



Emulsifying properties of a glycoprotein extract produced by a marine *Flexibacter* species strain TG382

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ABSTRACT

We report, for the first time, on the production of an emulsifying polymer produced by a *Flexibacter* species (designated strain TG382). This polymer, E-382, was produced extracellularly during growth of the organism in a marine broth amended with glucose. After cold ethanol precipitation, extensive dialysis and lyophilization, a chemical analysis of the resultant dried polymer revealed it to be a glycoprotein composed of 10.9% protein, 23.3% carbohydrate and a 5.5% uronic acid content. At relatively low concentrations (0.02%, w/v), E-382 was found to form oil-in-water emulsions against hydrocarbon and food oils under neutral pH and acidic conditions. The most stable emulsions were formed against the oils sunflower, vegetable and ground nut under neutral pH conditions. Aqueous solutions of the polymer were viscous, and its reduced viscosity (η_{red}) was determined to be 0.54 m³/kg. Although proteins and uronic acids may possess surface-active properties, the viscosifying effect of this polymer, which is a typical feature of some commercial hydrocolloids, is more likely to confer its high emulsion-stabilizing qualities.

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1. Introduction

Surface-active agents are chemical compounds possessing both hydrophobic and hydrophilic domains, allowing them to interact between water-soluble and non-soluble phases [1,2]. Ecologically, these compounds perform important functions in microbial and multicellular systems. Examples include the synthesis of the surfactant hyaluronic acid within the alveoli of human newborn infants for the normal functioning and early development of the lungs [3]; the activities of biosurfactants on the swarming behavior of some bacteria and in promoting their colonization or biofilm formation on biological and non-biological surfaces [4–6]; and the influence of biosurfactants on the dissolution of hydrophobic substrates and in enhancing their bioavailability to hydrocarbon-degrading bacteria [7,8].

Microbial extracellular polymeric substances are one class of surface-active compound that can help form and stabilize emulsions [9]. However, microbial strains found to produce polymeric emulsifiers in low quantities are often given little attention, or discarded as uninteresting candidates for biotechnological development. Such polymers, though, can exhibit very high surface-

activities at relatively low concentrations, which may be attributed to the presence of multi-reactive chemical groups with high affinities for hydrophobic substances. Bioenergetically, microorganisms capable of synthesizing highly reactive polymers would not necessarily need to produce these in large quantities since their function is likely to be met at lower concentrations, consequently saving up to 70% of their energy expenditure [10].

For biotechnological applications, there are obvious economic advantages for using polymers exhibiting high functional activities at low concentrations—a property commonly expressed as a high ‘yield value’ [11]. Polymeric emulsifiers, such as gum arabic, have found extensive uses in a wide range of biotechnological and industrial applications, from healthcare and cleaning products to foods, beverages, pharmaceuticals and textiles [1,2]. However, relatively high concentrations of these polymers may be needed (up to 20%, w/v of gum arabic, for example) in order to achieve optimal functionality [12,13]. The search for new types of amphipathic polymers has gained increasing momentum over recent years, due mainly to a need for ingredients with improved or novel functionalities compared to the current commercial inventory. Those derived from natural sources are of particular interest since they exhibit lower levels of toxicity, higher biodegradability, and are under increasing consumer-demand as natural alternatives compared to their synthetically produced counterparts [1,14].

In this study, we describe the production of a high molecular-weight extracellular emulsifying agent produced by a marine bacterium belonging to the Bacteroidetes phylum, *Flexibacter* sp.

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TG382. The chemical composition, viscosifying effect and ability of this polymer to emulsify a range of oils were investigated with a view to its potential biotechnological application.

2. Materials and methods

2.1. Isolation, screening and identification

Bacteria were isolated by spreading 100 μ L of 10-fold serial dilutions of seawater on solid synthetic seawater (SSW) medium [15] supplemented with NH_4NO_3 and *n*-hexadecane supplied via the vapour phase as the sole carbon source. Colonies were grown in the dark for 3 weeks at 28 °C. Bacteria displaying distinct colony morphologies were streaked onto ZM/10 agar [16] and stored at –80 °C with 30% glycerol. A total of 22 bacteria were isolated and screened for the production of surface-active agents. For this, each isolate was grown in ZM/10 (low nutrient) and ZM/1 (high nutrient) liquid media supplemented with glucose (1%, w/v). Samples of cell-free supernatants (13,000 \times g; 10 min) were taken periodically for tensiometric analysis and emulsification assay (see below). Growth was monitored by absorbance of the cultures at 540 nm (OD_{540}). One isolate, TG382, was selected for further study based on its production of high emulsifying activity. This organism was identified by DNA sequencing of its 16S rDNA genes as described previously [16].

2.2. Emulsifier production and extraction

Strain TG382 was grown in 2 L Erlenmeyer flasks containing 750 mL of ZM/1 medium amended with glucose (1%, w/v). The cultures were incubated (28 °C; 150 rpm) until the cell-free culture liquid produced maximum emulsification index values ($\text{EI} = 100\%$) when tested against *n*-hexadecane. The cells were then removed using vacuum filtration (0.2 μ m; Millipore) and the cell-free filtrate treated with 2 volumes of cold 99% ethanol. The precipitated material was recovered by centrifugation, dialysed (1 kDa) against 20 L of distilled water, and then lyophilized. The resultant dried material was used in all subsequent chemical and physical characterization experiments.

2.3. Emulsification assays

For initial screening, a modified version of the method described by Cooper and Goldenberg [17] was used to measure emulsifier production during the growth of each strain in liquid culture, as well as to measure the ability of the extracted polymers in forming water-in-oil (W/O) emulsions. Samples (0.5 mL) to be tested were introduced into acid-washed (0.1 N HCl) screw-cap glass tubes (100 mm \times 13 mm), and then overlaid with a 0.45 mL volume of *n*-hexadecane. The tubes were manually shaken (15 s) and vortexed (15 s) vigorously to homogeneity, allowed to stand for 10 min, shaken again as before, and then allowed to stand at 21 °C. The height of the emulsion layer was then measured after 24 h and expressed as a percentage of the total original height of oil in the tube—i.e. emulsification index (EI_{24}).

The production of the E-382 emulsifier during growth and its ability, to form oil-in-water (O/W) emulsions was determined using a modified version of the method described by Cirigliano and Carman [18]. For this, cell-free culture liquid samples or aqueous solutions of the emulsifier (0.02%, w/v) were mixed with the test oil (15%, v/v) in the same way as described above. The mixtures were allowed to stand for 10 min prior to taking turbidity measurements of the bottom layer using a spectrophotometer at 540 nm. Triplicate readings were taken every 10 min for up to 60 min, and the log of these plotted against time. The slope of the curves generated was calculated and expressed as the decay constant (K_d) which denotes the stability of the emulsions formed, as previously described [19]. The emulsifying activity (A_{540}) was recorded after allowing the emulsions to stand for 24 h. All K_d and A_{540} values were expressed as the average of triplicate experiments. Activities were compared under neutral (0.1 M potassium phosphate buffer, pH 7.0) and acidic (0.1 M sodium acetate buffer, pH 3.5) conditions. Emulsifying activities were also determined after the polymer was treated at 121 °C for 15 min prior to emulsification with the various oils.

2.4. Chemical analysis

Total carbohydrate was assayed as described previously by Dubois et al. [20]. Total protein concentration was determined using the BCA protein assay kit (Sigma, St. Louis, MO) with bovine serum albumin as the standard. Lipid analysis was performed by GC-MS, as described previously [21]. Total uronic acid content was determined using the method of Cesaretti et al. [22].

2.5. Measurement of surface tension and viscosity

A NIMA DST-9005 surface tensiometer (NIMA Technologies, Coventry, UK) was used to measure the surface tension of cell-free supernatant samples taken during the growth of TG382 in liquid culture, as well as on solutions of the extracted polymer, E-382, dissolved in water at a final concentration of 1 mg/mL. A 5 mL volume of each solution to be measured was put into a clean well of a Teflon carousel and placed onto the tensiometer platform. A platinum wire ring was submerged into the solution and then slowly pulled through the liquid–air interface, to measure the

surface tension (mN/m). Between each measurement, the platinum wire ring was rinsed with water, chloroform and then allowed to dry. The viscosity of E-382 was measured using an Ostwald PST viscometer (Dannon-Fenske). All determinations were carried out at 21 °C using a concentration of 1 mg/mL of the polymer dissolved in distilled water.

2.6. Statistical analysis

For the emulsification assays, a one-way analysis of variance (ANOVA) was used for each pH and oil type combination (Tukey's test) to determine if any significant difference ($p \leq 0.05$) was measured in the activity and stability values produced by E-382.

3. Results and discussion

During screening for surface-active agents in marine bacteria, strain TG382 was selected for its ability to produce excellent emulsification against *n*-hexadecane during its growth in liquid medium. This strain was characterized as a gram-negative heterotrophic bacterium that produces irregular round, convex orange colonies that were semi-mucoid to sticky on solid medium. 16S rRNA gene sequencing identified TG382 to belong to the Bacteroidetes phylum within the genus *Flexibacter* [23]. Its GenBank accession number is FJ502131.

TG382 grew abundantly in ZM/1 medium, but high emulsification values were only produced when this medium was supplemented with glucose. As shown in Fig. 1, emulsifier production and a gradual increase in the pH of the medium were coupled to growth. Although emulsifier production occurred during growth, maximum emulsification values were only obtained at late stationary phase of growth (56 h). The emulsions formed, which were produced against *n*-hexadecane, remained stable, even after leaving them standing for several weeks at room temperature (results not shown). Since the surface tension of the culture medium remained relatively constant at 67.4 ± 1.5 mN/m, it was concluded that, under the growth conditions used, TG382 did not produce extracellular

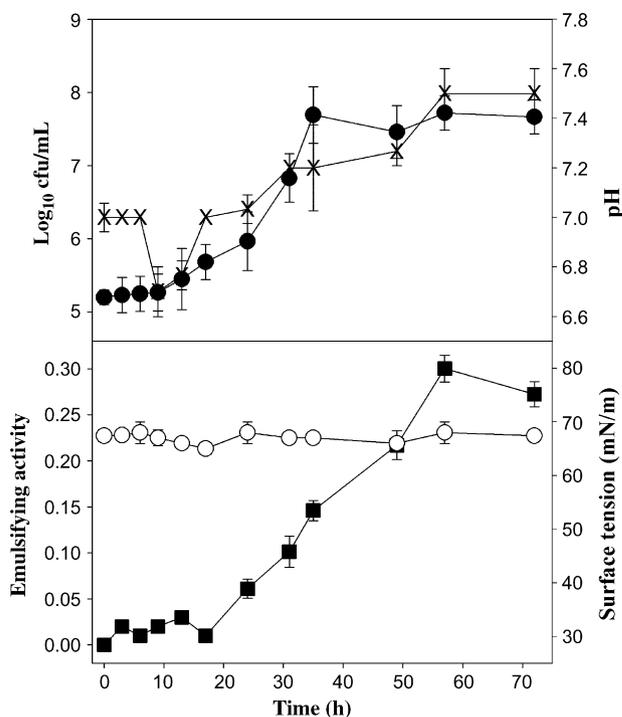


Fig. 1. Growth and emulsifier production by strain TG382 in ZM/1 liquid medium amended with 1% (w/v) glucose. Emulsifying activities and surface tension values were derived from cell-free culture broth after the removal of cells by centrifugation. Solid circles, Log_{10} cfu/mL; \times symbols, pH; empty circles, surface tension; solid squares, emulsifying activity.

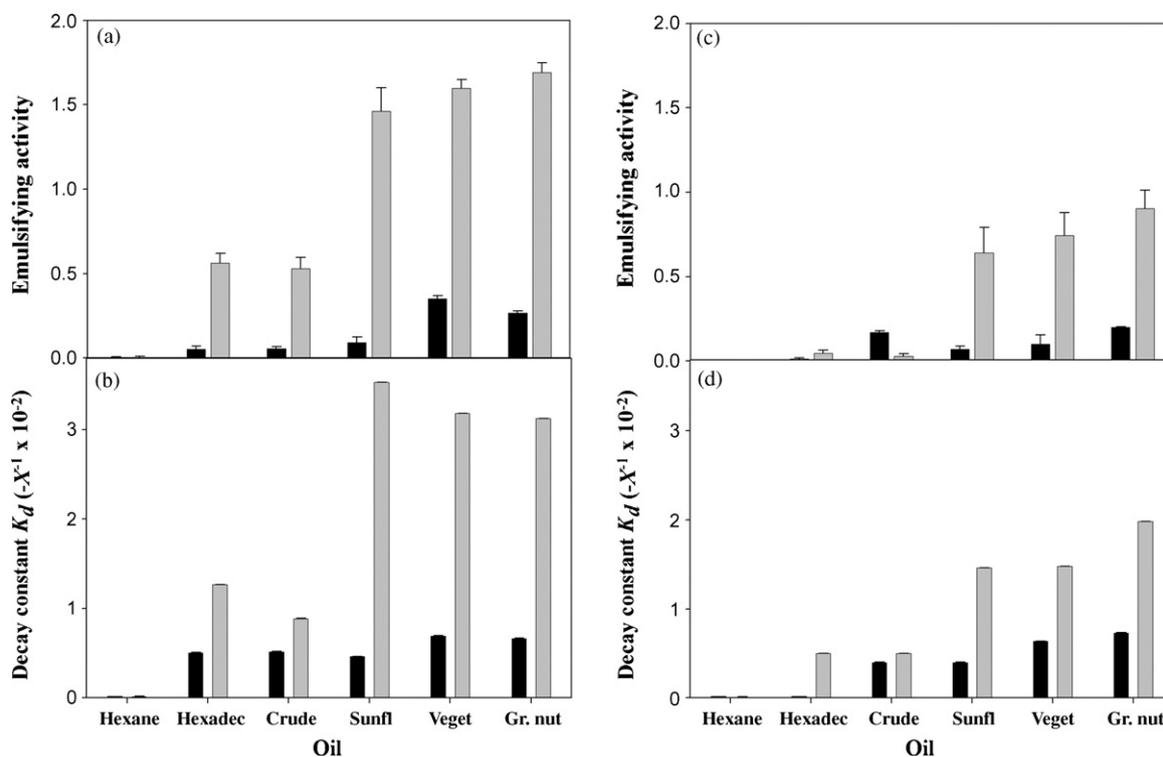


Fig. 2. Emulsifying activities and corresponding decay constants at pH 7.5 (a and b) and pH 3.5 (c and d) for O/W emulsions prepared using E-382 against the oils hexane, hexadecane, light crude oil, sunflower, vegetable and ground nut. Solid bar, untreated control; grey bars, E-382.

products exhibiting surfactant activity. Some bacteria produce low molecular-weight surfactants that lower the interfacial and/or surface tension of liquids [24], whereas others, like TG382, are found to produce high molecular-weight surface-active agents, or emulsifiers that primarily act to form stable oil–water emulsions [9]. This extracellular emulsifying agent was easily extracted from the cell-free spent medium by cold ethanol precipitation. After extensive dialysis and subsequent lyophilization, the average dry-weight yield of this emulsifying agent, E-382, was 0.32 g/L under non-optimized conditions.

The total percent carbohydrate and protein content of E-382 were found to be 23.3% and 10.9%, respectively, indicating that the polymer is a class of glycoprotein. No fatty acids were detected by lipid analysis. Interestingly, approximately 65% of the polymer remained unaccounted for in our chemical analysis. This feature, which is not uncommon to other bacterial exopolysaccharides, may be attributed to the presence of uronic acids [25,26] or glycosidic linkages of hexosamines [27] that can render polysaccharides highly resistant under acid hydrolysis conditions.

Concentrations of E-382 (0.01–0.2%, w/v) dissolved in water did not have any effect on the surface tension of water (72.1 mN/m at 21 °C). However, the viscosity of water was markedly increased. At concentrations of 0.02%, the reduced viscosity (η_{red}) was measured to be 0.54 m³/kg, representing a higher viscosity than that of widely used hydrocolloid emulsifiers such as gum arabic (0.17 m³/kg) and carboxymethyl cellulose (0.39 m³/kg) when measured under the same experimental conditions [28,29]. The ability of E-382 to emulsify oils whilst also increase viscosity suggests its potential to be developed as a surface-active thickening agent in the production of certain foods, drinks and healthcare products [30,31]. One main advantage of E-382 is its ability to dissolve readily in water and in various buffered solutions of different pH (3.0–7.5) to produce solutions of very good clarity (not shown). Some commercial emulsifiers, particularly those with a lipid component (e.g. lecithin), tend to form cloudy solutions that have limited their application.

Fig. 2 shows the emulsifying activities and corresponding decay constants (K_d) of E-382 when tested against different oils under neutral (a, b) and acidic (c, d) conditions. Multiple one-way ANOVA analysis was used to identify significant differences ($p \leq 0.05$) in emulsification activities and K_d values as contributed by the type of oil used and pH treatment. The polymer produced significantly higher emulsifying activities against the three food oils (sunflower, vegetable and ground nut) under neutral pH conditions when compared to their respective untreated (no polymer) controls (Fig. 2a). These activities were approximately 16.0-, 4.6- and 6.4-fold higher against these oils compared to their respective controls. The K_d values measured for these emulsions were also significantly higher (Fig. 2b), indicating the emulsion-stabilizing properties of E-382. Compared to their respective controls, these values were 7.7-, 4.6- and 4.7-fold higher against sunflower, vegetable and ground nut oil. Using the same emulsifying assay method employed in this study, we previously reported on the emulsifying activities of the commercial hydrocolloids xanthan gum and gum arabic against these food oils [28]. Compared to xanthan gum, E-382 produced higher activities against sunflower and vegetable oil, whereas similar activities were produced by both these emulsifiers against ground nut oil. When compared with gum arabic, E-382 produced higher activities against sunflower oil, whereas vegetable and ground nut oil were similarly emulsified by both these emulsifiers. With respect to emulsion-stabilizing properties, E-382 produced similar K_d values to those previously reported for xanthan gum and gum arabic [28] when these emulsifiers were tested against sunflower and vegetable oil. However, when tested against ground nut oil, E-382 produced emulsions that were at least 3 times more stable than those produced by either of these commercial emulsifiers.

Acidic conditions, however, produced an overall negative effect on the polymer's ability to emulsify all the oils tested (Fig. 2c) and to stabilize these emulsions (Fig. 2d). These inhibitory effects on emulsification under low pH conditions have previously been reported

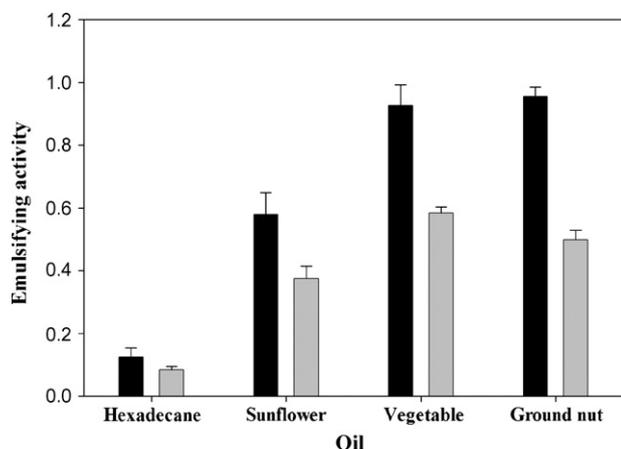


Fig. 3. Effect of heat treatment on the emulsifying activity of E-382 against hexadecane and some food oils. Black bars, non-heat treated control; grey bars, E-382 heated at 121 °C for 15 min prior to emulsification.

using other marine bacterial emulsifiers [32], and which may be explained by the protonation of carboxyl groups on these polymers [33] and/or of free fatty acids present in the food oils that can cause a reduction to the emulsifying potential of these polymers or ability of the oils to interact with them.

To test the heat stability of E-382, solutions of the polymer were treated to 121 °C for 15 min and then used to emulsify *n*-hexadecane and the three food oils. As shown in Fig. 3, heat treating the polymer produced a loss of 35%, 37% and 48% of its emulsifying activity against the oils sunflower, vegetable and ground nut, respectively. Although no significant reduction in these activities was measured against *n*-hexadecane, the activity of the control against this oil was already low. We observed that heat treatment at 121 °C had little effect on the viscosifying effect of the polymer (not shown). As discussed above, E-382's relatively good thermo-tolerance may be attributed to certain chemical groups that endow it with protection from hydrolytic degradation. This is a useful property for many commercial applications that involve surface-active or viscosifying agents in formulations subjected to high temperature treatments.

There are two general mechanisms to explain how water-soluble high molecular-weight molecules, like E-382, can impart an emulsion-stabilizing effect on oil-in-water emulsions: (1) they can act as non-adsorbing macromolecules, serving to increase the viscosity of the continuous phase or (2) they can adsorb to the oil–water interface, as may be mediated by amphiphathic proteins or hydrophobic moieties on the polysaccharide chain. The emulsion-stabilizing properties of xanthan gum, for example, are defined by process 1, whereas both mechanisms (1 and 2) help explain how the polysaccharide indican, a gum produced by *Beijerinckia indica*, is able to form highly stable emulsions [34]. The proteinaceous component of the plant-derived hydrocolloid gum arabic [12,35–37] and of the bacterial emulsifier emulsan [38] has also been shown to play an active role in the emulsification of oils. Uronic acids have also been suggested to contribute emulsion-stabilizing properties to polysaccharides, as has been reported for some galactomannans [39,40] and xanthan gum [41]. Although the content of these acids in E-382 (5.5%) was not as high as that often found in many exopolysaccharide fractions from marine bacteria (20–50%) [42,43], these acids may contribute some role in the emulsion-stabilizing properties of this polymer. Although further work will be required to elucidate the mechanisms conferring E-382 with its high emulsion-stabilizing properties, its viscosifying effect is likely to play an important role in this process.

4. Conclusion

We have shown that this new high molecular-weight glycoprotein exhibits potential for use in applications where surface-active ingredients and viscosity builders possessing unique, or enhanced, properties are in demand. The multi-functionality of E-382 as an emulsifier, stabilizer and viscosifying agent is advantageous commercially as it could help to reduce the number of ingredients needed to be added to process formulations. In addition, the ability of E-382 to dissolve in different solvents to form clear and non-cloudy solutions is also a useful property for the manufacturing of consumer goods that require a transparent end product—e.g. certain healthcare products such as gels.

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