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# Bacterial Exopolysaccharides as New Natural Coagulants for Surface Water Treatment

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**Abstract:** Coagulation-flocculation step is one of the most important steps during surface water treatment. Chemical coagulants are ordinary used such as alum. However, these chemical coagulants are dangerous to environment and human health. Natural coagulants derived from natural sources receive much attention during last years. This study aimed to isolate new bacterial exopolysaccharides from *Bacillus licheniformis*, *B. insolitus* and *B. alvei* to be used as natural coagulants during coagulation-flocculation process. Efficiency of extracted bacterial exopolysaccharides was examined through removal ability of bacterial indicators and some physicochemical parameters of River Nile water samples. Bacterial exopolysaccharides showed great removal percent when used as sole coagulant materials. Addition of alum to bacterial exopolysaccharides enhance removal efficiency.
**Keywords:** Bacteria, Exopolysaccharides, Coagulation-flocculation, Surface water treatment.

## Introduction

Coagulation-flocculation followed by sedimentation, filtration and disinfection, often by chlorine, is used worldwide in the water treatment industry before the distribution of treated water to consumers. Coagulation-flocculation process has some advantages in water treatment, since it doesn't require complex machineries, no energy consumption, and high ability for removing suspended, colloidal and soluble particles[1]. Coagulation /flocculation step which is considered as a vital stage during treatment of raw water such as surface water, domestic wastewater and industrial wastewater. It helps in removal of dissolved organic substances and turbidity from water through addition of chemical coagulants such as alum, ferric chloride and synthetic organic polymers. The efficiency of these chemicals as coagulant is well-recognized[2,3], however, there are some disadvantages for using these coagulants such as ineffectiveness in cold water[4], high procurement costs, complete or partial non-biodegradability, health effects on human, production of large volumes of sludge and significantly affect pH of treated water. In addition, it was proven the direct link between using these chemical coagulants and occurrence of Alzheimer disease[5]. In addition, partial degradation of chemical coagulants (synthetic polymers) produce intermediate substances which have some neurotoxic and carcinogenic effects[6]. Searching for alternative natural-based coagulants to avoid these disadvantages become an insistent issue. Natural coagulants were applied in water treatment and showed many advantages such as low cost, low toxicity (environmental friendly), biodegradability and small volumes of sludge[7,8]. During last years, there was a great attention to develop and usage of natural coagulants. These natural coagulants can be produced or extracted

from plant tissues, animal tissues and microorganisms[7,9]. Many natural coagulants produced from microorganisms such as bacteria, actinomycetes, algae, yeast and fungi. These microbial coagulants composed of bio-macromolecules such as polysaccharides, proteins, lipids, nucleic acids, etc.[10,11]. Most of studies focused on removal of only one type of pollutants using microbial coagulants such as heavy metals[12-14], or dyes[15], while no more reports about multiple pollutants removal[16]. In this study, new bacterial exopolysaccharide (EPS) produced by newly isolated strains from water and soil, *Bacillus licheniformis*, *Bacillus insolitus* and *Bacillus alvei*, had been discovered and applied as coagulants for surface water treatment.

## Experimental

### 1. Sampling.

Raw River Nile water samples were collected in sterile glass bottles according to the standard methods for the examination of water and wastewater[17], then immediately transferred to the laboratory within 4 h inside an ice box for further examination.

### 2. Bacterial examination.

All samples were examined for detection and enumeration of bacterial indicators of pollution, these indicators including; total coliforms (TC), fecal coliforms (FC) and fecal streptococci (FS).

### 2.1. Total viable bacterial counts (TVBC).

Plate count agar (Himedia, India) medium used for enumeration of total viable bacterial counts in water samples using poured plate method[17]. 10 mL of 2,3,5-Triphenyl Tetrazolium Chloride (TTC) sterilized solution added to plate count agar after sterilization at 121°C for 15 minutes for pigmentation of colonies with red color. Inoculated Petri dishes were incubated at 37°C and 22°C for 24 h and 48 h, respectively. After incubation periods, colonies were counted and represented as cfu.ml$^{-1}$.

### 2.2. Total coliforms.

Membrane filtration process was carried out for detection and enumeration of total coliforms, fecal coliforms and fecal streptococci according to Shash et al.[18]. m-Endo agar LES (Himedia, India) media used for detection and enumeration of total coliforms. After incubation at 35 ± 0.5ºC for 24 h, typical coliforms colonies appear as pink to dark-red color with a metallic surface sheen were counted and calculated as colony forming unit per 100 ml (cfu.100 ml$^{-1}$).

### 2.3. Fecal coliforms.

m-FC agar (Fluka, Switzerland) media used for detection and enumeration of fecal coliforms in water samples using membrane filtration technique as mentioned above. Light to dark blue colonies after incubation at 44-45°C in water bath for 24 h were counted as fecal coliforms (cfu.100 ml$^{-1}$).

### 2.4. Fecal streptococci.

m-Enterococcus agar (Himedia, India) media used for detection and enumeration of fecal streptococci (enterococci) in water samples using membrane filtration technique. Light to dark red colonies were counted as fecal streptococci (cfu.100 ml$^{-1}$) after incubation at 35°C for 48h.

### 3. Physicochemical examination.

Some physicochemical parameters were measured in collected water samples. These parameters including; turbidity, total dissolved solids (TDS), biological oxygen demand (BOD), sulfate ($SO_4^{+2}$) and nitrate ($NO_3^-$). All physicochemical parameters were measured according to standard methods for the examination of water and wastewater[17], as represented in Table (1).

**Table 1. Measured physicochemical parameters in water samples.**

| Parameter | Unit | Measuring method |
|---|---|---|
| Turbidity | NTU | Turbidimeter [10b] a portable Hanna turbidimeter (model: HI 93703) |
| Total dissolved solids (TDS) | $mg.l^{-1}$ | Evaporation test method |
| Biological oxygen demand (BOD) | $mgO_2.l^{-1}$ | Winkler s iodometric method |
| Sulfate ($SO_4^{+2}$) | $mg.l^{-1}$ | Turbidimetric method using UV/Vis spectrophotometer, Unicam model UV4-200 (UK): at wave length 420 nm. |
| Nitrate ($NO_3^-$) | $mg.l^{-1}$ | Technicon Auto Analyzer. |

## 4. Isolation and identification of exopolysaccharide (eps) producing bacteria.

### 4.1. Source of EPS producing bacteria.

Five samples from River Nile water and five samples from river sediment were collected for isolation of exopolysaccharide producing bacteria.

### 4.2. Bacterial isolation.

Serial dilution technique[17] was carried out for isolation of EPS producing bacteria. One mL of suitable serial dilutions ($10^2$ and $10^3$) was spread on the surface of sterilized plate count agar (Himedia, India) Petri dishes. Inoculated Petri dishes were incubated at 35°C for 24-48 h, after incubation, selected different colonies (depending on colony morphology) were picked up for purification and further experiments.

### 4.3. Bacterial identification.

Picked up bacterial isolates were identified using GEN III OmniLog® ID System (Biolog, USA).

## 5. Production and isolation of EPS from liquid culture.

Bacterial strains were screened for production of exopolysaccharide in a liquid medium composed of the following ingredients ($g.l^{-1}$): peptone (4.0), yeast extract (2.0), and sucrose (20.0). The ingredients were dissolved in 1000 mL distilled water. The medium was distributed in conical flask 250 mL. The flasks were autoclaved and inoculated using actively growing culture and incubated at 37ºC for 3 days. The culture medium was centrifuged at 5000 rpm for 10 min (Sigma-Laborzentrifugen, 2K15) to remove bacterial cells. Trichloroacetic acid (5%) was added and left overnight at 4ºC and centrifuged at 5000 rpm again. The pH of the clear solution was adjusted to 7.0 with NaOH solution and dialyzed three times. The supernatant was completed to four volumes with ethanol 95% and left overnight at 4ºC. The precipitated exopolysaccharides were separated by centrifugation at 5000 rpm, washed twice with acetone and dehydrated by ether[19].

## 6. Purification of exopolysaccharides.

### 6.1. Fractionation by ethanol.

All bacterial strains were grown aerobically for 96 h in a production medium at 37ºC on a rotary shaker (150 rpm). After incubation period, cells were removed by centrifugation at 5000 rpm for 10 min (Sigma-Laborzentrifugen, 2K15). Trichloroacetic acid was added (5%) and left overnight at 4ºC and centrifuged at 5000 rpm again to remove proteins. The pH of the clear solution was adjusted to 7.0 with NaOH solution and dialyzed three times. The supernatant was subjected to fractional precipitation by 1, 2, 3, and 4 volumes of absolute ethanol according to Whistler and Lauterbach[20], stirred vigorously and kept overnight at 4°C. Precipitate from ethanol dispersion was collected by centrifugation at 5000 rpm (Sigma-Laborzentrifugen, 2K15) for 15 min, re-suspended in distilled water and lyophilized to afford the crude EPS.

**6.2. Ion exchange chromatography (fractionation by DEAE-cellulose).**

***A. Activation of Matrix.***

Diethylaminoethyl-cellulose (DEAE-cellulose) was prepared as recommended by Whistler (1965). Dry DEAE-cellulose (20 g) was treated with an excess volume of 0.5 M HCl solution and left at room temperature for two hours. After treatment with acid, DEAE-cellulose washed with distilled water until became neutral then it was treated with 0.5 M NaOH solution for two hours. After that, it washed with distilled water several times until it becomes neutral.

***B. Column Operation.***

The method described by Whistler[21] used for ion exchange chromatography. In this method, fractions from the previous step were concentrated and dialyzed against distilled water then adsorbed on a DEAE-cellulose column (70×1.5 cm) equilibrated with distilled water. Non-absorbed exopolysaccharides washed with distilled water and the absorbed exopolysaccharides eluted with a stepwise of NaCl (0.2-3.0 M). A fraction of 1.0 mL/ min was collected and a small portion was analyzed by phenol-sulfuric acid method[22]. The active fractions were pooled together, dialyzed against deionized water, and precipitated with ethanol after concentration.

**7. Coagulation/flocculation test.**

Coagulation tests were carried out using a standard jar test apparatus according to Ramavandi[23]. Raw River Nile turbid water (300 mL) was filled into beakers (1L), and the standard procedure for the jar test was followed at a constant room temperature of 24°C. The standard procedure involved 1 min of rapid mixing (120 rpm) followed by 10 min of slow mixing (45 rpm) for flocculation. Then, the treated water was allowed to settle for 15 min, and the supernatant sample was withdrawn by a sterile syringe from approximately 2 cm below the liquid level for analysis. The same coagulation test was conducted with no coagulant as a blank. Coagulation test was carried out on two stages, first using only extracted exopolysaccharides as coagulants, and second using exopolysaccharides with alum (50:50 *w/w*) as coagulant.

## Results and Discussion

Coagulation-flocculation process using metal salts, such as aluminum or iron salts, effectively increase particle size in water, and then these particles can be easily removed by sedimentation. A disadvantage of this process, however, it forms large amounts of metal hydroxides which cause production of large amounts of sludge[24]. As mentioned previously, some previous studies focused on removal of one or two pollutants of water using natural coagulants, however, the present study aimed to extract bacterial exopolysaccharides (EPS) and using them as natural coagulants for removal of bacterial indicators and some physicochemical parameters.

**Isolation of exopolysaccharides producing bacteria.**

Different bacterial isolates (depending on colony morphology and appearance) which picked up from River Nile water samples using nutrient agar media as a growth medium were examined for their abilities for production of exopolysaccharides (EPS). The obtained results showed the ability of three bacterial isolates for production of measurable exopolysaccharides amounts. These three bacterial isolates were identified using Biolog GEN-III OmniLog® (Biolog, USA) microbial identification system. Identification results indicated that the three bacterial strains were; *Bacillus licheniformis*, *Bacillus insolitus* and *Bacillus alvei*. The extracted EPS were purified for coagulation-flocculation experiments.

**Characterization of raw River Nile water.**

Raw River Nile water samples were characterized through enumeration of bacterial indicators of pollution including; total viable bacterial counts (TVBC) at both 37°C and 22°C, total coliforms (TC), fecal coliforms (FC) and fecal streptococci (FS), and through measurement of some physicochemical parameters including; turbidity, total dissolved solids (TDS), biochemical oxygen demand (BOD), sulfate ($SO_4^{+2}$) and nitrate ($NO_3^-$). Table (2) summarize the average values (3 samples) of bacterial and physicochemical quality of River Nile water used in the study. Total viable bacterial counts (TVBC) were $10^4$ and $10^3$ cfu.ml$^{-1}$ at 37°C and 22°C, respectively. Total coliforms, fecal coliforms and fecal streptococci showed average counts of $10^4$, $10^3$

and $10^2$ cfu.100 $ml^{-1}$, respectively. Measured physicochemical parameters showed average values of 9 NTU for turbidity, 250 $mg.l^{-1}$ for TDS, 4 $mgO_2.l^{-1}$ for BOD, 26 $mg.l^{-1}$ for sulfate and 0.8 $mg.l^{-1}$ for nitrate. Hassanein et al.[25] reported a study for physicochemical and microbiological quality of River Nile in Sohag governorate, Egypt. They examined thirty six River Nile water samples during hot and cold seasons, they almost found the same values of measured bacterial and physicochemical parameters as described in Table (2).

**Table 2. Characterization of raw River Nile water.**

| Bacterial indicators | | | | | Physicochemical parameters | | | | |
|---|---|---|---|---|---|---|---|---|---|
| TVBC (cfu.$ml^{-1}$) | | TC (cfu.100 $ml^{-1}$) | FC (cfu.100 $ml^{-1}$) | FS (cfu.100 $ml^{-1}$) | Turbidity (NTU) | TDS ($mg.l^{-1}$) | BOD ($mgO_2/L$) | $SO_4$ ($mg.l^{-1}$) | $NO_3$ ($mg.l^{-1}$) |
| At 37°C | At 22°C | | | | | | | | |
| $2.8x10^4$ | $3.1x10^3$ | $7.2x10^4$ | $6.8x10^3$ | $5.2x10^2$ | 9 | 250 | 4 | 26 | 0.8 |

**Coagulation-flocculation experiment**

Microbial polysaccharides have been used for water and wastewater treatment as natural coagulants during coagulation-flocculation process due to their unique structure, great physicochemical properties, high selectivity and reactivity[10,11,26-28]. Li et al.[16] studied the new bioflocculant (exopolysaccharide) produced by *Paenibacillus elgii* B69 bacteria for treatment of industrial wastewater. The new exopolysaccharide showed great potential for treatment of kaolin clay, heavy metal ions and dyeing pigments. In the present study, jar test method used for studying the removal efficiencies of new natural bacterial exopolysaccharides extracted from *Bacillus licheniformis*, *Bacillus insolitus* and *Bacillus alvei* isolated from raw River Nile water and soil samples. For studying the efficiency, the removal percent (R%) for each of bacterial indicators and physicochemical parameters were calculated. The following formula used for calculation of removal percent:

$$\text{Removal percent (R \%)} = (A-B)/A * 100$$

Where A: is the parameter value before addition of coagulant (before treatment).

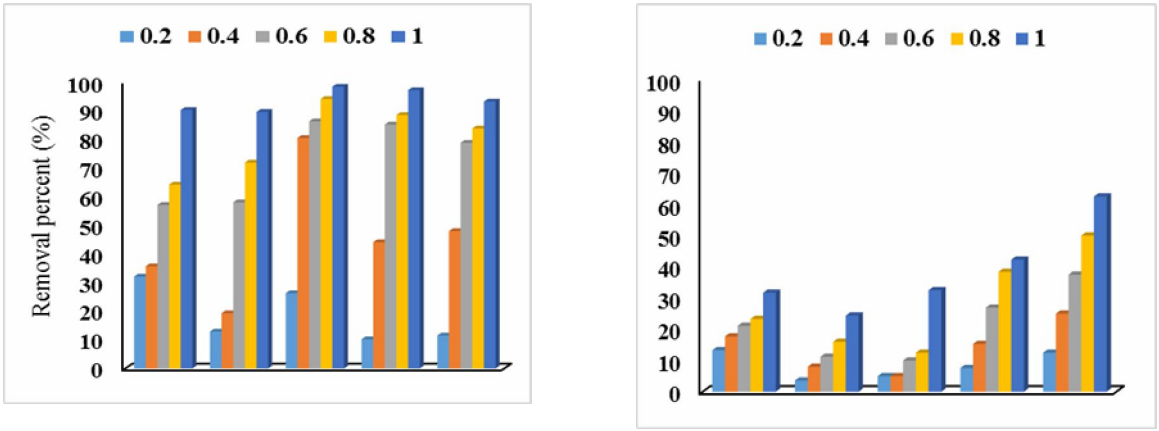
B: is the parameter value after addition of coagulant (after treatment).

Efficiencies of extracted bacterial exopolysaccharides (EPS) as natural coagulants were studied through two experiments, first by using of EPS as sole source of coagulation material with initial doses of 0.2, 0.4, 0.6, 0.8 and 1.0 $g.l^{-1}$ (Figure 1), and second through preparation of a mixture (50:50% w/w) of EPS with alum with initial doses 0.4, 0.8, 1.2, 1.6 and 2.0 $g.l^{-1}$ (Figure 2).

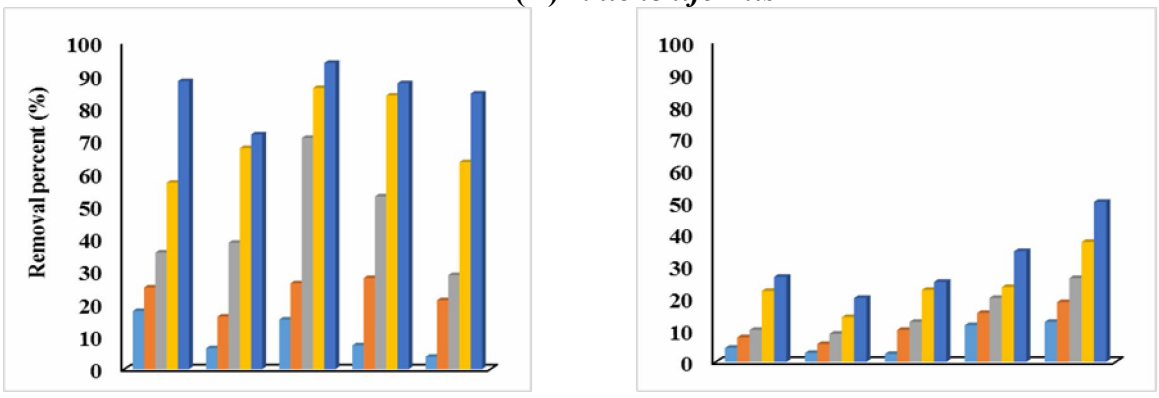
**Application of EPS as single coagulant material.**

The obtained results showed that, by increasing the bacterial exopolysaccharide (EPS) dose, removal percent increase for all of bacterial indicators and measured physicochemical parameters. Maximum removal percent observed at EPS dose of 1 $g.l^{-1}$ for all extracted bacterial EPS. *Bacillus licheniformis* EPS (Figure 1a) showed highest removal percent for both of bacterial indicators and physicochemical parameters, in comparison with *B. insolitus* and *B. alvei* EPS, since maximum removal percent were 90.3%, 89.6%, 98.4%, 97.2% and 93.2% for TVBC at 37°C, TVBC at 22°C, total coliforms (TC), fecal coliforms (FC) and fecal streptococci (FS), respectively. Also, it was clear that, *Bacillus licheniformis* EPS has low efficiency in removal of measured physicochemical parameters in comparison with the removal efficiency of bacterial indicators. Maximum removal percent were 31.7%, 24.4%, 32.5%, 42.3% and 62.5% for turbidity, total dissolved solids (TDS), biological oxygen demand (BOD), sulfate ($SO_4^{+2}$) and nitrate ($NO_3^-$), respectively. However, *Bacillus insolitus* EPS showed moderate removal efficiency (Figure 1b) for bacterial indicators. Maximum removal percent of bacterial indicators were 88.2%, 71.9%, 93.8%, 87.6% and 84.4% for TVBC at 37°C, TVBC at 22°C, TC, FC and FS, respectively. In addition, maximum removal percent for measured physicochemical parameters were 26.6%, 20%, 25%, 34.6% and 50% for turbidity, TDS, BOD, sulfate and nitrate, respectively. Moreover, EPS extracted from *Bacillus alvei* (Figure 1c) showed lowest removal efficiency for bacterial indicators, the maximum removal percent were 65.3%, 70.6%, 88.4%, 85.8% and 82.1% for TVBC at 37°C, TVBC at 22°C, TC, FC and FS, respectively. From the other hand, the maximum removal percent of *B. alvei* EPS for

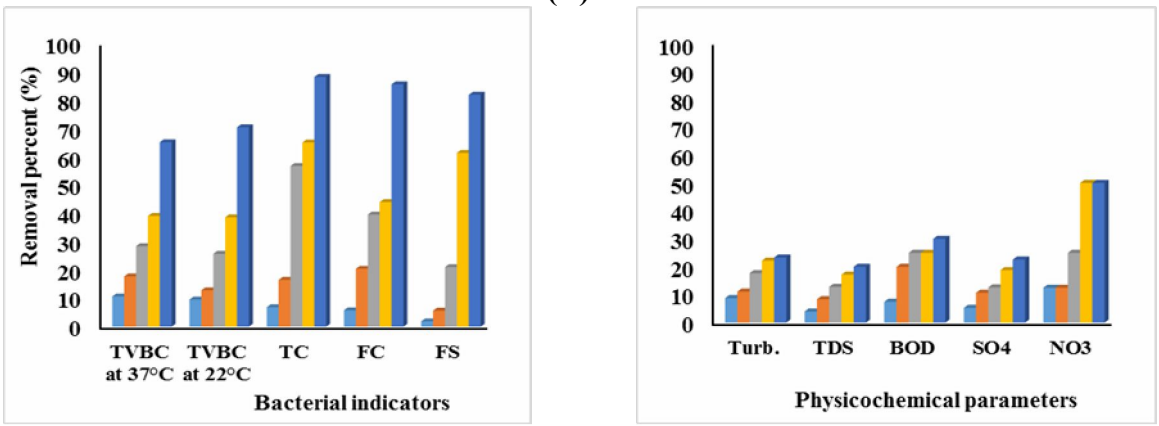
physicochemical parameters showed slight variations in removal percent, since maximum removal percent were 23.3%, 20%, 30%, 22.6% and 50% for turbidity, TDS, BOD, sulfate and nitrate, respectively. Many natural coagulants were extracted and applied as coagulant materials for water and wastewater treatment. These natural coagulants derived from plants such as *Plantago* species[29], *Phaseolus vulgaris*[8,30] and *Moringa oleifera* seeds[1]. Actinobacteria, a group of gram-positive bacteria, such as *Cellulomonas* and *Streptomyces* species were also used for production of natural coagulants[31-33]. Also, Recently, Choy et al.[34] summarized twenty one different plant types that produce natural coagulants, these plants were categorized as fruit waste (cactus, fungus, nuts, cereals and spice). In a more recent report, Choy et al.[35] provided a review of fourteen natural coagulants producing plants categorized as vegetables and legumes. The ability of natural coagulants for removal of bacteria and helminthes was reported by Zamudio-Pérez et al.[36]. Authors used natural gums such as Hydroxy-propyl triammonium chloride guar gum, for removal of coliforms and helminthes in municipal wastewater. They found removal percent of 82%, 94% and 99% for total coliforms, fecal coliforms and helminthes, respectively. They found also removal percent of 46% and 39% for chemical oxygen demand (COD) and turbidity, respectively.




**(A) *B. licheniformis***




**(B) *B. insolitus***




**(C) *B. alvei***

**Figure 1. Removal efficiencies of bacterial EPS as sole coagulant material.**

**Application of EPS and alum mixture as coagulant material.**

To study the removal efficiency of extracted bacterial exopolysaccharides (EPS) in case of mixing with alum, mixture of each EPS with alum (50:50%, *w/w*) were prepared. The obtained results (Figure 2) indicated that, by mixing EPS with alum, there were observable enhancement in the removal efficiencies of all bacterial indicators and physicochemical parameters. It was clear that, there was no clear variations in removal efficiencies for bacterial indicators, since maximum removal percent (2 $g.l^{-1}$ dose) of *B. licheniformis*, *B. insolitus* and *B. alvei* EPS mixed with alum, showed removal percent ranged from 97.1% to 99.8%. In contrast, the same behavior was observed in case of all measured physicochemical parameters removal efficiencies, except TDS and BOD, since *B. alvei* showed highest removal percent of TDS (76%) followed by *B. insolitus* (60%) and *B. licheniformis* (44%), and for BOD maximum removal percent were 75% (*B. insolitus*), 72.5% (*B. alvei*) and 65% (*B. licheniformis*) as represented in Figure (2 a,b,c). As mentioned previously, the present study represent usage of bacterial exopolysaccharides as natural coagulants for raw surface water treatment. Addition of alum to extracted bacterial exopolysaccharides (natural coagulants) enhanced the removal efficiencies of bacterial and physicochemical quality of raw surface water. Hu et al.[24] used chitosan as natural coagulant for treatment of high turbid surface water. They found that using of chitosan as single coagulant improved the turbidity removal (less than 50 NTU) and the amount of produced sludge was half (5 $mg.l^{-1}$) of using aluminum chloride as metal salt coagulant. They add a comparative low dosage of aluminum salt (13.5 $mg.l^{-1}$ as Al) with chitosan which improved the turbidity removal (less than 10 NTU). The treatment capability of EPS may be due to that, EPS contain many active functional groups such as carboxylate ($RCOO^-$), which carry negative charges, these negative charges bind to metal ions[19]. Heavy metals adsorption by microbial exopolysaccharides are widely reported such as *Bacillus firmus*[37] and *Paenibacillus validus* MP5[14]. The high removal efficiencies of studied bacterial exopolysaccharides as natural coagulants may be attributed to strong adsorption with positive charges carrying metals such as heavy metals, debris, oily particles, organics and mud leading to form large sized and heavy weight flocs. These new flocs increased in size during rapid and slow mixing of water. Bacteria, protozoa and other microorganisms are adsorbed on the surface of these flocs. This phenomena allow rapid degradation of organics in water which decrease levels of organic pollution in water, turbidity level and other related physicochemical parameters. In addition, decrease in counts of bacterial indicators also occurred due to collection of bacteria on the surface and inside flocs, which allow other protozoa and higher microorganisms to feed on these bacteria. Also, inside large sized flocs, aerobic condition decreased which is unsuitable for some aerobic bacterial growth. Moreover, mixing of natural bacterial EPS with alum enhances the removal efficiencies due to the known capability of alum as coagulant. On the other hand, produced sludge using EPS will be easily disposed, since it contains exopolysaccharides which act as nutrients for bacterial growth and fermentation.

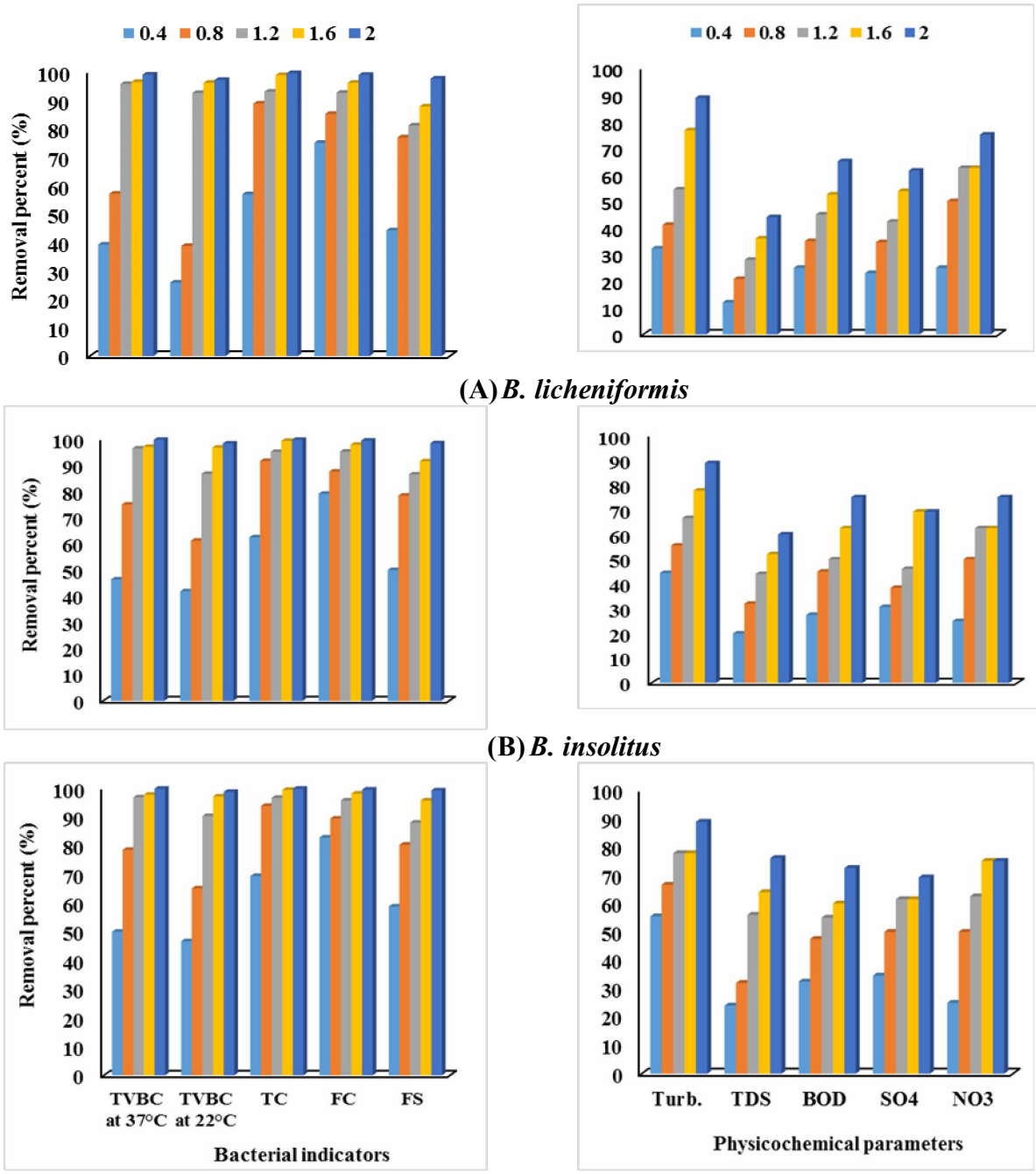



**Figure 2. Removal efficiencies of bacterial EPS mixed with alum as coagulant material.**

## Conclusions

From the present study, it can be concluded that;

1. The present study is the first study to investigate ability of *Bacillus licheniformis*, *Bacillus insolitus* and *Bacillus alvei* bacterial species to produce exopolysaccharides which have great potentiality to act as natural coagulants used in surface water treatment during coagulation-flocculation step.

2. Application of these new bacterial exopolysaccharides as natural coagulants showed measurable removal efficiencies for bacterial indicators and some physicochemical parameters of surface water.

3. By mixing these natural EPS with alum, removal efficiencies increase which decrease used amounts of alum leading to public health improvement.

4. Produced sludge can be easily biodegrade because presence of EPS which act as feed for microorganisms and low amounts of alum which decrease toxicity of sludge.

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