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Structural Analysis and Antioxidant Activity of a Biosurfactant Obtained from *Bacillus subtilis* RW-I

[*Bacillus subtilis* RW-I 'den Elde Edilen Biyosürfaktanın Yapısal Analizi ve Antioksidant Aktivitesinin Belirlenmesi]

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ABSTRACT

Aim: In this study, the antioxidant activity of a biosurfactant produced by *Bacillus subtilis* RW-I isolated from refinery wastewater was studied.

Materials and Methods: The crude biosurfactant was isolated from the cell free broth of *Bacillus subtilis* RW-I culture. The structural determination and further purification of biosurfactant was investigated with HPLC and FTIR analysis. Antioxidant activity of biosurfactant was tested with reducing power, ferrous ion chelating and 1,1- diphenyl-2-picryl hydrazyl (DPPH) scavenging activity.

Results: The peaks obtained in HPLC and FTIR spectrum were very similar to standard lipopeptide, surfactin. Ferrous ion chelating and DPPH scavenging activity of biosurfactant at 1.0 mg/mL concentration were found as 75.3% and 85.2%, respectively. These values were lower than those found in control test substance as butylated hydroxy toluene (BHT) and EDTA. Biosurfactant at 2.4 mg/mL concentrations possessed up to 1.19 weaker reducing power than BHT. These lower values can be caused by lower contents of hydroxyl groups than those present in the control.

Conclusion: The results indicate that biosurfactant obtained from *B. subtilis* RW-I has the antioxidant capacity to scavenge free radicals and that these results suggest that the biosurfactant could be used as alternative natural antioxidants after toxicological examination.

Keywords: biosurfactant, reducing power, ferrous ion chelating assay, 1,1-diphenyl-2-picryl hydrazyl scavenging activity

ÖZET

Amaç: Bu çalışmada rafineri atık sularından izole edilen *Bacillus subtilis* RW-I tarafından üretilen biyosürfaktanın antioksidan aktivitesi araştırılmıştır.

Gereç ve Yöntem: Biyosürfaktan izolasyonunda 72 saatlik *Bacillus subtilis* RW-I kültür sıvısı kullanılmıştır. Yapısal analiz ve ileri saflaştırma prosesleri HPLC ve FTIR analizleri ile gerçekleştirilmiştir. Biyosürfaktanın antioksidan aktivitesi indirgeme gücü, demir şelatlama ve 1,1-difenil-2-pikril-hidrazil süpürme aktivitesi incelenerek belirlenmiştir.

Bulgular: HPLC ve FTIR analizlerinde elde edilen pikler kontrol amaçlı kullanılan lipopeptid yapıdaki surfaktin ile oldukça benzerlik göstermektedir. 1.0 mg/mL biyosürfaktan konsantrasyonunda demir şelatlama ve 1,1-difenil-2-pikril-hidrazil süpürme aktivitesi sırası ile %75.3 ve %85.2 olarak bulunmuştur. Bu değerlerin kontrol test maddeleri olarak kullanılan bütillendirilmiş hidroksi toluen (BHT) ve EDTA ile elde edilen değerlerden daha düşük olduğu belirlenmiştir. 2.4 mg/mL konsantrasyonunda biyosürfaktanın indirgeme gücünün BHT'ye kıyasla 1.19 kat düşük olduğu belirlenmiştir. Bu sonuçlar biyosürfaktanın içerdiği hidroksil gruplarının kontrollere kıyasla daha düşük olması ile açıklanabilir.

Sonuç: Bu sonuçlar ile *Bacillus subtilis* RW-I tarafından üretilen biyosürfaktanın serbest radikalleri süpürebilecek antioksidan aktiviteye sahip olduğu belirlenmiştir ve bu sonuçlar toksikolojik değerlendirme sonrasında biyosürfaktanın alternatif doğal antioksidan olarak kullanılabileceğini göstermektedir.

Anahtar kelimeler: biyosürfaktan, indirgeme gücü, demir şelatlama analizi, 1,1-difenil-2-pikril-hidrazil süpürme aktivitesi

Introduction

The formation of potentially toxic compounds caused by the oxidative deterioration of lipids in foods is responsible for the decrease in food quality and safety. It is necessary to suppress lipid peroxidation in food in order to preserve nutritional value [1,2]. Antioxidant supplementation to foods is the most effective way for delaying the lipid peroxidation. Antioxidants can increase shelf life by retarding the process of lipid peroxidation when added to food products [3,4]. Butylated hydroxytoluene (BHT) and derivatives are widely used as synthetic antioxidant compounds. However, it has been suggested that these compounds have some side effects such as carcinogenicity [5,6]. Therefore, determination of the natural antioxidants source is important. A lot of studies have analyzed the antioxidant potential of a wide variety of substances, such as legumes [7], seeds [8], flavonoids [9] and teas [10-12]. Some of these natural products have well-proven antioxidant activities.

The microbial surfactants called biosurfactants are microbial compounds with a distinct surface activity that exhibit a broad diversity of chemical structures such as glycolipids, lipopeptides and lipoproteins, lipopolysaccharides, phospholipids, fatty acids and polimeric lipids [13,14]. Therefore, it is reasonable to expect diverse properties and physiological functions of biosurfactants such as increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing and biofilm formation [15]. A host of interesting features of biosurfactants have led to a wide range of potential applications in the medical field. They are useful as antibacterial, antifungal and antiviral agents, and they also have the potential to be used as major immunomodulatory molecules and adhesive agents [16]. Because there is limited information published to prove the biological activities of biosurfactants, in this research we attempt to determine the possible antioxidant activity of a biosurfactant isolated from *B. subtilis*. Antioxidant activity of biosurfactant was investigated with reducing power, ferrous ion chelating and 1,1- diphenyl-2-picryl hydrazyl scavenging activity. These methods are easy, rapid and practical for the routine measurement of total antioxidant activity. And also small amount of sample is needed for analyses and also the results are comparable with the reference [17].

Material and Methods

Microorganisms and biosurfactant isolation

B. subtilis RW-I was isolated from water samples collected from coasts of Kızılırmak contaminated with Kırıkkale Refinery wastes. The method of serial dilutions of the sample (0.1 mL) was inoculated on nutrient agar plates and incubated at 37 °C for 24 h. After this period the selected colonies with different properties were purified by repeated inoculation on Mac. Conkey Agar (MCA) at 30°C for 48 h. Some biochemical tests were applied to bacterial strains obtained from different colonies. By using biochemical tests results, *B. subtilis* RW-I was identified with API 20 E System (Api Biomérieux SA, France) & VITEK 2 analyzer (Biomérieux SA, France). Isolate was grown aerobically in 250 ml mineral salt medium, containing 0.1% (w/v) (NH4)2SO4, 0.025% (w/v) MgSO4, 1% (w/v) glucose, 0.5% (w/v) NaCl in 100 mM potassium phosphate buffer pH 7.0, and 0.1% (v/v) trace metals solution in 250-ml Erlenmeyer flask at 35°C and 200 rpm for 7 d [18,19].

To isolate the biosurfactant, *B. subtilis* RW-I culture was centrifugated and the supernatant was precipitated overnight at 4°C by adding concentrated HCl to achieve a final pH of 2.0, in order to precipitate lipids and proteins. Gray white pellets formed by precipitation were collected by centrifugation and the pellets were left to dry. The dried biosurfactant was resuspended in dichloromethane in a separating funnel and shaken vigorously; surfactin was recovered in the organic layer at the top. The extraction was performed twice, and the organic layers were pooled and evaporated. The residue was collected and stored at 4°C.

Analysis of biosurfactant

Fourier transform infrared spectra of the biosurfactant samples was obtained by using a FTIR spectrophotometer (Perkin Elmer Paragon 1000). The samples were mixed with KBr and pressed into a tablet form. The FTIR spectrum was then recorded. Isolated biosurfactant was solved and analyzed by High performance liquid chromatography (Schimadzu, Japan) equipped with a C18 column (5 µm). The mobile phase consisted of 20% trifluoroacetic acid and 80% acetonitrile. The absorbance of the eluent was monitored at 205nm and the flow rate was 1.0 ml/min. Commercial surfactin (Sigma) was used as a standard.

Reducing power

The reducing power of biosurfactant was determined as described by Oyaizu [20]. Different concentrations of biosurfactant in distilled water (0.1-2.5mg/mL) was mixed with equal volume of 0.2 M phosphate buffer and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Subsequently, 2.5 ml of trichloroacetic acid (10%) was added and the mixture was centrifuged at 3000 rpm for 10 min. The resulting supernatant was taken and mixed with 500 µL of dH_2O and 100 µL of 0.1% ferric chloride then incubated at 37°C for 10 min. The absorbance at 700 nm was measured. Increased absorbance indicated increased reducing power.

Determination of 1,1- diphenyl-2-picryl hydrazyl scavenging activity

The antioxidant potential of the biosurfactant was determined on the basis of their scavenging activity of the stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radi-

cal. DPPH method is widely used and the easiest method to determine the antioxidant activity of compounds [17]. The aliquots of the different concentrations of the biosurfactant were added to 5.0 ml of a 0.004% (w/v) solution of DPPH. Absorbance at 517 nm was determined after 30 min, and IC_{50} (the half maximal inhibitory concentration) was determined. IC_{50} value denotes the concentration of sample required to scavenge 50% of the DPPH free radicals [21].

The radical scavenging activity at different biosurfactant concentration was calculated by the Equation 1.

$S_{DPPH} = 100 \times (1 - A_{sample}/A_{DPPH})$

where A_{sample} indicates the absorbance of the solution in the presence of test samples, and A_{DPPH} indicates the absorbance of the DPPH solution in the absence of the test samples.

Ferrous ion chelating assay

The chelation of ferrous ion by the biosurfactant was carried out by the methods of Decker and Welch [22]. 1.0 mL of biosurfactant with a concentration range of 0.125-2.0 mg/mL was mixed with 3.7 mL of deionized water and then the mixture was reacted with ferrous chloride (2 mmol/L, 0.1 mL) and ferrozine (5 mmol/ L, 0.2 mL) for 20 min. The absorbance at 562 nm was determined spectrophotometrically (UVmini-1240, Shimadzu, Japan). EDTA was used as a positive control with same concentrations. Chelating activity on ferrous ion was calculated using the following Equation.

Chelating effect (%) = $Ab - As / Ab \times 100$

Where Ab is the absorbance of the EDTA and As is the absorbance in the presence of biosurfactant.

Statistical Analysis

All analyses were performed in triplicate. The data were recorded as means standard deviation and analyzed using SPSS v.11.5 for Windows. Paired samples T-test was performed and p values< 0.05 were regarded as significant.

Results

Preliminary characterization

The chromatograms of standard surfactin and biosurfactant recovered from *B. subtilis* RW-I were shown in Figure 1. HPLC analysis revealed the presence of three major peaks in the biosurfactant recovered from *B. subtilis* RW-I. These peaks also appeared in the standard surfactin chromatogram with a similar retention time. These findings show the significance of the similarity in molecular structure of our product with that of the surfactin standard.

HPLC fractions of biosurfactant with a retention time between 13.02-15.71 were collected which are the main peaks of chromotagram and similiar with surfactin. The fraction with a retention time of 20.19 was not collected due to the some impurities present in the peak. The collected fractions were subjected to infrared spectroscopy analysis. Figure 2 shows the infrared spectrum of the isolated biosurfactant and surfactin. The band centered at 1450-1455 cm^{-1} corresponds mainly to the CH2 bending of lipids [23]. Strong adsorption bands observed in the range of 1600 and 1700 cm^{-1} demonstrate amide I vibration for proteins. In addition, there was another characteristic peak at 3300 cm−1 which arises from N–H and O–H stretching vibration of peptides [24]. The band centered at 1541 cm^{-1} is assigned to the Amide II bonds arise from N–H bending and C–N stretching [25].

Reducing power

Figure 3 shows the reductive capability of the biosurfactant compared to BHT. Reducing power of biosurfactant was compared with BHT and it was found that BHT was 1.2 times more effective than biosurfactant at a concentration of 2.0 mg/mL. The reducing power increased as the biosurfactant concentration increased, indicating a dose-dependent response. Really the reductive capability significantly increased as the concentration of biosurfactant increased from 0.2 mg/mL to 2.5 mg/mL ($p<0.05$). However, the increase in the reductive capability between 1.5 mg/mL and 2.5 mg/mL biosurfactant was not significant ($p>0.05$).

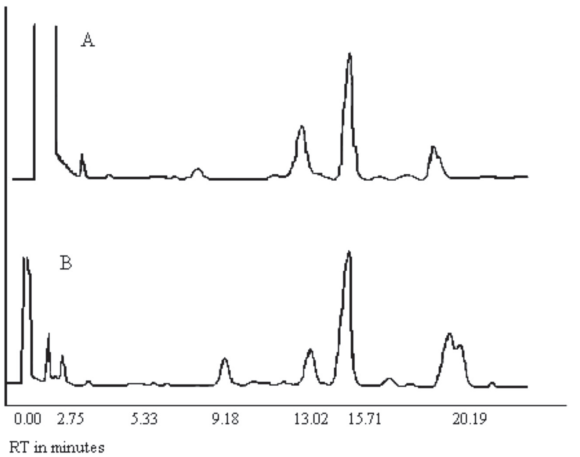

Figure 1. The HPLC chromatograms of standard surfactin (A) and purified biosurfactant by *B. subtilis* (B)

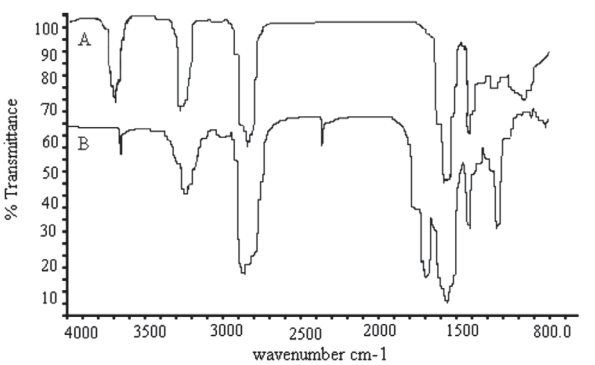

Figure 2. FTIR spectrums of A. biosurfactant from *B.subtilis*; B. Standard surfactin

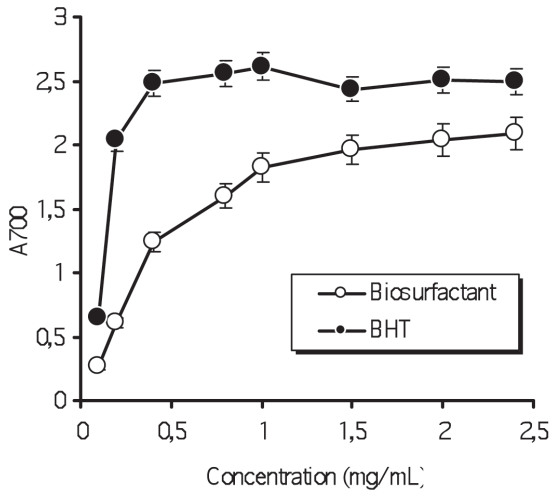

Figure 3. Reductive capability of the biosurfactant. Each value represents mean ± standard deviation (n=3).

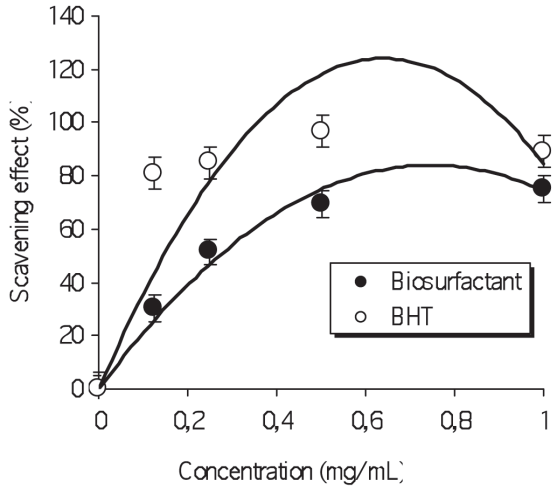

Figure 4. Scavenging effect of biosurfactant on 1,1 diphenyl-2-picrylhydrazyl (DPPH) radicals. Each value represents mean ± standard deviation (n=3).

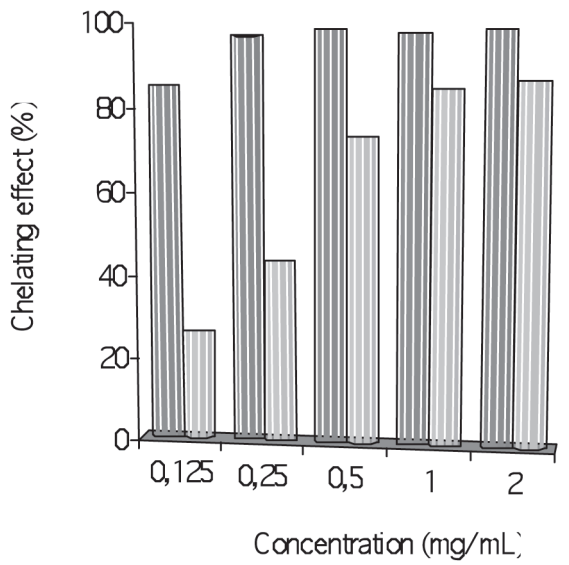

Figure 5. Ferrous ion chelating effect of biosurfactant. Each value represents mean ± standard deviation (n=3); EDTA and Biosurfactant

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) Scavenging Effect

It is well known that antioxidants can seize the free radical chain of oxidation and form stable free radicals, which would not initiate further oxidation. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) has been used extensively as a free radical to evaluate reducing activity of substances [17]. The DPPH scavenging activities of biosurfactant and BHT as control were summarized in Figure 4. Linear response curves were also obtained and the IC_{50} were estimated as 0.09 mg/mL for BHT and 0.25 mg/mL for biosurfactant. In the presence of 2.5 mg/mL biosurfactant, the DPPH scavenging activity was significantly higher than that of the 0.2 mg/mL concentration ($p<0.05$).

Ferrous Ion Chelating

Biosurfactant exhibited a concentration dependent scavenging effect on ferrous ion (Fig. 5). However, scavenging effect of EDTA (control) on ferrous ion was not concentration dependent. The ferrous ion scavenging effect of biosurfactant and EDTA was 85.2% and 98%, respectively at a concentration of 1.0mg/mL. The ferrous ion scavenging effect of biosurfactant was significantly increased with increasing the concentration from 0.125 to 1.0 mg/mL ($p<0.05$). But the increase in the range of 1.0-2.0 mg/mL was not significant.

Discussion

In the present study, we found that the biosurfactant obtained from *B. subtilis* RW-I exerts an antioxidant activity and has a protective role against oxidative stress. HPLC and FTIR results strongly suggest that the biosurfactant obtained in this study had a lipopeptide structure. Similar findings of a lipopeptide biosurfactant production by *Bacillus polyfermenticus* KJS-2 (BP-KJS-2) have been reported [26]. Antioxidant activity of biosurfactant was investigated with reducing power, DPPH scavenging and ferrous ion chelating assays. The antioxidant activities of natural components may have a reciprocal correlation with their reducing powers [27]. The reducing power increased as the biosurfactant concentration increased, indicating some functional groups present in biosurfactant structure were both electron donors and could react with free radicals to convert them into more stable products. Reducing power of biosurfactant was compared with BHT and it was found that BHT was 1.2 times more effective than biosurfactant at a concentration of 2.4 mg/mL. It has been shown that the antioxidant effect exponentially increased as a function of the reducing power, suggesting that the antioxidant properties are associated with the reducing power of components [28]. Also the ability of the biosurfactant to scavenge the DPPH free radicals was evaluated. Maximal scavenging effect of biosurfactant on DPPH was found in the range of 75-80% with a concentration of 1.0 mg/mL. IC_{50} of the BHT and biosurfactant were 0.09 and

0.25 mg/mL, respectively. The results indicate that biosurfactant showed the capacity to donate hydrogen; therefore they exhibit DPPH scavenging activity. The powerful scavenging effect of biosurfactant can be explained by the presence of substances with free hydroxyl moieties represented in FTIR spectra.

In ferrous ion reduction, the general ability of the biosurfactant to donate electrons is tested. The ferrous ion scavenging effect of biosurfactant and EDTA at 2.0 mg/mL concentration was 87.2 and 98.9%, respectively. Biosurfactant exhibited a concentration dependent scavenging effect on ferrous ion and possessed noticeable chelating activity of ferrous ion. However, scavenging effect of EDTA on ferrous ion was not concentration dependent. Likely, Zheng et al. [29] reported that the Fe^{2+} chelating activity of the EDTA showed a constant activity between 1.0 mg/ml -10 mg/ml concentration range. The lower reducing power, DPPH scavenging and ferrous ion reduction activities of the biosurfactant can be caused by lower contents of hydroxyl groups than those present in the control substances as BHT and EDTA.

Antioxidative properties of natural compounds are of great interest in both academia and the food industry, since their possible usage as natural additives has emerged from a growing trend to replace synthetic antioxidants by natural ones. Our results indicate that biosurfactant obtained from *B. subtilis* RW-I has the capacity to scavenge free radicals and that this product can protect cells against oxidative stres. These results suggest that the biosurfactant possess antioxidant properties and could be used as alternative natural antioxidants after toxicological examination. And also this is the first study that demonstrates the antioxidant activity of a biosurfactant produced by *B. subtilis* and also this study will provide bases for future studies in this area.

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