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Original article

Exopolysaccharide production from isolated *Enterobacter* sp. strain ACD2 from the northwest of Saudi Arabia

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ABSTRACT

Substantial interest has emerged on the use of marine microorganisms as producers of important macromolecules such as exopolysaccharides (EPSs), to apply them in areas including the food, pharmaceutical, and cosmetic industries, as well as other important sectors. In this study, thirty seven marine bacterial isolates were isolated from Haqel Beach in Tabuk region, Saudi Arabia. We found that only nine isolates have the ability to produce extracellular polysaccharides in medium. We selected the best producer and characterized it morphologically, biochemically, and molecularly using 16S rRNA analysis, revealing it to be *Enterobacter* sp. We also optimized the culture fermentation conditions to obtain the highest EPS yield. Our results showed that the optimal medium conditions were as follows: 15% sucrose, 0.5% peptone, presence of all fermentation medium salts, pH 7.5–8.0, incubation temperature 37 °C, and shaking at 150 rpm for 72 h, which conferred the highest EPS yield of 8.6 g/l.

1. Introduction

Natural Polymers such as exopolysaccharides (EPSs) have been considered to be essential sources of potential chemotherapy agents. Studies searching for new bioactive substances have been performed in marine habitats (Abdelhamid et al., 2020). Most bacterial species secrete slimy substances outside their cell walls when grown under specific culture conditions; this material is usually polysaccharide in nature (Sutherland, 2001a; 2001b), with a high molecular weight. These substances may be either capsular polysaccharides (Sutherland, 2001a) or EPSs, which are completely isolated from the cell (Sutherland, 2001b). The EPSs xanthan, gellan, cellulose, and succinoglycan derived from *Xanthomonas campestris*, *Sphingomonas paucimobilis*, *Acetobacter xylinum*, and *Rhizobium sp*., respectively, have been commercialized. Several marine bacteria, such as *Bacillus*, *Rhodococcus*, *Alteromonas*, *Enterobacter*, *Halomonas*, and *Klebsiella*, are also known as EPS producers (Nicolaus et al., 2000; Maugeri et al., 2002). Moreover, a study on *Zunongwangia profunda* SM-A87 isolated from deep-sea sediment performed by Liu et al. (2011) found that it can secrete significant quantities of EPS.

The discoveries of numerous types of EPS have been documented. However, only a few have achieved industrial and medical importance with considerable market appeal, particularly with regard to their use as biomaterials (Martínez-Cánovas et al., 2004).

Over a long period of time, marine plants, animals, and microorganisms have been revealed to be a huge source of bioactive metabolites. The biology of marine environments in particular is now progressively being recognized as a wellspring of potential natural products (Devesh et al., 2017; Wang et al., 2018).

Among marine bacterial biomolecules, novel EPSs produced by *Bacillus licheniformis* strain B3-15 (Maugeri et al., 2002), *Geobacillus thermodenitrificans* strain B3-72 (Nicolaus et al., 2000), and *B. licheniformis* strain T14 (Spanò et al., 2013) have been reported among the few compounds until now derived from marine bacteria with antiviral and immunomodulatory activity (Gugliandolo et al., 2014).

A marine Enterobacter cloacae, isolated from a sediment sample in India was reported to produce an acidic EPS71a, with high content of uronic acids (Iyer et al., 2005). This EPS showed emulsifying properties comparable to commercial gums.

In particular, the EPS produced by *Halomonas maura* (mauran), other than possessing interesting physicochemical and rheological properties (i.e., high viscosity, heavy metals binding, thixotropic

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and pseudoplastic features) showed immunomodulator effects and antiproliferative activities on human cancer cells, which are encouraging in pharmaceutical applications (Llamas et al., 2006).

Much of the aquatic microbial environment remains unexplored due to the enormity of the aquatic biosphere (Satpute et al., 2010), so focus has recently been placed on marine bacteria for the isolation of novel compounds (Viju et al. 2017). The study of marine bacteria and their role in the production of bioactive compounds is becoming an increasingly important research topic (Faulkner, 2001).

Numerous bacterial EPSs have important industrial applications as gelling and emulsifying agents. Curdlan (produced by *Agrobacterium*), xanthan (produced by *Xanthamonas campestris*), and gellan (produced by *Sphingomonas paucimobilis*) have been approved for use as food adjuncts by the United States Food and Drug Administration (McIntosh et al., 2005).

This study was established to isolate a marine bacterium with the ability to produce EPSs, identify it by a 16S rRNA technique as *Enterobacter* sp., and increase its EPS productivity by culturing it under optimized growth conditions.

2. Materials and methods

2.1. Sample collection

Five marine water samples were collected in sterile containers, labelled appropriately, and taken to the laboratory for analysis. All samples in the present study were collected from Haqel Beach, Tabuk region, Saudi Arabia.

2.2. Bacteriological isolation media

Nutrient agar and nutrient broth media were purchased from Oxoid Ltd.

2.3. Polysaccharide production medium (fermentation medium)

The medium used for polysaccharide production consisted of the following components: 3% glucose, 0.25% casein hydrolysate, 0.4% K_2HPO_4, 0.07% $MgSO_4{\cdot}7H_2O$, and 0.005% $ZnSO_4{\cdot}4H_2O$ This medium was buffered at pH 7.0 and all the medium constituents dissolved in marine water.

2.4. Isolation methods

The marine samples were cultivated and isolated in accordance with the work of Viju et al. (2016).

2.5. Characterization and identification of isolates

From pure cultures, the isolates were identified based on their cultural, morphological, physiological and biochemical characteristics.

2.6. Identification of bacterial isolate by 16S rRNA gene and sequencing techniques

The 16S rRNA gene technique was used for the genetic studies, in accordance with the work of Weisburg et al. (1991) and Coenye and Vandamme (2003).

2.6.1. DNA extraction and purification

DNA extraction was performed following the protocol of GeneJET Genomic DNA Purification Kit (Fermentas). The purified DNA was used immediately for PCR.

2.6.2. Purification of PCR product and DNA sequencing

The PCR product was cleaned up using GeneJET PCR Purification Kit (Fermentas). Alignment of the resultant sequence against the NCBI GenBank database was then performed.

2.6.3. Nucleotide sequence accession number

The 16S rRNA gene sequence was deposited in the NCBI Gene Bank nucleotide sequence database under accession number EF025542.

2.7. Maintenance of bacterial strains and fermentation

The investigated bacteria were maintained by monthly subculture on slants of nutrient agar media. The inoculated slants were incubated at 28–30 °C for 1–2 days and the resulting heavy growths were then used for the fermentation experiments or stored at 4 °C.

2.8. Testing the isolates for EPS productivity

The cells of the selected isolate growing on one slant were transferred to a flask containing the corresponding fermentation medium. After shaking at 150 rpm and 37 °C for 24 h, flasks containing the sterilized fermentation medium were inoculated with 1 ml aliquots of the 24-h-old broths. Production of bacterial EPS was achieved by fermentation in shaken conical flasks (250 ml), using Innova-4000, a rotatory shaker, at 150 rpm and 37 °C. During the fermentation period, the growing bacterial cultures were examined for their viscosity, EPS yield, and culture protein content of the culture filtrates.

2.9. Analytical methods

2.9.1. Determination of total carbohydrates

Total carbohydrate content was determined using the phenol–sulfuric acid method (Dubois et al., 1956) and the exopolymer will be precipitated as follows;

This was performed gravimetrically by isolation of the methanol-precipitatable material by centrifugation. 3.0 ml culture broth was added to 9.0 ml methanol and mixed thoroughly. The resulted precipitate was isolated by centrifugation (4000 rpm). After drying, the precipitate was weighed and designed as the "Alcohol-precipitatable material". This comprised bacterial cells and methanol-precipitatable biopolymers.

2.9.2. Determination of total proteins

The total proteins were achieved according to the method of Lowry et al. (1951).

2.9.3. Determination of viscosity

The Viscosity can be determined as the ratio between the viscosity of diluted EPS broth (1:20) against water and the viscosity was measured (10 ml) at 25 °C in an Ostwald viscometer.

2.9.4. Determination of the monosaccharide constituents of the produced EPS

Samples were analyzed for monosaccharides constituents by high-performance liquid chromatography (HPLC) according to Horwitz and Latimer (2005). Sugars were extracted into DMSO; the extract was passed through C_{18} Sep-Pak cartridge and stored under refrigeration till analysis. Standard solutions of individual sugars with analytical Glucose, Fucose, Mannose, Galactose, and N-acetyl glucose amine were prepared by diluting each analyzed sugar in deionized water. Injection volume of each standard was 20 μL.

2.9.5. Statistical analysis

Data were statistically analyzed using SPSS, version 10.00 for Windows (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation.

3. Results

3.1. Isolation of bacterial isolates from marine samples

A total of thirty seven marine bacterial isolates were isolated from Haqel Beach, Tabuk region, Saudi Arabia. Only nine of these were found to be EPS producers. The ESP-producing isolates were differentiated and identified by a 16S rRNA PCR technique as four isolates belonging to the genus *Enterobacter* and five belonging to different genera, as listed in Table 1.

3.2. Identification of the bacterial isolates

Identification of the isolates was achieved by studying their morphological characters and biochemical features, as recorded in Table 1, and confirmed genetically by using 16S rRNA PCR sequence techniques, the results of which are recorded in Table 2. We found that a large proportion of the isolates belonged to *Enterobacter* sp. (44.44%), while among the rest (55.55%) three belonged to the genus *Klebsiella* (33.33%), one was *Citrobacter* sp. RHBSTW-00944 (11.11%), and one was from the genus *Serratia* (11.11%).

A single marine isolate (*Enterobacter* sp.) was selected as the best EPS producer. According to the morphological and biochemical results (recorded in Table 2), the selected isolate was a Gram-negative bacillus that was motile and not a spore-former. These results shown in Table 2 coincide with the identity of *Enterobacter* sp.

Molecular characterization of the selected isolate was achieved by performing PCR for the 16S ribosomal RNA gene, followed by alignment of the sequence produced by the PCR with the GenBank database, which also showed high similarity to *Enterobacter* sp.

3.3. Selecting the isolate with the highest EPS production

All bacterial isolates (nine isolates) exhibiting a shiny mucoid appearance were examined for their ability to produce EPSs. The single isolate with the highest EPS productivity was selected. After morphological and biochemical identification of all isolates and confirmation of their identity by 16S rRNA PCR analysis, the selected EPS-producing isolate was identified as *Enterococcus* sp. and then used for the rest of the study.

3.4. Chemical composition of the produced EPS

The produced EPS was examined for its monosaccharide constituents by HPLC. This revealed that the structure of the produced EPS consisted mainly of fucose, galactose, glucose, and glucuronic acid, at a molar ratio of 2:1:1:1, as the monosaccharide components (data not shown).

Table 2
Morphological and biochemical characters of the isolated EPS-producing bacterium.

Character	Results
Gram stain	Negative
Spore stain	Negative
Motility test	Positive
Growth temperature	37 °C
Oxidase test	positive
Urease production	Positive
Gelatinase production	Negative
Indole production	Negative
Catalase test	Negative
Voges–Proskauer test	Positive
Citrate test	Positive
H_2S production	Negative

3.5. Studying the effect of the fermentation conditions on EPS productivity

In these experiments, we fixed all growth factors except for the studied factor, which was varied to examine its effect on the EPS productivity. The growth factors include two main groups: environmental factors (e.g., incubation period, initial pH, shaking speed, and incubation temperature) and nutritional factors (e.g., different carbon/nitrogen sources and fermentation medium salts).

The selected isolate *Enterobacter* sp. was subjected to further studies to optimize the cultural conditions to achieve the highest EPS yield. In each step of the optimization experiment, we tested one parameter, including environmental conditions (shaking speed, incubation temperature, pH, and incubation time), fermentation carbon sources (glucose, fructose, galactose, and sucrose), fermentation nitrogen sources (peptone, casein hydrolysate, triammonium citrate, and urea), and presence/absence of inorganic salts (K_2HPO_4, $MgSO_4$, and $MnSO_4$). For each parameter, we varied the applied conditions (Tables 3 and 4). The results of these experiments showed that the optimal medium composition was 15% sucrose, 0.5% peptone, 0.4% K_2HPO_4, 0.07% $MgSO_4$, and 0.001% $MnSO_4$, with rotation at 150 rpm, pH 7.5–8, and an incubation period of 72 h (Tables 1–4). Because of this optimization, we achieved a high yield of EPS production of 8.6 gm/l.

4. Discussion

In this study, thirty-seven bacterial isolates were isolated from Haqel Beach, Tabuk region, Saudi Arabia. After identification by the 16S rRNA gene technique, only nine isolates were found to be EPS producers, with almost half of the isolates belonging to *Enterobacter* sp. We selected the best EPS producer for further study and identified it by a 16S rRNA technique as *Enterobacter* sp. ACD2.

Table 1
Bacterial isolates identification by 16S rRNA partial sequence among the EPS isolates.

Isolate no.	Isolate code	16S rRNA partial sequence	Identity %	Isolate name
1-	I 1	CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAG	100	*Enterobacter* sp. strain ACD2
2-	I 2	TCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAA	100	*Enterobacter roggenkampii* strain RHBSTW-00002
3-	I 3	TCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAA	100	*Enterobacter* sp. strain M13
4-	I 4	TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTG	100	*Enterobacter roggenkampii* strain RHBSTW-00002
5-	I 5	ATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTG	100	*Klebsiella grimontii* strain RHBSTW-00039
6-	I 6	CGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTG	100	*Klebsiella aerogenes* strain G5_AM
7-	I 7	TGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTA	100	*Klebsiella quasipneumoniae* strain RHBSTW-00138
8-	I 8	ATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTG	99	*Citrobacter* sp. RHBSTW-00944
9-	I 9	TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGT	100	*Serratia plymuthica* strain YA-4

Table 3
Effect of the environmental culture conditions for *Enterobacter sp.* EPS production.

Environmental factor		Total carbohydrate (g/l)	Total protein (g/l)	Viscosity (s)
Initial pH	6.0	1.85	2.48	93
Incubation temperature	6.5	2.19	2.61	114
	7.0	3.86	1.86	159
	7.5[a]	5.50	1.74	204
	8.0	5.51	1.46	234
	8.5	4.96	1.55	233
	9.0	4.20	1.50	201
	25° C	3.9	1.44	167
	37 °C[a]	5.51	2.50	237
	45 °C	3.3	2.20	211
	60 °C	1.2	1.0	55
Incubation period	24 h	4.25	2.01	156
	48 h	5.48	1.63	159
	72 h[a]	5.51	1.51	237
	96 h	5.51	1.51	234
Shaking rate	50 rpm	3.74	2.57	156
	100 rpm	3.83	2.42	159
	150 rpm	5.51	2.01	207
	200 rpm[a]	4.96	1.95	234

[a] Control: the standard EPS production medium.

Table 4
Effect of the nutritional factors on the isolated *Enterobacter sp.* EPS production.

Nutritional factor			
1) Different carbon sources (g/l)	Total carbohydrate (g/l)	Total protein (g/l)	Viscosity (s)
Sucrose3%	3.17	2.71	207
5%	4.46	3.61	220
10%	5.41	3.38	314
15%	7.80	3.10	345
Glucose3%*	5.52	1.62	237
5%	4.51	1.51	204
10%	3.73	1.89	153
15%	2.36	2.10	93
Fructose 3%	2.25	2.30	204
5%	3.55	2.28	220
10%	4.28	2.42	201
15%	2.05	2.83	111
Galactose 3%	1.92	0.06	17
5%	2.04	0.08	17
10%	2.10	0.04	17
15%	2.12	0.01	17
2) Nitrogen source*(g/l) + 15% sucrose instead of 3% glucose.			
Peptone0.25%	3.2	2.51	115
0.5%	8.6	2.56	392
1.0%	3.4	3.2	154
1.5%	3.0	3.6	111
Yeast extract0.25%	4.3	2.77	232
0.5%	4.4	3.15	312
1.0%	4.1	3.50	221
1.5%	3.1	3.9	151
Soybean extract 0.25%	1.02	5.77	17
0.5%	1.22	6.15	17
1.0%	1.20	6.50	17
1.5%	1.12	6.9	17
Urea 0.25%	1.1	3.2	17
0.5%	1.0	3.7	17
1.0%	13	4.65	17
1.5%	1.2	5.30	17
Casein hydrolysate 0.25%*	5.51	1.51	237
0.5%	5.51	1.82	200
1.0%	5.00	2.1	155
1.5%	4.33	2.8	150
3) Medium salt elimination			
K_2HPO_4	4.3	1.45	215
$MgSO_4$	4.0	1.50	212
$MnSO_4$	4.1	1.51	215
All salts eliminated	1.10	1.44	17

* Control: the standard EPS production medium.

The results of this study proved that the EPS of the isolated *Enterobacter* sp. strain ACD2 chemically consisted mainly of fucose, galactose, glucose, and glucuronic acid, at a molar ratio of 2:1:1:1, as the monosaccharide components. The yield of EPS is related to many factors, such as the isolate type, the site of isolation, and environmental and nutritional factors.

Table 3 clearly shows the effect of the environmental factors such as initial pH and incubation temperature on EPS productivity. The optimal initial pH and incubation temperature to produce the highest EPS yield were pH 7.5–8.0 and 37 °C, at which the EPS yield and viscosity were high (5.51 g/l and 237 sec., respectively). On the other hand, higher or lower pH values or incubation temperatures would lead to decline in the EPS yield. These findings are in agreement with the work of Torres et al. (2012), who reported that the EPS productivity of *Enterobacter* A47 increased in the temperature range of 25–37 °C and pH range of 6.0–8.0.

The shaking speed of 150 rpm was associated with the highest EPS yield, which differs from the shaking rate of 100 rpm as used by Mata et al. (2006) with another organism. Moreover, the nutritional factors strongly affected the EPS productivity, such as supplementation of the EPS production medium with different carbon sources; specifically, this involved replacing the glucose medium with different carbon sources (Petry et al. 2000), including sucrose, fructose, and galactose, in addition to glucose. Table 4 clearly shows that the best carbon source that gave the highest EPS production was 15% sucrose (EPS 7.8 g/l), followed by 3% glucose as a control (EPS 5.51 g/l) and 10% fructose (EPS 4.28 g/l), while galactose sharply lowered the EPS yield and viscosity to minimal levels. These results agree with those of Lee and Lee (2005). From these results, this study proved that high concentrations of carbon sources had inhibitory effects on bacterial EPS production, in agreement with the work of Lin and Chen (2007) and Chen et al. (2008).

The other important nutritional factor was nitrogen source. Many researchers have reported the effect of nitrogen source on the production of EPS (Chen et al., 2008). In this study, four nitrogen sources were applied instead of the casein hydrolysate in the EPS production medium. These nitrogen sources are listed in Table 4 and included peptone, yeast extract, urea, and low-cost soybean. In this experiment, all other growth conditions were fixed, using 15% sucrose as the carbon source. The results showed that the optimal nitrogen source giving the highest EPS productivity was 0.5% peptone (8.6 g/l), followed by control 0.25% casein

hydrolysate (5.51 g/l), and finally 0.5% yeast extract (4.4 g/l). On the other hand, urea and soybean would decrease the EPS productivity sharply to 1.22 g/l, but elevate the culture filtrate T. protein to its highest level to reach 5.3 and 6.9 g/l, respectively. Soybean as a nitrogen source would enhance the cell growth but not the EPS productivity, in agreement with the work of Hussein et al. (2015).

Our results proved that the growth of isolated marine *Enterobacter* sp. ACD2 on the optimum medium conditions, which contains; sucrose 15%, peptone 0.5%, in addition of all fermentation medium salts will give the highest EPS yields (8.6 g/l) when grown at pH 7.5–8.0, incubation temperature 37 °C with shaking speed 150 rpm for 72 h and this exceeds than the results of Enterobacter cloacae subsp. Dissolvens (5.6 g/l EPS) when grown on its basal medium which contains; carbon (sucrose, molasses, paraffin and sunflower oil) and nitrogen sources, NaCl concentration, incubation time, pH (6–8) and incubation temperature 30 °C (Azari et al., 2017).

On other hand, *Halomonas ventosae* and *H. anticariensis,* which incubated on 32 ^{0}C, sea salt concentration of 7.5% w/v, 1% glucose as a carbon source and 100 rpm for 5 days, the EPS yields were 2.835, 2.895, 2.965 and 4.995 g/l for strains AI12T, AI16, FP35T and FP36, respectively (Mata et al., 2006).

The last nutritional factor affecting the EPS productivity was the fermentation medium salts. Table 4 illustrates that the absence of one salt among the production medium salts would decrease the productivity (EPS: 4.0 g/l), while the absence of all salts reduced the EPS productivity to 1.10 g/l. This agrees with the findings of Helal et al. (2012) and Cheirsilp et al. (2001).

5. Conclusion

From previous results, we concluded that the marine environment in Tabuk region contains a significantly low amount of microbial communities and is limited to only a few species, including a few bacteria. The isolated bacteria were identified morphologically and biochemically, and confirmed by 16S rRNA gene sequencing. Among the identified isolated bacteria, 44.4% were classified as *Enterobacter* sp. and the rest as other different species. We also measured the EPS productivity for all bacterial isolates and found that the maximum EPS production yield was from *Enterobacter* sp. strain ACD2. According to the optimization of the culture growth conditions, we can use modified EPS production medium consisting of sucrose (15%), peptone (0.5%), K_2HPO_4 (0.4%), $MgSO_4 \cdot 7H_2O$ (0.07%), and $MnSO_4 \cdot 4H_2O$ (0.005%), with incubation aerobically (shaking at 150 rpm) at 37 °C and pH 7.5–8.0, instead of the basal EPS production medium, to obtain an EPS yield equal to 8.6 g/l, which is greater than the level of EPS with basal fermentation medium (5.51 g/l). Further study of the produced EPS is recommended to test its possible applications and biological activities.

Author contributions

Mikhlid Almutairi was responsible for the study design. Mikhlid Almutairi and Mohamed Helal performed all practical experiments. Mohamed Helal performed data analyses and wrote the first draft of the manuscript. Mikhlid Almutairi wrote the final draft of the manuscript. All authors read and approved the final submitted manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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