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Optimization of the EPS production of a bacterial floc consortium using different parameters

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ABSTRACT

The capacity for exopolysaccharide (EPS) biosynthesis of a bacterial consortium culture was investigated. To obtain maximal EPS recovery, pH, carbon and nitrogen sources, and inorganic ions were optimized. At pH 8, the flocculation index (FI) percentage was 62.6%, viscosity was 1.38 ± 0.015 g $m^{-1}.s^{-1}$, and 10.3 g/L EPS was produced. However, the maximum total EPS production (12 g/L), FI percentage (70%), and viscosity (1.4 ± 0.01 g $m^{-1}.s^{-1}$) were achieved when ammonium sulfate was used as nitrogen source. Meanwhile, the highest EPS production was realized when dextrose was selected as carbon source with and without crude oil. Dextrose exhibited significant advantage over the four other carbon sources. After 5 days of incubation with crude oil, the dextrose enhanced EPS production to (14 g/L) with a high FI percentage (70.7%). Among the inorganic ions, the maximum EPS production (16.1 g/L) was obtained after adding 0.5% potassium chloride to the culture medium, with a high viscosity of 1.44 ± 0.005 g $m^{-1}.s^{-1}$.

1. Introduction

Microbial cells produce different types of exopolysaccharide (EPS) during their life cycle. The EPS is relatively has simple structure, polysaccharide structures in microorganisms contain various shape and rigidity, primarily capsule EPS is produced through the log phase of growth bacteria, whereas during the stationary phase produced slime EPS (Kumar et al., 2011). The chemical characteristics of EPS change with culture age, nutrient levels, and growth conditions (Kumar et al., 2007). EPSs are organic macromolecules with a high molecular weight and are naturally "sticky." Bacterial EPSs, which are chemically diverse, are important in the interaction between bacteria and their environment (Singha, 2012). The study of culture conditions during EPS production can be of interest in optimizing production. In wastewater treatment systems, most microbial aggregates, such as sludge flocs and biofilms, are present in the form of extracellular polymeric substances, e.g., EPSs (Sheng et al., 2010). EPSs have been found to occur in enormous variety of unique forms; EPSs are closely associated with cells in the form of distinct capsules or as slime that is separated from the cell surface. In microbial cells EPSs are frequently set up as the external structures of prokaryotic and eukaryotic. EPS production by bacteria is a highly complex process and it is affected by many different parameters (Vijayendra et al., 2008). EPS is consider to render many functions, such as promoting the first attachment of cells to firm surfaces, the forming and maintenance of microcolonies and grown biofilm structures, and enhancing biofilm impedance to ecological stress (Czaczyk and Myszka, 2007). Extracellular polymeric substances (EPS) produce from bacteria have highly hydrated that are mainly composed of proteins, polysaccharides and DNA, also protection against environmental stresses such as salinity and drought by support bacterial life in chemical reactions or nutrient entrapment (Costa et al., 2018).

Some bacteria produced EPS as aqueous solution and have high viscosity this behavior can be presence of carboxylic acid groups, this is an important role or requirement for the use of EPS in applications water treatment, (foods, biomedical, oil recovery application biomed cosmetics) (Fusconi et al., 2010; Daud et al., 2019).

Under all conditions bacteria can produce EPS; however, the quantity and structure of EPS are dependent and influenced by nutritional and environmental conditions (Xu et al., 2010). In general, EPS production that is influenced by environmental factors and structure (Kanmani1 and Yuvapriya, 2017), can be classified into two groups.

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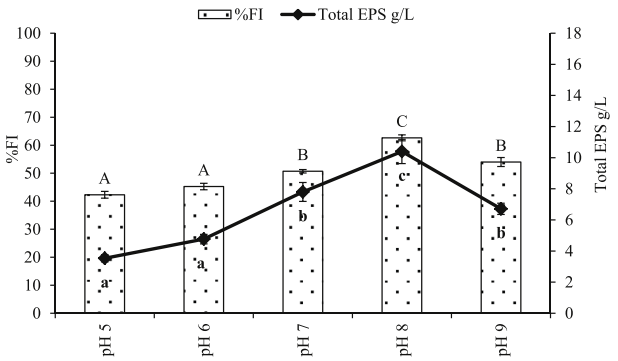

Fig. 1. Effects of different pH values on FI percentage and total EPS production of the consortium culture. Variations in upper case and lower case letters represent statistically significant differences in FI percentage and total EPS production at a specific pH value ($P < 0.05$).

Table 1
EPS production and viscosity from consortium culture after 5 days of incubation at 37 °C in 150 rpm on different pH.

pH	Bound EPS g/L ±SD	Free EPS g/L ±SD	Total EPS g/L	Viscosity $g.m^{-1}.s^{-1}$ ±SD
5	3.2 ± 0.26	0.33 ± 0.057	3.53[a]	1.21 ± 0.015
6	4.5 ± 0.3	0.26 ± 0.057	4.76[a]	1.22 ± 0.015
7	7.4 ± 0.64	0.36 ± 0.057	7.76[b]	1.3 ± 0.01
8	9.96 ± 0.35	0.31 ± 0.014	10.3[c]	1.38 ± 0.015
9	8.06 ± 0.81	0.43 ± 0.057	8.81[b]	1.30 ± 0.005

Letters a, b and c: different letters shows significantly different of Total EPS within different pH by Tukey's test ($P < 0.05$).

(1) The first category involves changes in stress conditions that cause a transfer in the microbial community. Subsequently, the number of EPS-producing bacteria will increase or decrease in the entire bacterial consortium, which has to adjust to the fresh environment. (2) In the second category, the existing bacterial community reregulates the pathway of metabolic EPS production as response or reaction to changes in conditions (Liu et al., 2004). The present study aims to determine the potential EPS production from a bacterial floc consortium isolated from the environment under different parameter conditions.

2. Materials and methods

2.1. Preparation of the consortium culture

Four single bacterial isolates, namely, *Bacillus cereus*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* 28, and *Pseudomonas aeruginosa* HNYM41, were used in gasoline degradation (Almansoory, 2015). The isolates as consirtium put in 100 mL nutrient broth (NB) to grow and incubated in an orbital shaker at 37 °C, 150 rpm for 24 h (Al-Baldawi et al., 2017). The cells were separated via centrifugation at 4000 rpm (Eppendorf centrifuge 5810R) 15 min, and the pellet was suspended in 10 mL sodium chloride (NaCl; 0.85g) normal saline for preparation of standard inoculums, standard inoculate were prepared for every growth the isolate was monitored by measuring the absorbance at 550 nm (A550) by using spectrophotometer device (Genesys 10 UV Spectronic Thermo, USA) to adjust its optical density 0.5, calculate for all isolates as 10% (v/v) of standard inoculate (Almansoory et al., 2019). The bacterial culture was inoculated in mineral salts medium (MSM), subsequently, mixed culture was prepared using the standard inocula of each bacterium at an appropriate volume. Then, 10% (v/v) of standard inoculate were transferred into 100 mL of MSM with 1% (v/v) tapis crude oil as the sole organic source and incubated for 7 days at 37 °C in shaker at 150 rpm(Al-Baldawi et al., 2015).

2.2. Determining the flocculation index (FI)

Consortium culture isolates were grown in 100 mL NB. Standardized inocula were prepared as described by (Almansoory, 2015). Then, 10% standard inoculum was inoculated into 25 mL NB and incubated at 37 °C at 150 rpm. After 24 h, the samples growth were allowed to stand and stable then measured after 5, 10, 30, and 60 min (indicated as A_t). The samples were centrifuged at force 650 g immediately for 2 min, and the optical density at 550 nm of the supernatant (upper layer) was measured and indicated as A_s. The percentage of FI (%FI) was calculated using Equation (1) (Malik et al., 2003; Sannasi et al., 2009). All tests were performed in triplicate.

$$\%FI = \frac{(A_t - A_s)}{A_t} \times 100 \quad (1)$$

2.3. Extraction of free and bound EPSs

Bacterial cultures were grown in MSM with 1% crude oil and then incubated at 37 °C at 150 rpm for 7 days. Bacterial cultures were cen-

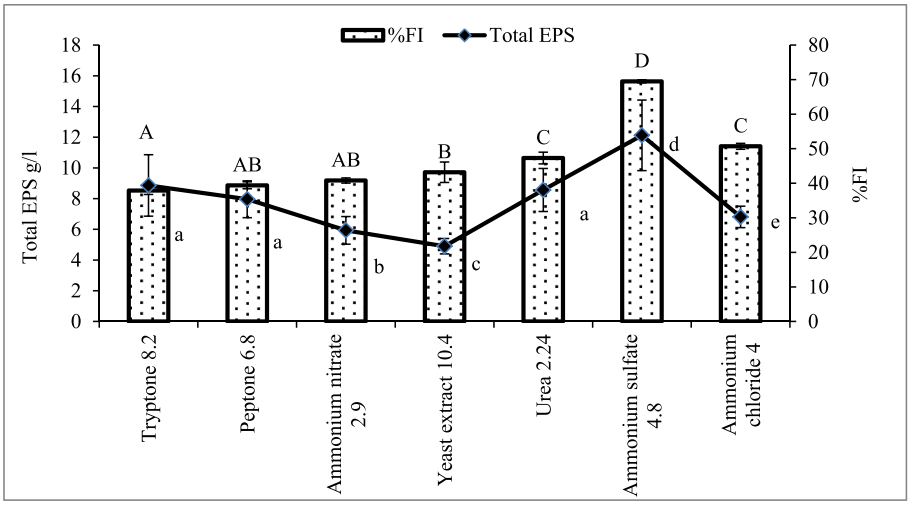

Fig. 2. Effects different sources of nitrogen on the FI percentage and EPS production of the consortium culture. Variations in upper case and lower case letters represent statistically significant differences in FI percentage and total EPS production ($P < 0.05$).

Table 2
EPS production and viscosity after 5 days of incubation under different sources of nitrogen.

Nitrogen sources	Bound EPS g/L ± SD	Free EPS g/L ± SD	Total EPS	Viscosity $g.m^{-1}.s^{-1}$ ± SD
Tryptone	6.06 ± 0.20	2.80 ± 0.06	8.86[a]	1.33 ± 0.01
Peptone	5.06 ± 0.15	2.90 ± 0.26	7.96[a]	1.30 ± 0.01
Ammonium nitrate	4.60 ± 0.10	1.33 ± 0.23	5.93[b]	1.29 ± 0.01
Yeast	3.50 ± 0.49	1.40 ± 0.36	4.9[c]	1.38 ± 0.01
Urea	6.13 ± 0.15	2.40 ± 0.06	8.56[a]	1.36 ± 0.01
Ammonium sulfate	8.36 ± 0.45	3.76 ± 0.15	12.12[d]	1.40 ± 0.01
Ammonium chloride	6.46 ± 0.07	0.35 ± 0.07	6.81[e]	1.29 ± 0.01

Letters a, b, c, d and e: different letters shows significantly different of Total EPS within different sources of nitrogen Tukey's test ($P < 0.05$).

Table 3
EPS production and viscosity after 5 days of incubation under different sources of carbon with crude oil addition.

Carbon sources With crude oil	Bound EPS g/L ±SD	Free EPS g/L ± SD	Total EPS g/L	Viscosity $g.m^{-1}.s^{-1}$ ±SD
Sucrose	7.5 ± 0.60	2.4 ± 0.26	9.9[a]	1.54 ± 0.01
Dextrose	11.1 ± 1.10	2.9 ± 0.20	14.0[b]	1.57 ± 0.03
Glucose	3.2 ± 0.20	2.2 ± 0.15	5.4[c]	1.35 ± 0.01
Fructose	2.8 ± 0.15	2.2 ± 0.10	5.0[c]	1.33 ± 0.01

Letters a, b and c: different letters shows significantly different of Total EPS within different sources of carbon with crude oil addition Tukey's test ($P < 0.05$).

trifuged under 10000 *g* for 15 min in temperature 4 °C, and 10 mL of the supernatant was precipitated with 30 mL ethanol by incubating at −20 °C for 24 h. Centrifuged the liquid under 10000 *g* for 15 min to remove the supernatant. Bound EPS was extracted from the cell pellet using the EDTA method (Sheng et al., 2005). The precipitate from the free and bound EPSs was dried at 105 °C for 24 h and weighed (Eboigbodin and Bigges, 2008).

2.4. Effects of pH levels, carbon and nitrogen sources, and inorganic ions on FI percentage and EPS production

The FI percentage, viscosity, and EPS production of the consortium culture were investigated after 5 days under the effects of carbon and nitrogen sources and operational parameters (i.e., pH and inorganic ions with 0.5% concentration). Different pH levels (5.0, 6.0, 7.0, 8.0, and 9.0) were initially tested and measured by pH meter (Memmert, Germany). The effect of different carbon sources (5%), such as glucose, dextrose, fructose, and sucrose, added to the culture medium was also investigated. To select the best carbon source that can produce the highest FI percentage and EPS amount, different nitrogen sources (e.g., ammonium sulfate, ammonium nitrate, tryptone, peptone, urea, yeast, and ammonium chloride) were explored. Moreover, the effects of various inorganic ions, such as potassium chloride (KCl), calcium chloride ($CaCl_2$), manganese chloride ($MnCl_2$), and zinc chloride ($ZnCl_2$), were investigated by adding 0.5% concentration of each ion to the culture medium. All experiments were conducted in triplicate to ensure the precision of the obtained data with and without the addition of crude oil. (IQ Scientific Instruments, Spectrum Technologies, Plainfield, USA).

2.5. Statistical interpretation

The effects of pH levels, nitrogen sources, and inorganic ions on FI percentage and total EPS production were statistically analyzed using IBM SPSS Statistics (version 21 for Windows, IBM Corporation, U.S.A.). Significant differences in the effects of various nitrogen sources on FI percentage and total EPS production were determined using one-way ANOVA ($P < 0.05$), whereas post-hoc Tukey's test was performed for multiple comparisons. Mean ± standard deviation (SD) was determined in triplicate for all the experiments. In addition, two-way ANOVA was adopted to compare the effects of carbon sources, including sucrose, dextrose, glucose, and fructose, on FI percentage and total EPS production with and without crude oil hydrocarbons. In addition, post-hoc multiple comparison of means was conducted using Tukey's method at 95% confidence interval or $P < 0.05$.

3. Results and discussions

3.1. Effects of different pH values on EPS production

Nouha et al. (2017) reported flocculation index of EPS has been one of the key properties for application biopolymer in different field and the studies are investigated available which have the important structure function relationship between EPS functional composition and flocculation index. The flocculation activity has been modeled by various mechanisms, and the flocculants activity of high-molecular weight EPS. As shown in Fig. 1, the consortium culture produced a high amount of EPS (10.3 g/L) at pH 8. EPS production increased with increasing pH value. In particular, increases of 62%, 54%, 44%, 24%, and 14% were recorded at pH 8 compared with at pH 9, 7, 6, and 5, respectively. These results indicate that the consortium culture prefer a basic environment to achieve maximum EPS production. Fig. 1 shows that an increase in EPS production increases FI percentage. The FI percentage at pH 8 was 62.6%, and 10.3 g/L EPS was produced this is agreement with Hereher et al. (2018) investigated increased EPS production with increased pH until arrival at 9. Therefore, FI is relevant to EPS production. On the basis of the results of Malik et al. (2003), the consortium culture used in our study exhibited good flocculation at pH 7, 8, and 9 (i.e., 50% or more), but only partial flocculation at pH 5 and 6 (20%–50%). Thus, pH 8 was selected in the present study to further investigate the EPS production of the consortium culture. A low EPS production by bacterial cells at a low pH (at 5 and 6) may increase the acidity of the medium, thereby causing acid stress on bacterial cells as reported by Zhang et al. (2011).

The activity of flocculation for EPS produced by the consortium culture at pH 9 (as 49.3% at 10 mg/L EPS) is better than that produced by *Pseudoalteromonas sp.* SM9913 isolated by Li et al. (2008). Flocculation activity is an important step in achieving good bioremediation of pollutants. In pollution treatment, the use of non-flocculating bacteria increases turbidity and decreases the efficiency of treatments (Malik et al., 2003). The effects of various pH values of the medium on EPS production and viscosity are presented in Table 1. A high viscosity was obtained at pH 8. The viscosity of the culture medium is dependent on EPS production. Increased EPS production increases the viscosity of the medium. Liu et al. (2011) reported that viscosity increases with pH because of the higher ionization degree of the carboxylate group in EPS. By contrast, a decrease in viscosity is related to the high accumulation of alkali on a negatively charged polysaccharide. Statistical analysis showed no significant difference at pH 5 and 6 in FI percentage and total EPS production ($P = 0.68$ and $P = 0.77$, respectively). However, significant differences were observed at pH 7, 8, and 9 ($P = 0.002$, 0.000, and 0.000, respectively). These results indicate that neutral to alkaline media are better for FI and total EPS production.

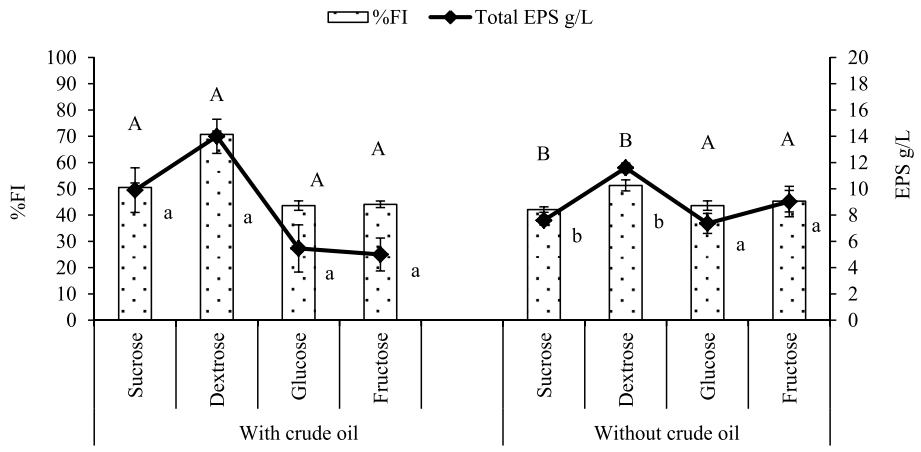

Fig. 3. Effects different sources of carbon on the FI percentage and EPS production of the consortium culture with and without crude oil. Differences in upper case and lower case letters represent statistically significant differences in FI percentage and EPS with and without crude oil using specific nutrients ($P < 0.05$).

Table 4
EPS production and viscosity after 5 days of incubation under different sources of carbon without crude oil addition.

Carbon sources Without crude oil	Bound EPS g/L ±SD	Free EPS g/L ± SD	Total EPS g/L	Viscosity $g.m^{-1}.s^{-1}$ ± SD
Sucrose	4.30 ± 0.15	3.26 ± 0.23	7.60[a]	1.39 ± 0.01
Dextrose	8.50 ± 0.30	3.10 ± 0.10	11.60[b]	1.40 ± 0.01
Glucose	4.36 ± 0.89	3.00 ± 0.26	7.36[a]	1.20 ± 0.01
Fructose	6 .00 ± 0.79	3.03 ± 0.56	9.03[c]	1.35 ± 0.01

Letters a, b and c: different letters shows significantly different of Total EPS within different sources of carbon without crude oil addition Tukey's test ($P < 0.05$).

3.2. Effects of different nitrogen on EPS production

The effects of seven sources from nitrogen on production of EPS and FI percentage are digested in Fig. 2. The maximum total EPS production (12 g/L) and FI percentage (70%) were achieved when ammonium sulfate was used as nitrogen source. Ammonium sulfate exhibited considerable advantage over the six other nitrogen sources. When ammonium sulfate was added as nitrogen source, total EPS production increased by 16.8% (Fig. 2) compared with that of the control at pH 8 (Fig. 1). Berekaa (2014) reported that using ammonium sulfate as nitrogen source can increase the EPS production of *Bacillus sp.* Moreover, increasing EPS production by adding ammonium sulfate increased FI percentage by approximately 9.9%. The viscosity of the culture medium also increased slightly with increased EPS production (Table 2) by adding ammonium sulfate. Nichols et al. (2009) investigated that the viscosity of a medium depends on sugar (carbon sources) and EPS contents. Statistical analysis showed a highly significant effect of ammonium sulfate as nitrogen source on EPS production and FI percentage ($P < 0.001$) compared with other nitrogen sources.

3.3. EPS production by consortium culture under carbon sources

A wide variety sources of carbon, including sucrose, dextrose, glucose, and fructose, were used with and without crude oil hydrocarbons to produce EPS. Fig. 3 shows the effects of adding different carbon sources on EPS production and FI percentage with or without 1% crude oil. The results indicated that after 5 days of incubation, dextrose yielded high EPS production (14 g/L) and FI percentage (70.7%). It demonstrated significant advantage through the four sources of carbon. Maintaining different parts of the floc structure depended of the specific role for layered structure, EPS on different layers, because flocs are shear sensitive, the separation of EPS on different parts of the floc is normally categorized (Ding et al., 2015).

EPS was increased by 14 g/L when dextrose was added as a carbon source. Many studies have demonstrated the influence of various sources of carbon on produced EPS (Miqueleto et al., 2010; Papinutti, 2010). Carbon source is very important factors that affect EPS production (Wang et al., 2006). The viscosity of the culture medium was also increased to 1.57 g $m^{-1}.s^{-1}$ by 14 g/L EPS (Table 3). The composition of the medium has been registered to play very important role in EPS production; moreover, flocculation activity can be affected by some factors, such as sources of carbon and nitrogen, incubation conditions, temperature, and pH (Yilmaz et al., 2012; Onbasili and Aslim, 2009). The addition of sucrose and dextrose as carbon sources for the two cases (with and without crude oil) yielded significantly different results ($P < 0.05$) on FI percentage, whereas the addition of glucose and fructose ($P > 0.05$) did not based on the results of the two-way ANOVA. With regard to EPS production, the addition of fructose and glucose as sources of carbon for the two cases (with and without crude oil) yielded significantly different results ($P < 0.05$), whereas the addition of sucrose and dextrose ($P > 0.05$) did not.

Fig. 3 shows the effects of adding different sources of carbon on produced EPS and FI percentage without crude oil hydrocarbons. The results indicated that after 5 days of incubation, dextrose still obtained high EPS production and FI percentage. However, EPS amount decreased by approximately from 14 g/L to 11.6 g/L, with an FI percentage of 51.3% in the absence of crude oil hydrocarbons Table 4. The results indicate the effects of crude oil hydrocarbons on EPS production as a carbon source.

Wang et al. (2018) investigated produced EPS by *Aerococcus Uriaeequi* under different sources of carbon; the highest yield was gated when D-glucose was used as the sources of carbon. Lee et al. (1997) reported that *Bacillus polymyxa* can use all the carbon sources (fructose, sucrose, lactose, glucose, galactose, and starch) to produce EPS. Under the aforementioned conditions, the maximum amount of EPS and broth viscosity reported 8.90 g/L and 6551 g $m^{-1}.s^{-1}$ respectively, which were the first showed high amount values of EPS from a bacteria. The EPS solution of exhibited more viscosity, interesting styling thinning possession, considerable tolerated high temperatures, high of pH, and a wide range salinity, this is agreement with Patel and Prajapati (2013) EPS produced in bacteria are play role in the protection of the bacterial cell against adverse condition in the environment such as toxic compounds desiccation, stress, antibiotics or (e.g. toxic metal ions, sulphur dioxide, and ethanol.

3.4. Influences of inorganic ions

The effects of different inorganic ions, such as KCl, calcium $CaCl_2$, $MnCl_2$, and $ZnCl_2$, were also investigated by adding 0.5% concentra-

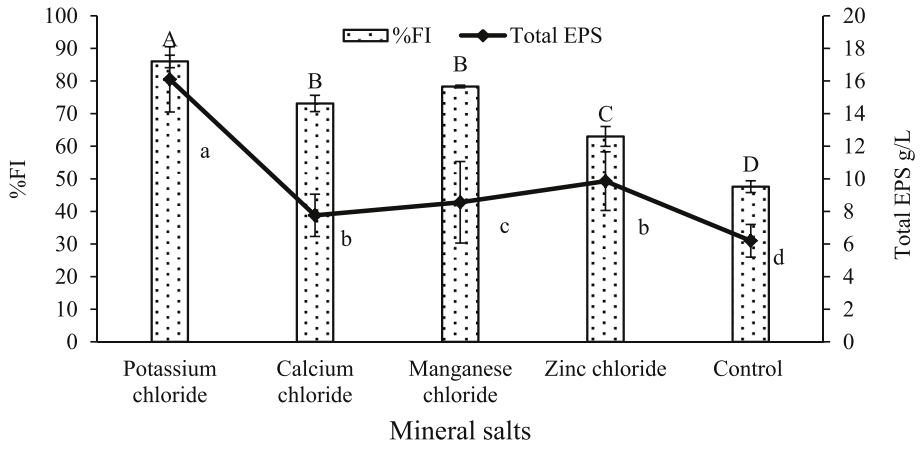

Fig. 4. Effects of 0.5% concentration inorganic ions on the FI percentage and EPS production of the consortium culture. Differences in upper case and lower case letters represent statistically significant differences in FI percentage and total EPS production with different mineral salts ($P < 0.05$).

Table 5
Effects of various inorganic ions on EPS production and viscosity after 5 days of Incubation.

Inorganic ion	Bound EPS g/L ±SD	Free EPS g/L± SD	Total EPS g/L	Viscosity $g.m^{-1}.s^{-1}$ ± SD
Potassium chloride	8.60 ± 0.95	7.50 ± 0.17	16.1[a]	1.44 ± 0.01
Calcium chloride	2.96 ± 0.15	4.80 ± 0.20	7.6[b]	1.29 ± 0.01
Manganese chloride	5.60 ± 0.60	2.96 ± 0.15	8.5[c]	1.33 ± 0.01
Zinc chloride	6.96 ± 0.51	2.90 ± 0.20	7.8[b]	1.35 ± 0.01
Control (MSM)	6.20 ± 0.36	0.48 ± 0.05	6.7[d]	1.30 ± 0.01

Letters a, b and c: different letters shows significantly different of Total EPS within different inorganic ions Tukey's test ($P < 0.05$).

tion of these ions to the culture medium. Maximum EPS production (16.1 g/L) was achieved when KCl was added to the culture medium (Fig. 4). We selected KCl on the basis of the result.

Wang et al. (2011) used different inorganic ions to enhance EPS production by *Bacillus thuringiensis* 27. In their study, *B. thuringiensis* 27 produced 15.36 g/L EPS when 0.5% NaCl was added to the culture medium. In our study, the consortium culture exhibited higher EPS production when KCl was used. In addition, FI percentage was increased to 86%, whereas Wang et al. (2011) obtained only 80.4%. Ions are known to considerably influence of the EPS viscosity. Viscosity decreased a little from 1.57 g $m^{-1}.s^{-1}$ to 1.44 g $m^{-1}.s^{-1}$. When 0.5% salts were added, the EPS viscosity decreased slightly. The apparent viscosity was 1.44 g $m^{-1}.s^{-1}$ when KCl was used (Table 5). The results showed that the EPS produced by the consortium culture could remain stable when different salts were added; thereby indicating that solution for EPS can exhibit perfect salt resistance (Liu et al., 2011). As shown in Fig. 4, the effects of different inorganic ions clearly presented statistically significant differences in FI percentage and total EPS production ($P < 0.05$).

In summary, produced EPS by *Streptococcus thermophilus* ST1 depended on chemical conditions, such as the first pH and composition of the culture and sources of carbon and nitrogen. Parameters appropriate for the growth of *S. thermophilus* ST1 were showed to be favorable for produced EPS. The optimization of chemical parameters for *S. thermophilus* ST1 significantly increased produces EPS by 130%. The viscous EPS solution nature by *S. thermophilus* ST1 (as reported in present study) indicates the possible application of EPS production to improving the physical properties (Zhang et al., 2011). Some researcher investigated high viscosity for EPS solution is strong behavior, good thermal and pH stability, and high salt resistance demonstrate good potential for applications in enviroment (Liu et al., 2011; Sheng et al., 2010).

4. Conclusions

In conclusion, this study used various parameters to enhance the EPS production of a bacterial floc consortium under different conditions. The addition of various substances, such as ammonium sulfate (as nitrogen source) and dextrose (as carbon source), increased total EPS production, FI percentage, and viscosity with and without crude oil. The maximum EPS production (16.1 g/L) was achieved when 0.5% KCl was added to the culture medium. In addition, FI percentage increased to 86%.

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