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# Evaluation of antioxidant activity of emulsan *in vitro*

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## 1. INTRODUCTION

Oxidative stress has been involved in the development of several pathologies, including vascular damage associated to myocardial and neurological degeneration, such as arteriosclerosis, cerebral ischemia among others; while diabetes, rheumatoid arthritis, inflammation, cancer-initiation, and acceleration of the aging processes are also reported (Coyle and Puttfarcken, 1993; Margail *et al.*, 2005). The toxicity of oxidative stress is believed to be caused by Reactive Oxygen and Nitrogen Species (ROS and RNS, respectively) that can initiate a wide range of oxidative toxic reactions in biological systems (Cuzzocrea *et al*., 2001). Typical radicals and radical-related toxic molecules produced by ROS are hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$), and superoxide anion radical ($O_2^{\bullet -}$) (Figure 1). The RNS toxic reactants include nitric oxide ($NO^{\bullet}$) and the most powerful oxidant peroxynitrite anion ($ONOO^-$). Biological antioxidant mechanisms described in the literature are many, but with different efficiencies implicating enzymatic as well as non-enzymatic activities. Some of the most relevant antioxidative biocatalysts are superoxide dismutase, and catalase. In addition, other specialized enzymes such as glutathione peroxidase and γ-glutamylcysteine synthetase can be involved in deactivation mechanisms (Figure 1). Well-known non-enzymatic antioxidants are β-carotene, glutathione, melatonin and vitamins C and E, (Valko *et al*., 2006). However, excessive and persistent formation of free radicals could be main factors of genotoxic effects.

Antioxidants can be defined as molecules that either directly scavenge or indirectly reduce reactive species to inactive or at least less reactive molecules (Berg *et al*., 2004). Prevention of oxidative reactions in foods, pharmaceuticals and cosmetics and the management of oxidative stress-related diseases are some of the potential applications of antioxidants. The common antioxidants used to prevent the oxidation of lipids in foods are butylated hydroxyanisole, propyl gallate and 2-tert-butylhydroquinone (Moure *et al*., 2001). However, there has been growing concern over the safety of

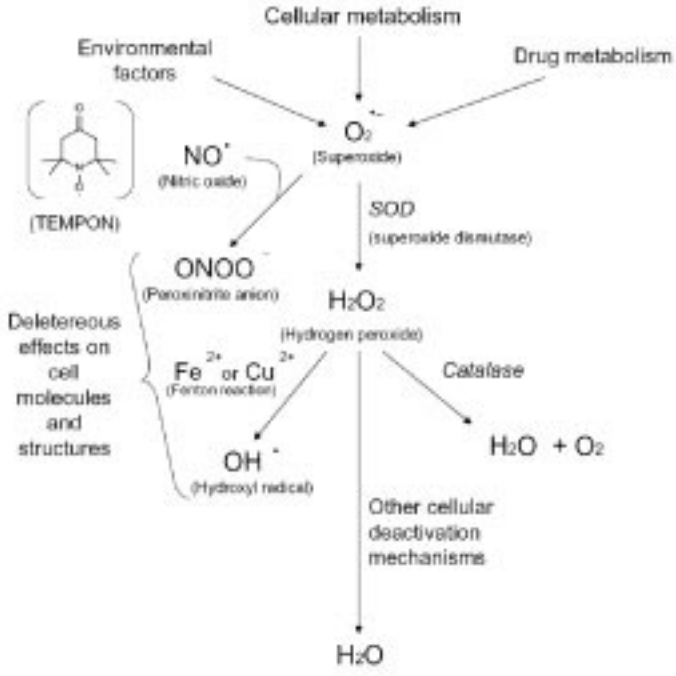


**Fig. 1:** Molecules involved in the oxidative stress of the cell and main detoxification mechanisms.

synthetic antioxidants because of issues about carcinogenic potential have been reported (Witschi, 1986; Williams *et al.*, 1999). Therefore, interest in alternative and safe antioxidants from natural sources is increasing for use in foods as well as for preventive and therapeutic medicines.

Biopolymers are increasingly in demand at the industrial level for use in many products and a wide ranges of applications such us pharmaceuticals, foods, plastics, cosmetics, remediation of toxic compounds, among others. The major reasons for biopolymers for these uses are: (a) they are based on annually renewable sources and abundantly available in the nature; (b) they are biodegradable and their production, use and disposal do not present environmental problems; (c) they tend to be safe for human use as they are natural materials; (c) the production could be scaled up without costly changes of infrastructure and equipment (Kaplan, 1998). Currently, a number of microbial polysaccharides are produced at large scale from different sources, from micro-organisms to marine algae, and plants. The most important microbial polysaccharide at industrial scale, xanthan, is synthesized by *Xanthomonas campestris*, but other relevant polymers from microbial origins include dextran, alginate and pullulan. Generally, exopolysaccharides produced by micro-organisms at large scale are widely used in food, pharmaceutical and chemical industries, and are currently used as bioflocculants, bioabsorbents, heavy metal removal agents, food stabilizers and thickeners, drug delivery agents, and related needs (Steinbóchel, 2004). Particularly, emulsan is an anionic exo-lipopolysaccharide complex synthesized by *Acinetobacter venetianus* RAG-1, a Gram-negative bacterium. The polysaccharide complex includes amino sugars, and O-acyl and N-acyl bound fatty acid side chains with a length of 10 to 20 carbons, representing

5-23 % of the polymer. The combination of hydrophilic anionic sugar main chain repeat units, along with the hydrophobic side groups leads to the amphipathic behaviour of the emulsan complex and, therefore, its ability to form stable oil-in-water emulsions. During the past years, studies in our laboratory have demonstrated many potential applications of emulsan as an adjuvant in vaccination (Panilaitis *et al.*, 2002), as a smart-carrier for protein delivery and triggered release, and as immune enhancer (Castro *et al*., 2006 and 2007), and for protein adsorption (Castro *et al*., 2008). Also, the ability to tailor the biopolymer and concomitantly change its properties using simple feeding procedures is another relevant advantage of emulsan (Panilaitis *et al*., 2007).

In the present work, the potential scavenging activity of emulsan *in-vitro* was explored using ROS and RNS produced by the Fenton reaction, and TEMPON radicals respectively, and coupled with Rose Bengal as a dye reporter. The effect of pH, temperature, ionic strength (NaCl) and emulsan concentration were also evaluated.

## 2. MATERIALS AND METHODS

### 2.1. Reagents

Chemicals and solvents were of analytical grade, and other reagents and microbiological media were of highest available grade obtained form Aldrich or Sigma (St. Louis, MO), and Difco (Franklin Lakes, NJ) used as provided. Rose Bengal diacetate (4,5,6,7-tetrachloro 2',4',5',7'-tetraiodo diacetate, a derivative of fluorescein) was purchased from Molecular probes.

### 2.2 Radical formation

The Fenton reaction ($Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + {}^{\bullet}OH + OH^-$), a well-known ${}^{\bullet}OH$ generator was utilized to examine whether emulsan scavenge ${}^{\bullet}OH$ (Zang *et al.,* 1998). The reaction mixture contained $FeSO_4$ (100 μM), and $H_2O_2$ (10 mM), in the presence or absence of emulsan at a final volume of 1.00 ml. For RNS scavenging activities, solutions of 4-oxo-2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPON) were dissolved in osmosis purified distilled $H_2O$ or 50% methanol filtered through 0.22 μm pore membrane and used immediately. The presence of radicals in solution was detected spectrophotometrically by 0.1 % Rose Bengal (RB) dye as reporter at 547 nm.

### 2.3 Bacterial cultivation and emulsan production

*Acinetobacter venetianus* strain RAG-1 (ATCC 31012) was maintained on Luria Bertrani (LB) agar slants covered with LB medium to prevent cell dehydration at 5ΊC. Emulsan synthesis by *A. venetianus* strain RAG-1 was produced in saline medium supplemented with ethanol. Extracellular emulsan was purified by centrifugation, ammonium sulfate precipitation; phenol extraction followed by extensive dialysis as previously reported (Panilaitis *et al*., 2006).

### 2.4 Statistics

Two or three independent experiments were conducted with a minimum of duplicates (N = 2) or triplicates (N = 3) for each data point. Data for these measurements were analyzed using a Student's

t-test. Statistically significant values were defined as $p < 0.05$. Data in the graphs represent the mean ± standard deviation.

## 3. RESULTS AND DISCUSSION

In preliminary experiments, screening one dozen dyes sensitive to radicals able to absorb in the visible range showed no interaction with emulsan was performed (data not shown). Rose Bengal (RB) was selected because maximum absorption wavelength in the green zone of the electromagnetic radiation spectrum, and no changes of absorbance at 547 nm, were observed when 1,500 mg/ml of emulsan was added to the dye solution. In the presence of radicals produced by the Fenton reaction or TEMPON, the absorbance of RB at 547 nm decreased with time, producing a colourless solution (data not shown). It has been previously reported that the anion radical $RB^{\bullet -}$ disproportionates in the presence of light to the colorless reduced form of RB (Singh *et al*., 1995). Similarly, RB reacts with of ROS or RNS in solution to give a colorless solution, which can be attributed to the reduced form of RB.

Figure 2 shows the dose-dependent inhibition of RB-induced degradation of adsorption at 547 nm by emulsan. RB dye absorption showed hyperbolic decay versus time in the presence of Fenton reagents and the absence of emulsan. Emulsan scavenged radicals in a concentration-dependent manner revealed by the RB absorption decay that was inversely proportional to the biopolymer concentration. Emulsan concentrations equal or higher than 500 µg/ml quenched all radical activity, keeping the RB absorbance almost constant (Figure 2).

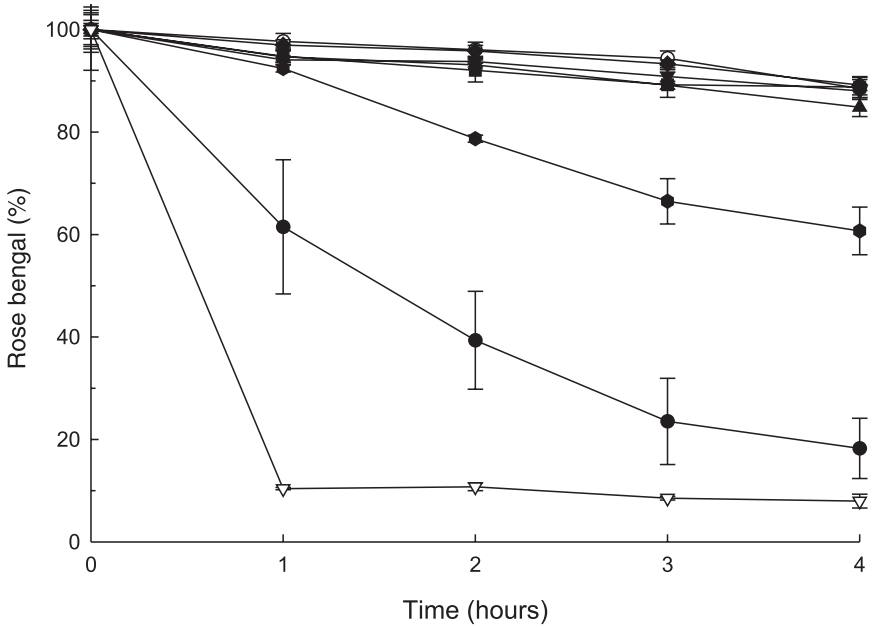


**Fig. 2**: Effect of emulsan concentration on scavenging activity of Fenton reaction using Rose Bengal as probe. Symbols: ○, RB dye (control); emulsan: ●, 50 µg/ml; ⬢, 250 µg/ml; ▲, 500 µg/ml; ◆, 750 µg/ml; ■, 1,000 µg/ml; ▼, 1,500 µg/ml; ▽, Fenton reaction without emulsan

Dependence of the scavenging activity of emulsan on pH using the Fenton reaction was performed in the range of pH 4.50 to 5.75 with and without 250 μg/ml emulsan at 37ºC for 4 h of incubation (Figure 3). RB adsorption was almost stable along the studied range in the presence of emulsan, keeping the dye concentration between 82.5 to 99.8% compared to the control (91.8 to 95.7%) at time zero. However, The Fenton reaction renders dye decay between 82.3 to 96.8% depending on pH. At pH 5.75, the RB decay was about 30.2 % in absence of emulsan, indicating that the Fenton reaction is strongly influenced by the pH.

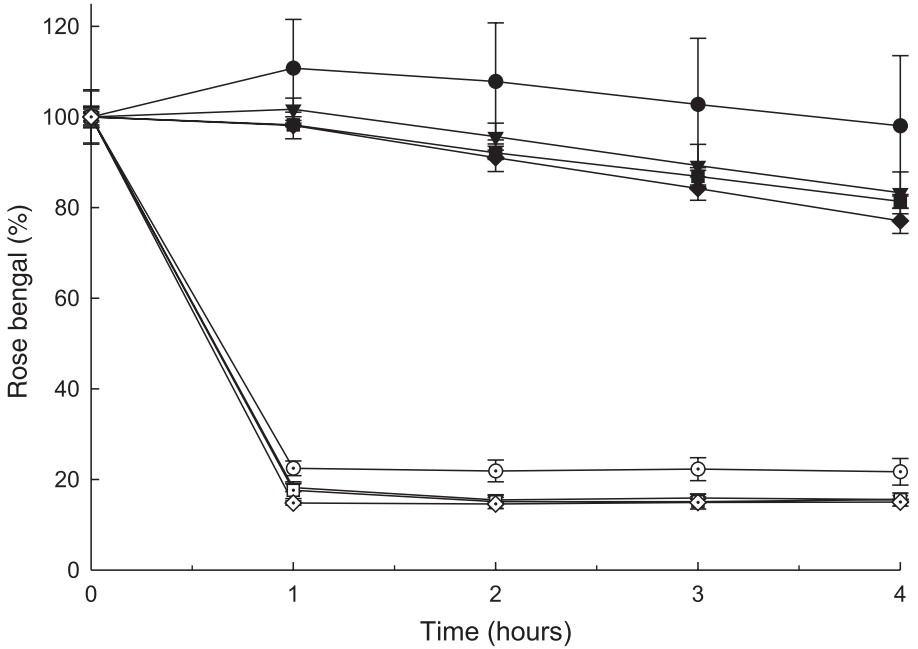


**Fig. 3**: Effect of pH on emulsan (1.00 mg/ml) scavenging activity of Fenton reaction using Rose Bengal as probe. Symbols: ○, RB dye (control); ■, emulsan added; ▽, Fenton reaction without emulsan.

The effect of temperature was studied in the range of 20 to 42ºC at pH 5.0 over 4 hours of incubation in presence or absence of emulsan (500 μg/ml). During the first two hours of incubation no differences were found between RB control (no Fenton reaction) and RB in the presence of emulsan at all temperatures. At temperatures higher than 34ºC and incubations longer than 3 hours, absorption differences between RB control (no Fenton reaction) and RB containing emulsan increased with the temperature in the range of 3.7 to 17.6%, indicating spontaneous dye decay to colorless molecules, probably a reduced form of RB (Singh *et al*., 1995). The decay of RB in the presence of emulsan increases about 0.9 to 1.3 % per degree centigrade after 34ºC, and 0.1% per minute after 3 hours incubation. Meanwhile, discoloration of the RB solution by the Fenton reaction without the protective action of emulsan was about 90% or higher at all temperatures tested (data not shown). The better scavenging activity of emulsan could be explained by the increase of polymer flexibility, by changing polymer conformation and exposing reactive groups from inside the molecule.

The effect of 10 to 1,000 mM sodium chloride on the RB inactivation by Fenton reaction was analyzed in presence or absence of emulsan (1.00 mg/ml) at 37ºC and 4 hours of incubation (Figure 4). The net effect of increasing the ionic strength of the solution enhances the stabilizing effect of emulsan on the RB tracer against the Fenton reaction. This effect could be interpreted as the decrease of the number of radical effective collisions to RB with the increase of ionic strength. The difference is more notable in vials containing emulsan, and could be attributed to enhanced hydrophobic interactions between the dye and the hydrophobic moieties of emulsan.

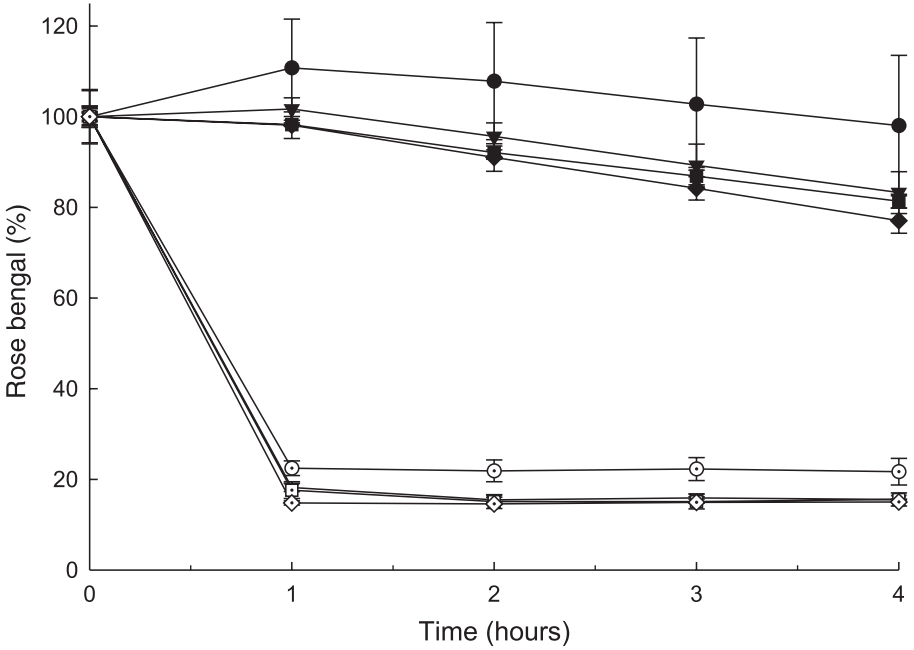


**Fig. 4**: Effect of NaCl (0 to 1.0 M) on emulsan (1.00 mg/ml) scavenging activity to the Fenton reaction using Rose Bengal as probe. Symbols: ●, ◆, ■, ▼, emulsan plus NaCl 1.0 M, 100 mM, 10 mM, 0 mM respectively; ○, ◇, □, ▽, Fenton reaction without emulsan plus NaCl 1M, 100 mM, 10 mM, and 0 mM respectively.

In order to study the capability of emulsan to scavenge RNS, TEMPON was used as model for the production of $NO^{\bullet}$ (nitroxile radical) (Bunyatova, 2004). Figure 5 displays the radical scavenging activity of emulsan on TEMPON traced by RB. The increase of emulsan concentration can be correlated with RB absorbance stability. However, a similar profile of RB decay was observed when the emulsan concentration was kept constant at 250 μg/ml and the TEMPON concentration was in the range of 1.0 to 1,000 mM (data not shown).

The effects of temperature between 20 to 42ºC, and ionic strength (1.0 to 1,000 mM of NaCl) on the scavenging activity of emulsan on TEMPON showed similar hyperbolic decay profiles to those displayed

in Figures 4 and 5 with the Fenton reaction. In contrast, the presence of emulsan in solution stopped the dye decay produced by the radicals (data not shown).

In conclusion, emulsan was able to scavenge both types of radicals ROS and RNS (and also inorganic and organic ones) effectively. Emulsan activity is probably based in three different types of (ion-radicals)-emulsan mechanisms: free radical scavenging and ion chelation ($Fe^{+3}$, TEMPON) (Zosin *et al*., 1983; Castro *et al*., 2008); peroxidation of fatty acids covalently linked to the biopolymer as in the cell membrane (Dix and Aikens, 1993); and breakage of the emulsan backbone (amino-sugar oxidation) as previously reported for other polymers (Schweikert *et al.*, 2000). Our results indicate that in addition to the known immunopotentiating activity of emulsan (Panilaitis *et al*., 2002), it could play a role in reducing genotoxicity induced by radical-producing anti-neoplastic drugs during cancer chemotherapy. Combining the advantages of tailoring, immunomodulation activity, adsorption of proteins and high sensitivity to environmental factors, as well as scavenging activity makes emulsan a useful biopolymer candidate for biomedical applications such as adjuvant in vaccination and drug delivery.

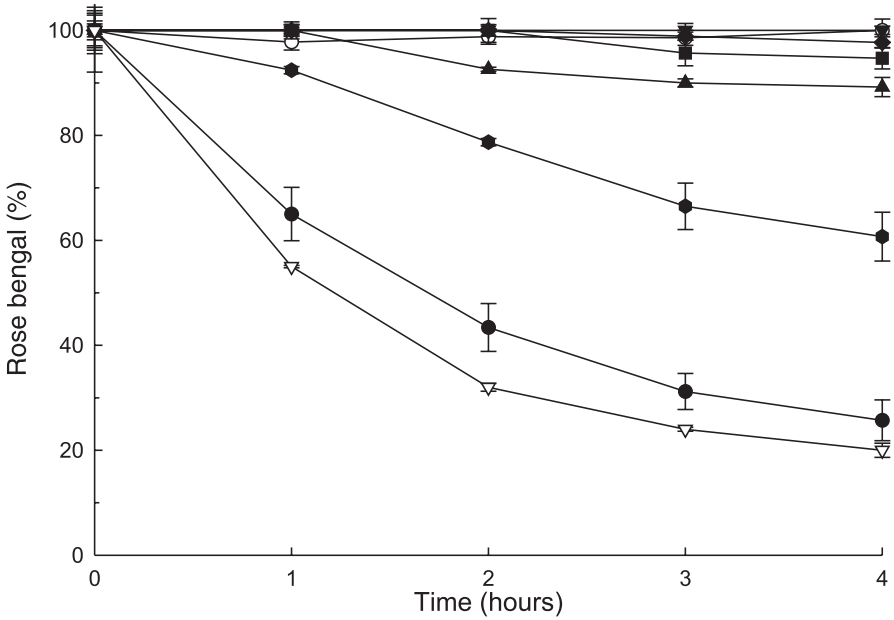


**Fig. 5**: Effect of emulsan concentration on scavenging activity of TEMPON reaction using Rose Bengal as probe. Symbols: ○, RB dye (control); emulsan: ●, 50 µg/ml; ⬢, 250 µg/ml; ▲, 500 µg/ml; ◆, 750 µg/ml; ■, 1,000 µg/ml; ▼, 1,500 µg/ml; ▽, TEMPON without emulsan.

## 4. SUMMARY

The scavenging activities of emulsan against reactive oxygen and nitrogen species (ROS and RNS, respectively) were investigated *in vitro* using Rose Bengal as reporter. Emulsan, a biopolymer complex produced by *Acintobacter venetianus* strain RAG-1 was produced, purified and supplemented to Rose Bengal solutions containing ROS produced by the Fenton reaction or Tempon as an RNS model. The

effect of pH, temperature and ionic strength was studied in both ROS and RNS systems. Emulsan was found active in scavenging ROS and RNS in a dose dependant manner. The results show that emulsan scavenging activity is consistent under broad range of experimental conditions.

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