PRODUCTION AND CHARACTERIZATION OF EXOPOLYMERIC SUBSTANCES WITH EMULSIFYING ACTIVITY BY THE MARINE BACTERIUM KOCURIA ROSEA

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Abstract - An extracellular polymeric substance (EPS) producing marine bacteria, identified and characterized asKocuria rosea strain 23Bbased on 16S rRNA sequencing and phylogenetic analysis(99 % similarity), was isolated fromCoast of Jalisco (Mexico). Glucose (1 %) was the most efficient carbon source for growth and EPS production. The highest EPS production (6 g/L) was yielded in fermenter culture at 250 rpm after 48 h of incubation.The EPS produced was capable of stabilizing water emulsions with kerosene and hexane.Results showed surface tension reduction and emulsion activity valuesfrom 58.80 ± 1.86 mN/m and 40 ± 0.34 to 52.32 ± 0.07%, respectively.Analysis of extracted EPS using Fourier transform infrared spectroscopyrevealed “finger sprint” to polysaccharides and protein.

Keywords - Marine bacteria, Kocuria rosea, extracellular polymeric substances, emulsifying activity

I. INTRODUCTION

In the recent years, there is an increased focus to search for novel biopolymers from microorganisms. Over the last few decades, many microorganisms isolated from diverse geographical locations and niches across the world have been explored as promising resources of novel exopolymeric substances. This exopolymeric matrixhas several basic functions, such as adhesion to surfaces, aggregation of the cells, formation of biofilms, protective barrier for cells against environmental stress and storage of reserve carbon source[1].This extracellular material comprises a heterogeneous group of structurally diverse polymeric substances, consisting of nucleic acids, lipids, proteins and polysaccharides.Particularly, the exopolysaccharides (EPS) are of biotechnological interest due to their structural diversity, biodegradability and safety due their non-toxicity for human and environment.Moreover, strain physiologic and metabolic characterization is extremely useful to understand and optimize microbial production. In marine environment many bacteria produce EPSto cope with strong environmental stress and to survive adverse conditions, and could exploited for the production of biopolymers with industrial applications such emulsifiers. Some EPS-producing marine bacteria are Alteromonas, Pseudoalteromonas, Shewanella and Vibrio species [2]–[5]. EPS production by microorganisms has the advantages compared to the plants[6].

II. DETAILS EXPERIMENTAL

2.1. Materials and Procedures

Strain isolation and conservation
The strain used in this study was isolated frommarine sediments at Jalisco coasts, in Mexico. The strain isolated was purified on Difco Marine agar at 30°Cand was conserved in cryogenic vials with Difco Marine broth (MB) and 20 % glycerol at -80°C.

Identification of bacteria strain.
Amplification of the 16S rDNA gene: Genetic material was obtained from fresh and pure cultures by cell lysis at 95 ° C for 10 min. The amplification of the 16S gene was carried out according to Marchesi et al. 1998 using JUMPSTART and the 63F primers (5'-CAG GCC TAA CAC ATG CAA GTC-3 ') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') in 20 μL of reaction mixture.

Sequencing of the amplified DNA fragment: The PCR products were observed on agarose gels (1% w / v) with GelRed, purified with the GENELUTE kit and quantified with Nanodrop at 260 nm. Both chains were sequenced by MACROGEN USA CORP, the consensus sequences were obtained with CLC MAIN WORKBENCH 7.1.

Phylogenetic analysis. The sequence obtained was compared with the sequences of the 16S rDNA genes available in the NCBI databases. The phylogenetic tree wasconstructed with the Maximum Likelihood method.

Quantitative Biofilm Assay

The biofilm formation is often mediated by extracellular polymeric substances, and hence, biofilm formation by the three EPS producing isolates was determined according to the method described earlier[7]. In liquid medium Difco MB, a colony of strain 23B was planted and left at 30 ° C during 24 h. The overnight broth culture of the strain were adjusted to 0.29 O.D.600 and inoculated to 50 µL on MG and MY agar (2 % p/v) media (Table I).

Table I. Chemical composition of the culturemedia used

Compound (g/L)	MY	MG
Glucose	10	10
NaCl	51.3	20
Marine salts (Red Sea)	-	2
Peptone	5	5
Yeast extract	3	1
Malt extract	3	-
$MgSO_4 \cdot 7H2O$	13	20
KCl	1.3	-
$MgCl_2 \cdot 6H_2O$	9	-
NaBr	0.15	-
$CaCl_2 \cdot 2H_2O$	0.2	-
$FeCl_3 \cdot 6H_2O$	traces	-

pH adjusted 7.0

The cultures were incubated at 30 °C for 72 h under static conditions and the biofilm was gently washed with saline solution.After weighing the biomass, ~ 2 mL of isotonic solution was added, centrifuging at 10,000 rpm for 30 min. The supernatant was recovered, and the obtained pellet was resuspended again in 1 mL isotonic solution (up to three times).The final pellet was weighed,and lyophilized to obtain cellular dry weight. On the other hand, the supernatants (~ 2.5 mL) were precipitated with cold ethanol (~ 7.5 mL) and were left at -22 ° C for 24 h. EPS was recovered, centrifuged at 3,800 rpm, 4 ° C, for 30 min and lyophilized. After 24 h the lyophilized biomass pellet and the recovered EPS pellet were weighed. Finally, by biomass / EPS ratio, yield was obtained.

Kinetic for Biomass, and EPS production

A inoculum (1% v/v) was prepared from the strain frozen stock in 125 mL Erlenmeyer flask containing 20 mL of medium MB and incubated at 250 rpm and 30 °C during 24 h. Then, 200 µL of the bacteria culture were harvested and inoculated into 125 mL Erlenmeyer flask with 19 mL Mg medium.

Growth was determined by OD at 620 nm and correlated with dry cell weigh. For this purpose, 20mL of culture broth were centrifuged and thepellet was washed twice with physiologic solution (sterile 0.9% NaCl solution), dried, and weighted. The sugar concentration was determinedusing DNS technique [8].

For EPS recovery, free cells-supernatant was precipitated using an equal volume of 96% acidified ethanol and kept at -20 °C overnight. Thereafter, the exopolysaccharide was separated by centrifugationanddriedforweight determination; EPS was also frozen and lyophilized for further characterization.

EPS surface tension and emulsifying activity

The cell-free supernatant was used to evaluated;surface tension by the Du Nouy ring method, using tensiomer DataPhysics DCAT, (Dataphysics Instruments, Raiffeisenstraße, Germany) and emulsion activity (E_{24}). Briefly, for the surface tension measurements were carried out at room temperature after dipping the platinum ring in the solution for a while in order to attain equilibrium condition.For calibration of the instrument, the surface tension of distilled water was first measured. The measurement was repeated at least three times, and an average value was used to express the surface tension of the sample.

For E24 assay: in a glass test tube (75 x 10 mm) a mixture of supernatant and kerosene at a constant ratio (1:1) was vigorously homogenized for 60 s and then left to stand 24 h at room temperature. The E_{24}(Eq. 1) was defined as the percentage of the ratio of the height of an emulsion to the total height of the mixed liquid after standing for 24 h. Culture media without inoculum was used as a negative control.

$$E_{24}, \% = \frac{\text{emulsion height (mm)}}{\text{total height (mm)}} \times 100 \quad (1)$$

Fourier-transformed infrared spectroscopy (FTIR)

FTIR spectra of EPS from Kocuria rosea 23B, were obtained using a FTIR spectrometer (Spectrum Two, Perkin Elmer; Boston, MA)equipped with a Golden-gate single reflection attenuated total reflectance (ATR) accessory (SensIR Technologies). The FTIR spectral region used was 4000–600 cm^{-1}. Spectra were collected by co-adding 35 scans at a resolution of 4 cm^{-1}. Base line and the spectrum data were processed with the Spectrum Molecular Spectroscopy program (Perkin Elmer, Boston, MA).

III. RESULTS AND DISCUSSION

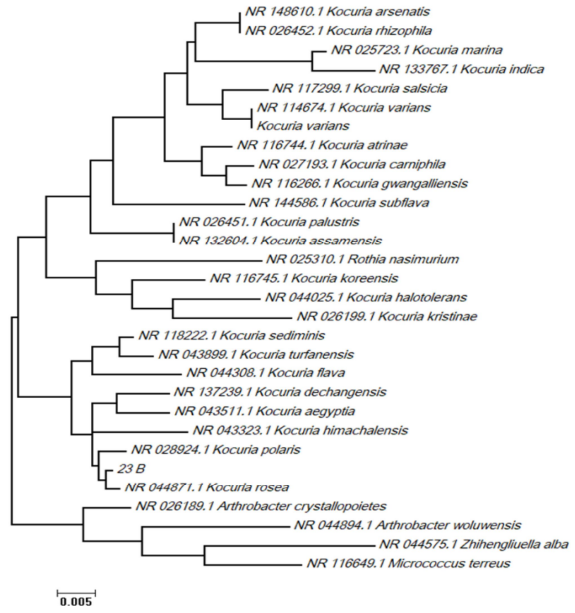
3.1 Identification of bacteria strain

Twenty-six bacterial strains isolated at Jalisco coasts in México were screened for EPS production and emulsifying activity. One of these strains, 23B, was positiveand selected for further tests due to its yield to produce EPS and emulsification activity.

The 16S rRNA gene sequence (NCBI accession number NR044871) from 23B was determined and compared with all sequences currently available for members of the genus Micrococcus: Kocuriaand related taxa. The results were used for the construction of a phylogenetic treeusing the method

of Maximum Likelyhood with the closest NCBI strains obtained by a BLASTn for 16S ribosomal RNA sequences (Bacteria Archea)(Fig.1). Showing that strain23Bwas a member of the genus Kocuria, and was clustered closely with speciesKocuria rosea, Kocuria aegyptia, Kocuria polaris and Kocuradechangensis with an identity of 99%. Based on 16S rRNA gene sequence and the phylogenetic tree, the strain 23B is identified as Kocuria rosea 23B. Now in days exist information on EPS producers from genusKocurialike of Kocuriaerythromyxa 32f and Kocuriarosea 32d, which produced EPS with biosurfactant property[9], and Kocuriaturfanesis strain-J which produced a biosurfactant that emulsified pesticides at extreme environmental conditions[10]. Also, Kocuria rosea strain BS-1, which produced an exopolysaccharide (designated as Kocuran) exhibiting in vitro antioxidant and immunosuppression properties[11].

Fig.1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences, showing the positions of strain 23B.The tree was obtained using the maximum-likelihood algorithm. The bar indicates the relative sequence divergence (0.005 nucleotide substitutions per site).



3.2Biofilm Assay

It was observed that strain 23 B was able to growth and produce exopolymeric substance on MG and MY medium. The highest yield (0.15 g/L) of EPS was when Kocuria rosea 23B, growth on MG medium, at 72 h incubation (Table II). The Fig. 2 shows the profile growth and EPS production from Kocuria rosea, of biofilm assay.

Table II. Biomass and product formation yields in biofilm assay of Kocuria rosea, growing on MG and MY medium (two replicates).

Medium	Time (h)	Biomass (g/L)	EPS (g/L)	$Y_{EPS/X}$	$Y_{EPS/S}$
MG	48	2.27	0.22	0.10 ± 0.02	0.022 ± 0.05
	72	2.49	0.37	0.15 ± 0.03	0.037 ± 0.03
MY	48	0.65	0.01	0.02 ± 0.01	0.001 ± 0.01
	72	1.11	0.05	0.05 ± 0.00	0.005 ± 0.00

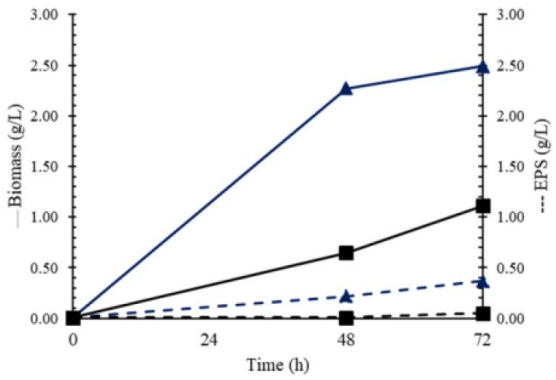


Fig. 2.Profileof growth and slime production of Kocuria rosea growing on MG (▲) and MY (■) agar plate media.

3.3Growth and EPS production

The aim of present study was to isolate and characterize marine bacteria able to produce exopolymeric substance with emulsifying activity. One very noteworthy result was that the bacteriumcould grow and produce its EPS on liquid MG media supplemented with glucose (1%). Fig.3shows the appearance of strain 23B during the exponential growth phase after about 24h incubation in MG medium containing 1% w/v glucose at 30 °C. As can be seen, the EPS was in close contact with the cell during this growth phase, but later it began to diffuse fairly evenly throughout the medium as an amorphous slime.

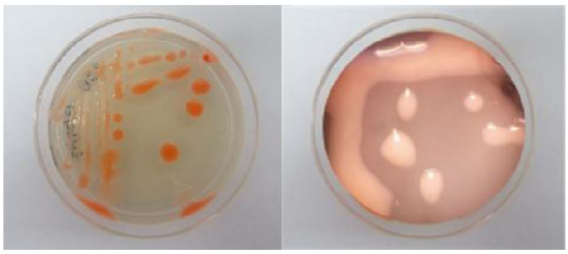


Fig.3. Growth Kocuria rosea 23B on Mg medium agar with glucose (10 g/L).

When the culture was centrifuged, the supernatants were viscous and formed stringy precipitates with cold ethanol and to enhance the precipitation, the samples were stored at 4°C for 24 h. The addition of two volumes of cold ethanol showed a better precipitation and recovery of the biopolymer.EPS exhibited good solubility in distilled water.

We found that the maximum quantities of EPS were reached when strain 23B was grown in MG medium containing 5% w/v total salts and an initial glucose concentration of 1% w/v, incubated at 30 °C for 4 days at 250 rpm. Under these conditions, OD_{600} was 1.47±0.04 and EPS production 6.08 ± 0.04 g/L of medium (Fig. 4a).Incubation time was the major factor influencing the quantity of EPS produced.The kinetics of EPS production by strain 23B showed thatthe polymer was produced mainly during the exponential growth phase and continued, although to

a much lesser extent, in thestationary phase. Production began to wane after 48 h incubation, when residual glucose was close to 6 g/L.The growth assessment with respect to EPS production as a function of time indicated that K. rosea strain 23B was a prominent EPS producer.

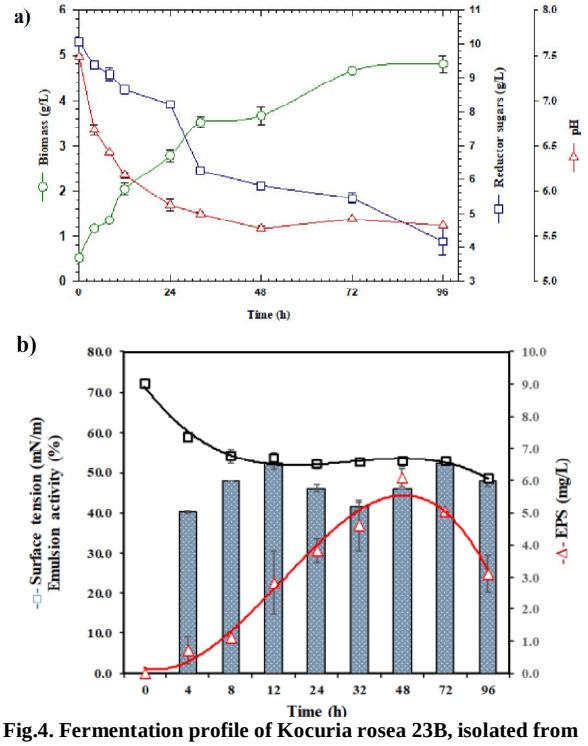


Fig.4. Fermentation profile of Kocuria rosea 23B, isolated from Jalisco coasts, growing in Erlenmeyer flasks on Mg medium with glucose (10 g/L). (a) Growth (green circle empty), sugar consumption (blue empty squares) and pH evolution (triangle empty). (b) Surface properties: surface tension (black squares empty) and Emulsion activity (bars) and EPS production (red triangle empty).

Emulsifying activity of EPS

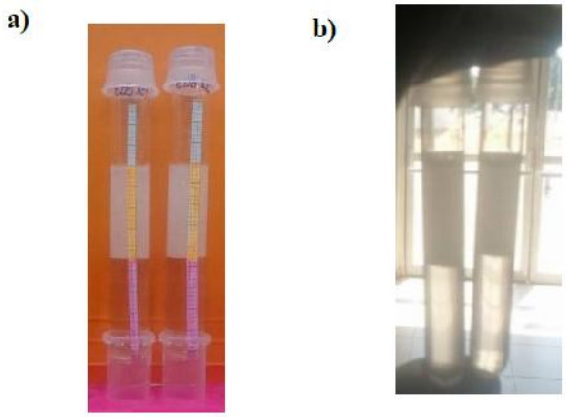


Fig.5. (a)The emulsification assay performed by mixing kerosene, with an equal volume of cell-free supernatant obtained from a culture of a bacterial strain that synthesizes extracellular. (b) Emulsion activity of EPS after precipitation using n-hexane as hydrophobic compound.

During growth of the bacterial strainKocruia rosea 23Bin flask, an important step was to monitor for the production of the bioemulsifier in order to determine the maximal bioemulsifier yields reached and then isolating the molecule. Supernatant from Kocuria rosea was capable of stabilizing mixtures of kerosene and n-hexane (Fig. 5). The Fig. 4b shows the profile of emulsification activity of supernatant and kerosene, it was observed that emulsifying activity start at 4 h of incubation; nonetheless, at 12 h of cultivation and 72 h it reaches its local maximum, both close to 50%.Also, it was found EPS 23B was capable to reduce the surface tension of the water from 72 to ~48 mN/m at 96 h of incubation.These results are similar to reported by Karnwal (2017), for K. rosea strain VBI, growing at 5% glycerol concentration and reducing the surface tension near ~50 mN/m [12].

FT-IR analysis

FTIR spectroscopy has been extensively applied to determine the functional groups within a polymeric structure. The FTIR analysis indicated the presence of different functional groups in the EPS excreted by Kocuria rosea after precipitated and washing several times with ethanol and dried. FTIR spectrum of the EPS is shown in Figure 6. Two spectral regions were chosen to analyze obtained spectra of EPS, namely the functional group region (4000–1500 cm^{-1}) and the finger print region for polysaccharides (1800-800 cm^{-1}).

The broad absorption bands exhibited between 3700 and 3050 cm^{-1} are attributed to O-H stretching vibration of hydroxyl groups and bands at ca. 3200 cm^{-1} are assigned to the vibrations hydrogen bonding. Few and small bands from 2850 to 2950 cm^{-1} are assigned to the methyl and methylene signals, it is shown in the peaks around 2960 cm^{-1} and 2926 cm^{-1} indicated the presence of asymmetric stretch C-H of CH_3 group and symmetric stretch C-H of CH_2 group, respectively. Bands present at 1750 and 1250 cm^{-1} that might represent O-acetyl ester linkage bonds. Further, the presence of bands at 1625 cm^{-1}, an asymmetrical stretching peak, which corresponds to carboxyl groups and minor at 1560 cm^{-1}, could be assigned to amide-I and amide-II stretching vibration bands. The absorption associated with the amide I band leads to stretching vibrations of the C=O bond of the proteins, whereas absorption associated with the amide II band leads primarily to bending vibrations of the N–H bond and the peak medium at ca. 1250 cm^{-1} can be assigned C-N stretching coupled with N-H bending [13], [14], which corroborates the presence of protein in the EPS.

The region from 1150-800 cm^{-1} has been attributed to the stretching vibrations of the COH side group and the glycosidic bonds, C-O-C stretching and are also attributed with formation of polymeric chains (CH_3 deformation). It is considered as the ‘fingerprint’ region for carbohydrates, which allows the identification of major chemical groups in polysaccharides, nevertheless the position and intensity of the bands are specific for every polysaccharide [15], [16]. The peak at 1421-1370

cm^{-1} confirmed the presence of C=H stretch. A peak of medium intensity show at 1250 cm^{-1} indicated C–C stretch of skeletal vibrations. Broad and intense band at ca. 1035 arises from stretching O-H coupled with bending C-O [17], [18]. Additionally, the band centered near 1628 cm^{-1} reveals the presence of a small shoulder at ca. 1735cm^{-1}arises from vibration stretching of carboxilic suggesting that neither glucuronic acid and the bands occur at about at 1000 - 1135 cm^{-1} range ascertain the presence of uronic acid, o-acetyl ester linkage bonds. Overall and based on the above, these data suggested a polysaccharide structure for this biopolymer, due to observed similarity in the 'fingerprint' region may indicate that the sample have the basic structure of polysaccharide with presence of protein.

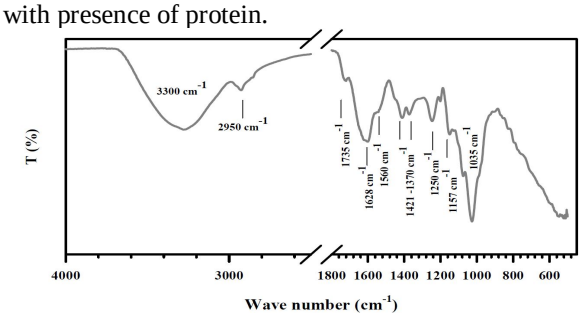


Fig. 5.FTIR spectra for purified exopolysaccharide from Kocuria rosea strain 23B

CONCLUSIONS

From the results previously presented, can be concluded that the wide diversity of microorganisms found in marine environment offers vast potential resources for biotechnological applications. Among them, Kocuria rosea strain 23B, isolated in coasts at Jalisco, México, has proved to be a versatile bacterium able to produce high amounts of EPS.Moreover, this these results refer to the strong character of the biosurfactante/bioemulsifying activityand its usefulness for industrial applications. Work is ongoing to elucidate this EPS complete chemical structure and its potential application in different biotechnology fields.

ACKNOWLEDGMENTS

M.A.L.O. Author to thank CONACYT for the fellowships granted for PhD studies (CVU number 412346).

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