and protein content in EPS: The carbohydrate and protein content of EPS producing bacterial isolates were listed in Table 2. The highest carbohydrate was found to be present in EPS extracted from *B. Subtilis* and the protein was higher in the EPS of *B. Cereus.* Total carbohydrate concentration was higher than the total protein in all the extracted EPS from the bacterial isolates which is in agreement with the previous investigations of Shahnavaz *et al.* (2015) who also showed the dominance of carbohydrate in EPS than protein. This increased concentration of carbohydrate than protein plays a major role in sludge settling by forming the bridges between the negatively charged groups and divalent cations present in the sludge (Higgins and Novack 1997b). However, proteins also help in the floc formation (Urbain *et al.* 1993) contributing to the binding strength and stability, by hydrophobic interactions and polyvalent cation bridging (Jorand *et al.* 1998).

Flocculation and emulsifying activity of EPS: The flocculation ability and emulsifying activity of seven EPS producing bacterial isolates were analysed. The maximum flocculation percentage observed in *Bacillus cereus* (40.18%) and this may be de due to the fact that EPS with long polymeric chains holds more active sites for binding when compared with the EPS produced by the other bacterial isolates. Also the maximum flocculating ability can be related to the highest production of EPS by the *Bacillus cereus*. The higher flocculating ability has been previously reported in *Bacillus cereus* isolated from biofloc culture ponds of Pacific white leg shrimp (93%) by Ismail and Amin (2018). *Bacillus megaterium* was found to have the highest emulsifying activity (63.53%) which was probably due to the higher levels of uric acid content in the extracted EPS of this specific isolate. This is in agreement with the studies of Yun and Park (2003) as they reported the polysaccharide produced by *Bacillus* sp. CP912 has a

greater potential to use as an emulsifier. Results from the previous findings correlated a significant increase in the emulsifying activity of EPS showed higher biodegradability and lower solubility in water. Thus the glycol-protein nature of the EPS can be used as excellent emulsifying agents with various medical and environmental applications (Cameotra and Makkar 2004, Rosenberg and Ron, 1999).

Sludge volume index: The Sludge volume index of EPS producing bacteria were listed in Table 2. The EPS of *B. infantis* showed lower sludge volume index when it is treated with sludge water collected from Nile tilapia biofloc culture ponds (15.38 ml/g). Sludge Volume Index was found to be below 150 mL/g for all the bacterial isolates which is required for a good sludge settling (APHA, 2005). Similarly Subramanian *et al*. (2010) isolated *Pseudomonas* species from waste water sludge and proved the ability of these bacterial strains in good sludge settling which is in accordance with our study as *Pseudomonas balearica* has the second lowest SVI followed by *Bacillus infantis*.

Fourier Transform Infra-red Analysis (*FTIR*): FTIR spectra of the extracted EPS from bacterial isolates were obtained. The peaks for *Bacillus subtilis* 3,432.05/cm (Fig. 1), *B. Megaterium* 3449.20/cm (Fig. 2), *B. infantis* 3,438.42/cm, *B. cereus* 3,450.06/cm, *Pseudomonas balearica* 3,453.52/cm, *P. mendocina* 3,453.25/cm, and *P.*

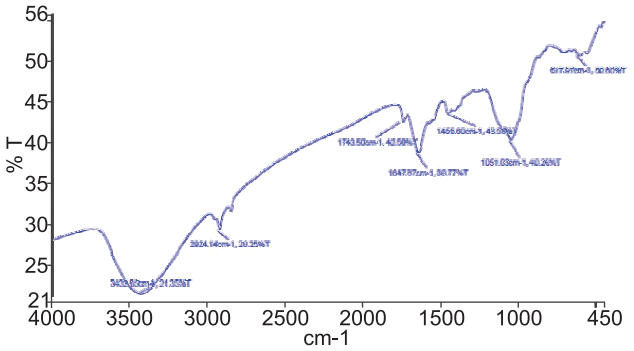

Fig. 1. FTIR spectra of the EPS from *Bacillus Subtilis.*

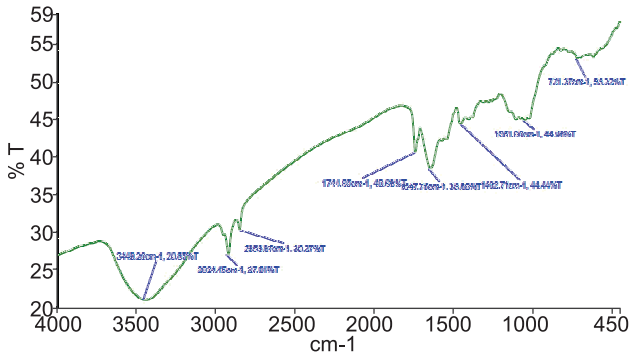

Fig. 2. FTIR spectra of the EPS from *Bacillus megaterium.*

Alcaligenes 3,417.87/cm were obtained indicating hydroxyl group from carbohydrate and water molecules present in the EPS (Liang *et al.* 2010). The peaks for alkyl groups were recorded at 2,924.14/cm for *Bacillus subtilis,* 2,924.45/cm for *B. Megaterium, 2*,922.68/cm for *B. infantis,* 2,925.05/cm for *B. cereus, 2*924.35/cm for *Pseudomonas balearica, 2*924.08/cm for *P mendocina,* and 2924.58/cm for *P. Alcaligenes* (Brian-Jaisson *et al.* 2016). The peaks for carboxylic groups were recorded at 1,647.67/cm for *Bacillus subtilis,* 1,647.76/cm for *B. megaterium,* 1,644.45/cm for *B. infantis,* 1,647.86/cm for *B. cereus,* 1,647.35/cm for *Pseudomonas balearica,* 1,644.95/cm for *P mendocina,* and 1,645.20/cm for *P. Alcaligenes* (Caruso *et al.* 2018). FTIR peaks also proved the characteristic bands arising from the functional groups present in the EPS which was also previously confirmed from the studies of Brian-Jaisson *et al.* (2016). In the present study, EPS producing isolates were screened from the culture ponds of Nile tilapia. The various characteristics of the EPS secreted by the isolates proved that it not only helps in the floc and filament formation in biofloc culture ponds but also in sludge settling as a mode of aquaculture wastewater treatment.

The current study proved the presence of EPS producing bacteria in the biofloc ponds providing the optimal environment for the flocculation and sludge settlement. The identified isolates can be mass cultured and deployed in the bioremediation of aquaculture effluents. This study also elucidates the advantage of adopting biofloc technology in aquaculture and more indepth insights on the mechanism in the EPS formation and spatial distribution will help for the broad range of applications in aquaculture.

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