*Journal of Food Biosciences and Technology*,
Islamic Azad University, Science and Research Branch, Vol. 7, No. 2, 9-18, 2017

# Evaluating the Production Yield of an Exopolysaccharide Produced by *Enterobacter cloacae* subsp. Dissolvens in Seawater

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Received: 19 July 2016 Accepted: 21 January 2017

ABSTRACT: The aim of this study was to investigate the influence of different parameters on exopolysaccharide (EPS) production from *Enterobacter cloacae* subsp. dissolvens using seawater as a basal medium. For this purpose, the effect of carbon (sucrose, molasses, paraffin and sunflower oil) and nitrogen sources, NaCl concentration, incubation time, pH as well as seawater dilution or enrichment with distilled water or *Aloe vera*, *respectively* on EPS production was investigated. Incubation of bacteria at 30°C in the seawater for 24 hours resulted in 0.56% EPS production. Increasing the incubation time from 1 to 4 days led to a decrease in biopolymer production. EPS yield was not affected by the addition of sucrose, pH (6-8) or by bacteria inoculation in brine (1-4% NaCl). Dilution of seawater with different ratios of distilled water (1:2, 1:1 and 2:1) or seawater enrichment with various concentrations of *Aloe vera* leads to EPS yield reduction. Polymer production was drastically increased with sunflower (to 5%) or paraffin (to 3%) concentration. The interactions between different parameters on EPS production in the formulated mediums with distilled water were studied. The results showed that EPS production was reduced significantly in the formulated mediums containing different concentrations of water, NaCl, nutrient broth, glucose, ammonium sulfate and potassium phosphate. Among different formulations, the highest yield (0.27%) was achieved with the formulation of distilled water, 3.4% NaCl, 1% paraffin and 0.05% $KH_2PO_4$, indicating that simulation of seawater using formulated distilled water didn't provide comparable results. Therefore, paraffin and sunflower oil would be the matter of choice due to their higher productivity.

**Keywords**: *Biopolymer Production Yield, Enterobacter Cloaca, Exopolysaccharide, Seawater*.

## Introduction

Bacteria produce diverse biopolymers with varied chemical properties. There are various types of extracellular polymers which may be grouped into four major classes; polysaccharides, inorganic poly-anhydrides (such as polyphosphates), polyesters, and polyamides (Nwodo *et al.*, 2012). However, only a few types of polysaccharide-producing bacterial strains have been used in industrial applications to date (Shimada *et al.*, 1997). Some types of commercialized EPS are produced from *Xanthomonas campestris* (xanthan), *Sphingomonaspauci mobilis* (gellan), *Acetobacter xylinum* (cellulose) and *Rhizobium* sp. (succinoglycan). It is still necessary to isolate new microbial polysaccharides with useful chemical, physiological, and rheological properties (Shimada *et al.*, 1997). Marine microorganisms may be able to produce novel metabolites which are not often present in microbes of terrestrial origin. To

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date, various marine microbial sources are known for production of EPS (Satpute *et al.*, 2010). *Enterobacter* sp. is considered as one of the most important species of EPS producing bacteria. Several polysaccharide-producing *Enterobacter* spp. have been identified. *Enterobacter cloacae* produce an acidic polysaccharide composed of fucose, galactose, glucose, glucuronic acid, pyruvate and acetate (Shimada *et al.*, 1997). In this study, a subsp. of *E. cloacae* (*dissolvens* PTCC 1798) was used to produce EPS from seawater as the cultivation medium. Therefore, the objective of this study was to use seawater as an inexpensive substrate for production of EPS as a valuable bio-emulsifier (Abbasi & Amiri, 2008) and also to evaluate the effect of different factors (pH, carbon source, agitation and …) on EPS production yield.

## Materials and Methods

- *Materials*

*Enterobacter cloacae* subsp. dissolvens PTCC 1798 was purchased from Persian Type Culture Collection (PTCC). Seawater was obtained from Persian Gulf (south of Iran). Sucrose, liquid paraffin (code No. 107162) and $(NH_4)_2SO_4$ were purchased from Merck (Darmstadt, Germany).

- *EPS production*

The bacteria were cultivated in nutrient broth at 30°C for 16 h and then decimally diluted to give an absorbance of 0.35 at 600 nm (a concentration of $10^7$CFU/ml). 0.4 ml of prepared bacterial suspension was added to 40 ml of sterilized seawater. The mixture was incubated at 30°C for 24 h. Afterwards cell debris was removed by centrifugation at 10,000×g for 30min. Four volumes of acetone were added to the supernatant and the mixture was leaved for 2 h and then was centrifuged at 10,000×g for 10 min at 4°C to precipitate the EPS. The precipitate was dried at 105°C to reach a constant weight. Finally, the precipitate was weighed to determine the EPS production yield. This procedure was constantly performed in all experiments except for factors that were considered as variable in each experiment.

- *The effect of incubation time and inoculum count on EPS production yield*

To evaluate the effect of incubation time and inoculum count on EPS production yield, samples of seawater were inoculated with $10^6$, $10^7$ and $10^8$ CFU/Ml of *Enterobacter cloacae* suspension and were incubated for 12, 24, 48, 72 and 96 h and the EPS production yield was estimated for each interval time.

- *The effect of nutrients, pH and temperature on EPS production in the seawater*

The influence of different carbon sources [sucrose (1, 2, 3 and 4% w/v), liquid paraffin (0.1, 1, 2, 3, 4, 5, 7, 9, 11 and 13% w/v), sunflower oil (0.1, 1, 2, 3, 4, 5, 7, 9, 11 and 13% w/v), and molasses (0.05, 0.1, 0.5 and 1%)] on the EPS production yield (%) were studied, while seawater was considered as the basal medium. The prepared mediums were sterilized at 121°C for 15 min. and then inoculated with bacterium according to the above procedure, and at the end, the yield of EPS production was calculated. In another group of experiments, enrichment of the seawater with *Aloe vera* extract was performed at 19:1, 18:2, 15:5 and 10:10 seawater: *Aloe vera* ratios to evaluate the influence of basal medium enrichment.

In order to evaluate the effect of nitrogen enrichment and its concentration on the EPS production, different concentrations of an inorganic nitrogen source $(NH_4)_2SO_4$ (0.05, 0.5 and 1%w/v) were added to the seawater medium. The effect of potassium phosphate was also tested, but as the salt began to precipitate immediately after its addition into the seawater, this test was aborted.

In order to evaluate the effect of pH on the EPS production yield, pH of seawater was adjusted at 6, 7 and 8. The inoculation

was performed and the EPS production yield was determined. The effect of pH in the presence of nitrogen source (0.5% w/v $(NH_4)_2SO_4$) was also evaluated.

In order to investigate the effect of dilution, seawater was diluted with distilled water at different ratios of 1:1, 1:2 and 2:1 and enriched with 1% glucose and 0.05% nutrient broth.

The effect of temperature was also evaluated at 20, 30 and 50°C during incubation time. The other investigated parameter was agitation speed that was performed at 100 and 200 rpm at 30°C during 24 h after inoculation. The effects of temperature and agitation were only evaluated for the best result concerned with the formulations to optimize the EPS production conditions.

- *The effect of different components and nutrients on EPS production in simulated mediums with distilled water*

Osmotic pressure is considered as an important factor in the production of EPS (Abbasi & Amiri, 2008). Different concentrations of NaCl in distilled water (1, 2, 3 and 4%) were prepared and EPS production yields were estimated. Thereafter, distilled water containing NaCl, was considered as the basal medium and enrichment of this medium with different sources of carbon, nitrogen or phosphate, *Aloe vera* extract and etc. was performed according to the Tables 6 and 7 and, the efficiency of formulated mediums was evaluated for EPS production.

- *Experimental design and statistical data analysis*

The design of experiment was conducted based on the "One factor at a time" design. According to this design, the experiments were performed on a constant medium basis (sterilized sea water, inoculum size of 0.4 mL for 40 mL of sea water, inoculum count of $10^7$ CFU/mL, incubation time of 24 hr. and incubation temperature of $30^oC$). The influences of different variables were evaluated at each time while other variables were kept constant and at the second step, the best results were selected and other variables were applied and evaluated at the selected conditions to optimize the conditions.

The results (EPS production yields) were analysed by analysis of variance method (ANOVA) and Duncan multiple range test was applied for further differentiation of the groups($\alpha<0.05$). The statistical software SPSS 16.0 (SPSS Inc., New Jersey, USA) was used for analyses.

**Results and Discussion**

It has been suggested that the bacterial polymer could be biosynthesized through either a block or a monomeric mechanism. Almost all of the *Enterobacteriaceae* is anticipated to synthesize the EPS via the second mechanism (Prasertsan *et al.*, 2008). After it has been synthesized, the polysaccharide would be released into the culture broth.

- *The effect of incubation time and inoculum count on EPS production yield*

Time course studies were conducted on EPS production by *E. cloacae* in a basal medium (sea broth) for 4 days at 30°C (Table 1). The results showed that the bacterium produced EPS within the first 12h. This finding is in contrast to Prasertsan *et al.* (2008) results whom reported that the highest EPS yield (0.22%) was obtained after 3 days for *E. cloacae* WD7 in a basal medium containing glucose, $(NH_4)_2SO_4$, polypeptone, yeast extract, $K_2HPO_4$, $MgSO_4.7H_2O$ and NaCl, (pH 7.0). Pawar *et al.* (2013) found that three days of incubation period was to be optimum for the production of maximum yield of EPS in nutrient broth containing 4% sucrose for an isolated bacterium from saline soil, but Muhammadi and Afzal (2014) research

showed that the maximum yield of EPS (0.54%) was obtained from 10 days old culture from *Bacillus* strain CMG1421. They suggested that this is due to the availability of carbon source till 10th day, but after that the prolonged incubation starvation stimulated depolymerization of synthesized EPS to liberate consumable carbon source for bacterial growth.

In this work, the EPS production yield was decreased with increasing the time to 48 or 72h (Table 1). However, EPS production in higher population of $10^8$ was lower than the lower populations of $10^7$ and $10^6$. Therefore, it seems that bacterial cell number is an effective parameter in EPS production. The association of low cell mass with greater polysaccharide production has been explained by Sutherland (1990) that stated the synthesis of cell wall polymer became slower when cells grew slowly. This made more isoprenoid phosphate available for exopolymer synthesis (Prasertsan *et al.*, 2008).

*- The effect of nutrients, pH and temperature on EPS production in the seawater*

Cell growth and EPS production usually depends on carbon sources which affect the quality, sugar component and/or molecular weight of EPS (Prasertsan *et al.*, 2008). The data for the effects of carbon sources on EPS production from *E. cloacae* showed the addition of sucrose at 1, 2, 3 and 4% and molasses at 0.05, 0.1, 0.5 and 1% (Table 2) did not improve the EPS production. The addition of these carbon sources stimulated cell growth and increased the biomass that resulted in turbidity of the medium and did not improve the EPS production. As mentioned earlier, the association of low cell mass with greater EPS production has been explained by Sutherland (1990). Muhammadi and Afzal (2014) found that carbon sources such as fructose, glucose and sucrose in coupled with nitrogen sources supported the growth of *Bacillus* strain CMG1403. However, this result was not in agreement with the results of Joo *et al.* (2004), Pawar *et al.* (2013), Shimada *et al.* (1997) and Prasertsan *et al.* (2008) who

**Table 1**. The effect of time and bacterial count on EPS production of *E. cloacae* in a basal medium (seawater) at 30°C

| Cell count (CFU/mL) | EPS production yield (%) | | | | |
|---|---|---|---|---|---|
| | 12 h | 24 h | 48 h | 72 h | 96 h |
| $10^8$ | $0.45^{a}$* | $0.45^{a}$ | $0.42^{a}$ | $0.36^{b}$ | $0.37^{b}$ |
| $10^7$ | $0.54^{a}$ | $0.55^{a}$ | $0.50^{a}$ | $0.45^{b}$ | $0.40^{b}$ |
| $10^6$ | $0.59^{a}$ | $0.59^{a}$ | $0.50^{b}$ | $0.34^{c}$ | $0.31^{c}$ |

* Values with different small letters in each row are significantly different ($P$<0.05).

**Table 2.** The effect of sucrose and molasses concentrations on EPS production by *E. cloacae* in basal medium (seawater) at 30°C.*

| Sample | EPS production yield (%) | Sample | EPS production yield (%) |
|---|---|---|---|
| Control (seawater) | $0.56^{a}$ | Control (seawater) | $0.56^{a}$ |
| Sucrose 1% | $0.54^{a}$ | Molasses 0.05% | $0.60^{a}$ |
| Sucrose 2% | $0.52^{a}$ | Molasses 0.1% | $0.57^{a}$ |
| Sucrose 3% | $0.58^{a}$ | Molasses 0.5% | $0.59^{a}$ |
| Sucrose 4% | $0.54^{a}$ | Molasses 1.0% | $0.58^{a}$ |

* Values with different small letters in each column are significantly different ($P$<0.05).

showed that sucrose was used by *Sarcodonaspratus*, an isolated bacterium from saline soil, *Enterobacter* sp. and *Enterobacter cloacae* WD7, respectively for EPS production.

According to Figure 1, increasing the sunflower oil to 5% resulted in an increase in EPS yield to 1.1%. More addition of oil (more than 7%) resulted in a decrease in EPS production. Figure 2 shows the effect of paraffin concentration on EPS production yield. According to Figure 2 by increasing the paraffin concentration to 3%, the EPS production yield increased significantly ($p<0.05$) to 1.5%. More addition of paraffin did not improve the EPS production yield ($p>0.05$). Therefore, the optimum sunflower and paraffin concentrations for EPS production by *E. cloacae* were about 5 and 3% w/v, respectively.

The addition of sources such as sunflower oil or paraffin that are insoluble in water makes large interfaces between the oil and aqueous phases. According to the previous work (Abbasi & Amiri, 2008), the EPS produced by *E. cloacae* is a surface active biopolymer, which means that the produced EPS tends to migrate into oil/water interface and keeps the EPS concentration in seawater at low levels. This probably stimulates the bacteria to produce more EPS for compensation.

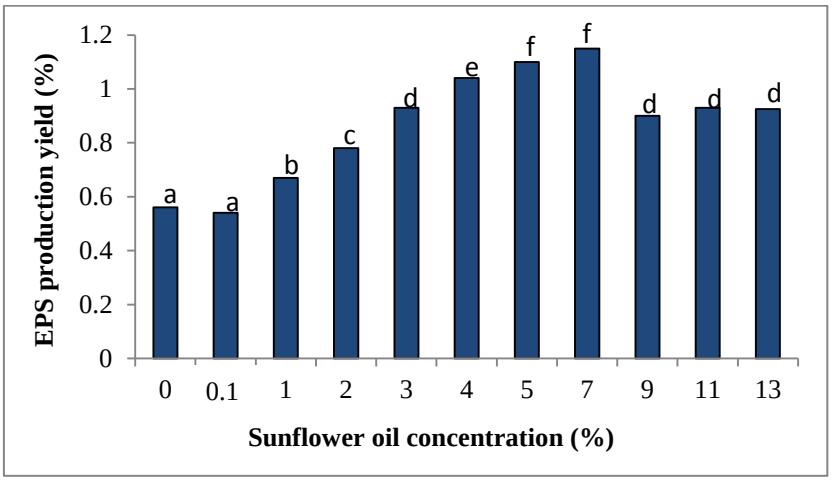


**Fig. 1.** The effect of sunflower oil concentration on EPS production by *E. cloacae* in basal medium (seawater) at 30°C.Values with different small letters in each column are significantly different (P<0.05).

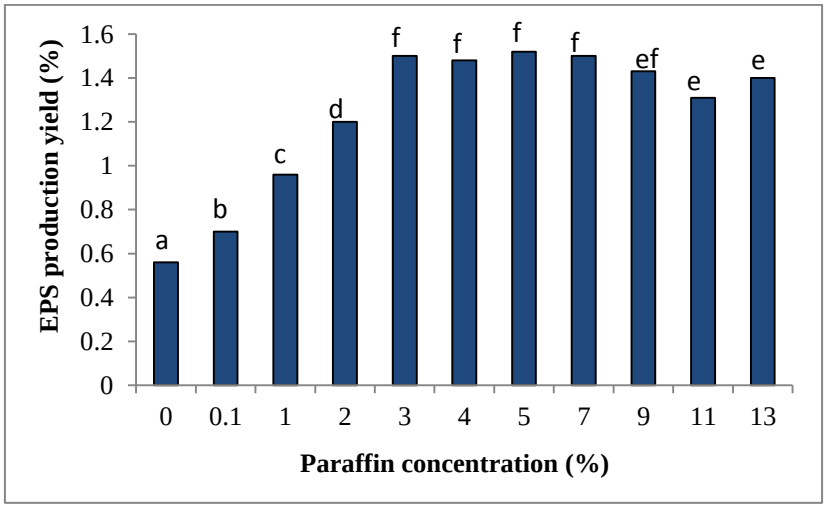


**Fig. 2.** The effect of paraffin concentration on EPS production by *E. cloacae* in basal medium (seawater) at 30°C.Values with different small letters in each column are significantly different (P<0.05).

The *E. cloacae* sp. has the potential to be used in microbial enhanced oil recovery (Bordoloi & Konwar, 2008). In this process, the microorganisms with the biosurfactant production ability are injected into the oil reservoirs to reduce the interfacial tension via biosurfactant production and subsequently, release the crude oil trapped in mature oil formations (Bordoloi & Konwar, 2008). The origin of *E. cloacae*, used in this research, was oil reservoir brine in Gachsaran region in Iran.

Enrichment of the seawater with *Aloe vera* extract at 19:1, 18:2, 15:5 and 10:10 seawater: *Aloe vera* ratios (Table 3) showed that the EPS yield from *E. cloacae* decreased with the addition of *Aloe vera* and by increasing the concentration of *Aloe vera*. This can be due to the cell growth which increased with the enrichment of the medium with *Aloe vera*. This result was in agreement with the results of Prasertsan *et al.* (2008) who performed enrichment of the medium with yeast extract in the range of 0–0.2% for the production of EPS from *E. cloacae* WD7.

The EPS production yield was not affected by the pH as there were not significant differences between the EPS yield (0.52, 0.52 and 0.57%) for pH of 6, 7 and 8 (the natural pH of seawater), respectively ($p>0.05$). This result was in contrast to other researchers who reported that pH had more influence on polysaccharide production than on cell growth; as at a specific pH, the synthesis of those enzymes responsible for EPS production are affected. The initial pH of 7.0 was found to be optimum for both cell growth and EPS production by *E. cloacae* WD7 (Prasertsan *et al.*, 2008). This optimum pH for EPS production from an isolated bacterium from saline soil was 7.5 (Pawar *et al.*, 2013). Muhammadi and Afzal (2014) found that the extreme pH profiles of the medium (pH<4.0 and pH>9) reduced not only the bacterial growth but also inhibited the biosynthesis of EPS from *Bacillus* strain CMG1421. They reported that the optimal pH for the highest productivity of EPS was about 7. It is reported that the initial pH affected the molecular weight of the product. For xanthan production by *X. campestris*, the pH must not be lower than 5.0 asacidic conditions may hydrolyze the polymer, and therefore give lower yields (Pawar*et al.*, 2013).

- *The effect of nitrogen source and concentration*

The addition of $(NH_4)_2SO_4$ as a nitrogen source was found to increase the production yield of EPS, with the addition of 0.05 to 1% $(NH_4)_2SO_4$ (Table 3). It seems that

**Table 3**. The effect of ammonium sulfate concentration, diluting or enrichment of seawater on EPS production by *E. cloacae* in basal medium (seawater) at 30°C

| Samples | EPS production yield (%) | Samples | EPS production yield (%) |
|---|---|---|---|
| Ammonium sulfate (0.05%) | $0.7^{a}$* | Ammonium sulfate (0.50%), pH=6 | $1.08^{a}$ |
| Ammonium sulfate (0.50%) | $1.0^{b}$ | Ammonium sulfate (0.50%), pH=7 | $1.02^{a}$ |
| Ammonium sulfate (1.00%) | $1.4^{c}$ | Ammonium sulfate (0.50%), pH=8 | $1.00^{a}$ |
| Seawater: *Aloe vera* (19:1) | $0.46^{c}$ | Seawater: distilled water (1:1)+1% glucose+0.05% nutrient broth | $0.045^{a}$ |
| Seawater: *Aloe vera* (18:2) | $0.45^{c}$ | Seawater: distilled water (2:1) +1% glucose+0.05% nutrient broth | $0.078^{b}$ |
| Seawater: *Aloe vera* (15:5) | $0.38^{b}$ | Seawater: distilled water (1:2)+1% glucose+0.05% nutrient broth | $0.230^{c}$ |
| Seawater: *Aloe vera* (10:10) | $0.28^{a}$ | | |

* Values with different small letters in each column are significantly different ($P<0.05$).

(0.2% $NH_4NO_3$), urea and potassium nitrate could also increase the production of EPS from *Erwinia herbicola*, and the production of bioabsorbent by *Alcaligenes latus* B-16 (Nohata & Kurane, 1994). Increasing the yeast extract concentrations as the sole nitrogen source also gave higher pullulan yields (11.0g$L^{-1}$ EPS) from a mixed culture of *Aureobasidium pullulans* and *Kluyveromyces fragilis* (Shin *et al.,* 1989).

- *The effect of dilution*

Dilution of seawater with distilled water (even when the inoculation medium was enriched with glucose and nutrient broth), resulted in significant EPS yield reduction when compared to the basal medium (Table 3). It seems that seawater medium is a key environment for EPS production, as diluting or enrichment of the medium resulted in declining the EPS yield.

- *The effect of temperature and agitation speed*

The combined effect of paraffin/agitation or paraffin/temperature were evaluated to optimize the EPS production yield in paraffin containing samples as the best formulation with the highest EPS yield (Table 4). Using higher or lower temperatures rather than the optimum growth temperature of the bacterium (30 $^oC$) significantly decreased the EPS production yield of samples containing paraffin while the effect was not significant for sea water without paraffin ($p<0.05$). In other words, the optimum condition of bacterium growth is coincided on EPS production conditions.

The significant effect of incubation temperature on polysaccharide biosynthesis has been documented in literature (Joo *et al.,* 2004; Prasertsan *et al.,* 2008). The optimum temperature for polysaccharide production depends on the type of microorganism (Prasertsan *et al.,* 2008). According to Sutherland (2001) reduction of the cultivation temperature by 10°C below optimal level inhibits the EPS biosynthesis by microbial cells (Muhammadi & Afzal, 2014).

The EPS yield was not changed when the agitation speed was increased from 100 to 200rpm in the basal medium, but the addition of paraffin, the EPS production was increased significantly (Table 4). It means that the increase in EPS production was observed for mediums containing oil. As paraffin is not soluble in water and separates at the top of water, agitation can increase the mixing of paraffin with the medium and thus increases the interface of paraffin-water that results in an increase in EPS production yield as discussed earlier. The other function of agitation is increasing the dissolution of oxygen in the medium, but this factor was not effective as the control sample (basal medium) didn't show any alteration in EPS yield. Our results were in agreement with the research carried out by Prasertsan *et al.* (2008) and Bandaiphet and Prasertsan (2006) who reported 200 rpm as the selected agitation speed. However, these researchers showed that the EPS yield decreased as the

**Table 4**. The effect of temperature and agitation speed on EPS production yield of paraffin-containing seawaters by *E. cloacae*

| Sample | 20°C | 30°C | 50°C | 100rpm | 200rpm |
|---|---|---|---|---|---|
| Control (seawater) | $0.54^{aA}$ | $0.56^{aA}$ | $0.54^{aA}$ | $0.55^{aA}$ | $0.55^{aA}$ |
| Paraffine 3% | $1.10^{bA}$ | $1.27^{bB}$ | $1.23^{bB}$ | $1.54^{bA}$ | $1.66^{bB}$ |
| Paraffine 5% | $1.28^{cA}$ | $1.58^{cB}$ | $1.35^{cA}$ | $1.60^{cA}$ | $1.74^{cB}$ |
| Paraffine 7% | $1.30^{cA}$ | $1.63^{dB}$ | $1.39^{cA}$ | $1.77^{dA}$ | $1.90^{dB}$ |

* Values with different small letters in each column are significantly different ($P<0.05$).

** Values with different capital letters in each row are significantly different ($P<0.05$).

agitation speed increased from 200 to 800rpm. Also, they reported that the oxygen supply at 200rpm was adequate for the tested bacterium (*E. cloacae* WD7). Muhammadi and Afzal (2014) mentioned that the maximum yield of EPS (0.54%) was obtained only from static culture for *Bacillus* strain CMG1403 and EPS yield decreased with increasing the agitation speed.

- *The effect of different components and nutrients on EPS production in simulated mediums with distilled water*

EPS production was not observed with the addition of salt to distilled water (Table 5). Therefore, the brine solutions were enriched with glucose (1%), nutrient broth (0.05%), phosphate and nitrogen sources (according to Table 5) to provide the required nutrients

**Table 5.**The effect of different formulations on EPS production in simulated mediums with distilled water

| | **Samples** | **EPS production yield (%)** |
|---|---|---|
| 1 | Distilled water | 0.00[a] |
| 2 | Distilled water +1% NaCl | 0.00[a] |
| 3 | Distilled water +2% NaCl | 0.00[a] |
| 4 | Distilled water +3% NaCl | 0.00[a] |
| 5 | Distilled water +4% NaCl | 0.00[a] |
| 6 | Distilled water +1% NaCl+1% glucose+0.05% nutrient broth+1% $K_2HPO_4$ + 1% $(NH_4)_2SO_4$ | 0.00[a] |
| 7 | Distilled water +2% NaCl+1% glucose+0.05% nutrient broth+1% $K_2HPO_4$ + 1% $(NH_4)_2SO_4$ | 0.00[a] |
| 8 | Distilled water +3% NaCl+1% glucose+0.05% nutrient broth+1% $K_2HPO_4$ + 1% $(NH_4)_2SO_4$ | 0.00[a] |
| 9 | Distilled water +4% NaCl+1% glucose+0.05% nutrient broth+1% $K_2HPO_4$ + 1% $(NH_4)_2SO_4$ | 0.00[a] |
| 10 | Distilled water +1% NaCl+0.05% nutrient broth | 0.03[b] |
| 11 | Distilled water +2% NaCl+0.05% nutrient broth | 0.03[b] |
| 12 | Distilled water +3% NaCl+0.05% nutrient broth | 0.01[b] |
| 13 | Distilled water +4% NaCl+0.05% nutrient broth | 0.02[b] |
| 14 | Distilled water +3.4% NaCl+1% glucose | 0.00[a] |
| 15 | Distilled water +3.4% NaCl+1% glucose+0.05% $KH_2PO_4$ | 0.05[c] |
| 16 | Distilled water +3.4% NaCl+1% glucose+0.05% $(NH_4)_2SO_4$ | 0.08[d] |
| 17 | Distilled water +3.4% NaCl+1% glucose+0.05% $(NH_4)_2SO_4$+0.05% $KH_2PO_4$ | 0.14[e] |
| 18 | Distilled water +3.4% NaCl+1% paraffin | 0.00[a] |
| 19 | Distilled water +3.4% NaCl+1% paraffin+0.05% $KH_2PO_4$ | 0.27[g] |
| 20 | Distilled water +3.4% NaCl+1% paraffin +0.05% $(NH_4)_2SO_4$ | 0.11[e] |
| 21 | Distilled water +3.4% NaCl+1% paraffin +0.05% $(NH_4)_2SO_4$+0.05% $KH_2PO_4$ | 0.10[e] |
| 22 | Distilled water +3.4% NaCl+1% paraffin+0.05% glycine | 0.00[a] |
| 23 | Distilled water +3.4% NaCl+1% paraffin+0.05% glycine+ 0.05% $KH_2PO_4$ | 0.20[f] |
| 24 | Distilled water +3.4% NaCl+1% glucose +0.05% glycine | 0.00[a] |
| 25 | Distilled water +3.4% NaCl+1% glucose +0.05% glycine+0.05% $K_2HPO_4$ | 0.04[c] |
| 26 | Distilled water: *Aloe vera* (19.9:0.1) | 0.00[a] |
| 27 | Distilled water: *Aloe vera* (19.9:0.1) | 0.00[a] |
| 28 | Distilled water+ *Aloe vera* (1%) | 0.00[a] |
| 29 | Distilled water+*Aloe vera* (2.5%) | 0.00[a] |
| 30 | Distilled water+ *Aloe vera* (5%) | 0.00[a] |
| 31 | Distilled water+ *Aloe vera* (7.5%) | 0.00[a] |
| 32 | Distilled water: *Aloe vera* (19:1) +3.4% NaCl | 0.005[a] |
| 33 | Distilled water: *Aloe vera* (18:2) +3.4% NaCl | 0.03[b] |
| 34 | Distilled water: *Aloe vera* (15:5) +3.4% NaCl | 0.03[b] |
| 35 | Distilled water: *Aloe vera* (10:10) +3.4% NaCl | 0.04[c] |
| 36 | Distilled water: *Aloe vera* (15:5) +2% NaCl | 0.08[d] |
| 37 | Distilled water: *Aloe vera* (15:5) +5% NaCl | 0.15[e] |
| 38 | *Aloe vera* +3.4% NaCl | 0.10[e] |
| 39 | *Aloe vera* | 0.03[b] |

* Values with different small letters in column are significantly different ($P$<0.05).

of the bacterium; but enrichment of NaCl solutions were not effective as no EPS production was observed. It seems that the simulated mediums gave higher cell growth. Therefore, glucose, phosphate and nitrogensalts were omitted to make a medium less suitable for cell growth and just NaCl and nutrient broth were maintained according to Table 5 (rows 10 to 13). The results showed that EPS was produced under the simulated formulations, but the yield was decreased significantly to lower than 0.05%. Therefore, in another set of experiments, nutrient broth was omitted from the formulations and either glucose, phosphate or nitrogen sources were maintained in NaCl solution (3.4%) as the basal medium (Table 5, rows 14 to 17). The addition of glucose as a carbon source in combination with 3.4 % (w/v) NaCl solution was not effective, but addition of phosphate salt to such medium increased the EPS production yield to 0.05%. The addition of nitrogen source made a better result (0.09%), and with the addition of both ammonium and phosphate salts, the EPS production yield was improved to 0.14% (the best result between above different formulations).

As the results were not comparable with seawater, 3.4% NaCl solution was enriched with either paraffin, ammonium or phosphate salts (Table 5, rows 18 to 21). According to the results, EPS was not produced when only paraffin was added to the brine, but fortification with ammonium or phosphate salts, gave better results (the best result was 0.27% EPS yield when phosphate salt was used with paraffin). Formulation of 3.4% NaCl solution with either paraffin or glucose in combination with ammonium and (or) phosphate salts and glycine did not provide good results.

The application of *Aloe vera* extract as the cultivation medium or fortification of either distilled water or 3.4% NaCl solution with different concentrations of *Aloe vera* extracts increased biomass and not EPS production yield (Table 5, rows 26- 39).

As described earlier, the optimum formulation for EPS production was distilled water, 3.4% NaCl, 1% paraffin, 0.05% $KH_2PO_4$that resulted in 0.27% EPS yield. This result was much lower than that of seawater as the basal medium (0.56% EPS yield). Based on our findings, formulation of distilled water with different compounds was not successful. However, the results of simulated mediums were completely comparable with the results of other researchers (Prasertsan *et al.*, 2008; Pawar *et al.*, 2013; Bandaiphet and Prasertsan, 2006).

## Conclusion

This research showed that the best basal medium for the production of exopolysaccharide by *E. cloacae* subsp. *Dissolvens* PTCC 1798 is seawater. The addition of different types of carbon (except paraffin and sunflower oils), nitrogen, sulfur and phosphorous sources into the basal medium and also enrichment with nutrient broth or aloe vera extract, did not improve the EPS production in almost all cases. The addition of nutrients into distilled water didn't provide an optimal medium for EPS production by bacteria. EPS was produced in some simulated seawater formulations, but the yields were significantly lower than using seawater. The most important finding of this research was that the addition of carbon sources such as paraffin or sunflower oil to the seawater highly increases the EPS production yields up to near three folds.

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