Characterization and Production of Extracellular polymeric substances (EPS) by *Bacillus pseudomycoides* U10

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Abstract Extracellular polymeric substances (EPSs) are important for industrial and medical relevance with significant commercial value. Different strategies are applied to reduce cost of production such as using cheaper substrates, improving product yield by optimizing fermentation conditions, or developing higher yielding strains via mutagenesis, and/or genetic and metabolic manipulations, and optimizing downstream processing. We aimed to determine the effect of whey, different pH values, temperature and incubation time on bacterial EPS production by *Bacillus pseudomycoides* U10. Maximum EPS production was obtained when 1 g/L whey was added to the growth medium. The optimum pH level was 7.0 and the highest EPS production was observed at 37 ºC. According to X-ray diffraction, thermogravimetric EPSs have poorly crystalline nature and exhibit two step degradations, corresponding to weight loss of moisture and/or carboxyl group and pyrolysis of EPS, without distinctive changes in different media conditions. SAXS data indicate the layer thickness of the bacterial EPS is from 12.04 to 14.07 Å for whey and dissolved LB conditions, respectively. It was found that EPS structures changed with whey addition, such as, higher d-values, lower weight losses which seemed to be related to increasing durability and/or stability.

Keywords: *Bacillus pseudomycoides*, EPS, Whey, XRD, SAXS, SEM

1. Introduction

Extracellular polymeric substances (EPSs) are the high-molecular-weight secretions from microorganisms into their environment. Their synthesis is favored by environmental stresses of microorganisms. Carbohydrates and proteins are found extensively in EPS (Sheng *et al*. 2010). Humic substances, uronic acids, lipids, nucleic acids, and some inorganic components are also reported by previous studies (Frolund *et al*., 1996; Dignac *et al*., 1998). EPSs have received considerable research attention recently because of their use in many applications and it is very important to understand the structure of EPS in order to use them in these areas. Many of the physicochemical properties of EPS are closely related to its three-dimensional structure, which has been studied in different model systems and by applying different spectroscopic techniques.

The main objectives in present investigation are as follows: (i) The ability of EPS production of *B. pseudomycoides* U10 and the total amount of carbohydrate, protein, and uronic acid in EPS were also analyzed. (ii) The lyophilized EPS was also analyzed by the SAXS, XRD (X-Ray Diffraction) and TGA (Thermogravimetric Analysis), (iv) We also aimed to gain preliminary data regarding the arrangement in space of EPS obtained from different growth media by SAXS analysis.

2. Material and methods

2.1. Bacterial strain and culture media

In present study, *Bacillus pseudomycoides* U10 was used local isolate from our culture stocks in Bacteriology Laboratory of the of the Biology Department in Pamukkale University. The composition of the Luria Bertani (LB) included the following materials (g/L): Tryptone 10.0, NaCl 10.0 and Yeast extract 5.0. Tryptic Soy Broth (TSB, g/L) was composed of peptone from casein 17.0 g/L; peptone from soymeal 3.0, D(+) glucose 2.5, NaCl 5.0 g/L; K_2HPO_4 2.5. Nutrient Broth (NB) was as follows (g/L): peptone from meat 5.0 and meat extract 3. Glucose and whey were added 1 g/L in growth medium for determination of effect on production.

2.2. Quantification, Purification and analysis of EPS

The amount of EPS in culture was measured by ethanol precipitation (Frengova *et al*. 2000). The total carbohydrate concentration in each sample was calculated by the phenol-sulfuric acid method (Dubois *et al*., 1956). The method of Hung *et al*. (2005) was used for purification of EPS from *B. pseudomycoides* U10. The amount of total protein was determined using a modified Lowry method (Hartree, 2004). Total uronic acid concentration was measured by a spectrophotometric method (Hung and Santschi, 2001).

2.3. X-Ray Diffraction (XRD) and Thermogravimetric Analyses (TGA)

XRD studies were performed on the Rigaku Miniflex model XRD with Ni-filtered CuKα radiation (λ = 1.54056 Å), running conditions of 40 mA, 40 kV, scan-speed 0.005°, time/scan 0.1 sec and 0.2 mm slit using LynxEye detector at Batman University (Turkey). Diffraction peaks were plotted as 2θ value and diffracted X-rays were calculated with Bragg's law d = λ / 2 Sinθ. TGA method was carried out on a Perkin Elmer SII-Diamond TG-DTA Instruments thermal analysis system in a dinitrogen atmosphere, applying a heating rate of 10 ºC min−1 across a temperature range of 30–1000 ºC at the Izmir Institute of Technology (IYTE-MAM, Turkey).

2.4. Small-angle X-ray scattering (SAXS)

A micro-line collimation Hecus SWAXS System3 was used with a conventional X-ray source (MoKα) and ISO-DEBYEFLEX 3003 generator (50kV-50mA) during the scattering measurements at 23°C, with measuring time of 700 seconds. A simultaneous measurement of SAXS range is possible in this system with a linear-position-sensitive detector used with 1024-channel resolution. Distances between the
channels and the sample-detector were 54 μm and 31.5 cm, respectively.

3. Results and Discussion

3.1. Effect of different media, initial pH, temperature, glucose and whey on production of EPS

Our bacterium was 100% identical to *Bacillus pseudomycoides* U10 (GenBanks: KF720933.1, JQ729679.1, EF210306.1, JX994092.1 and GU171377.1) (Life Sciences Research and Application Center, Gazi University). Physical and chemical growth conditions such as carbon sources, temperature, pH, incubation time and the composition of medium have been shown to affect EPS production in bacteria (Larpin *et al*., 2002; Doğan *et al.*, 2015). The bacterial EPS production in LB was higher than NB and TSB. On the other hand, the highest EPS yield was reached in LB. Because of this, we used LB in our experiments. In this study, the effects of pH and temperature on production EPS of strain U10 were evaluated before the experiments of EPS production (data not shown). EPS production by U10 was evaluated at five different pH values. The maximum production of EPS by U10 occurred at pH 7.0. On the other hand, EPS production decreased with increasingly alkaline conditions in the growth medium. Maximum EPS production was at 37 ^{o}C and it was found that EPS production was reduced effectively at 45 ^{o}C. In our study, we clearly observed that the growth of U10 at 45 ^{o}C was weaker than those of other temperatures. Glucose and whey generally increased the production EPS of U10 when compared to LB (control) at 37°C. This implied that glucose and whey significantly promoted the bacterial EPS production (Table 1). In previous studies, some researchers reported that organic acids stimulated bacterial EPS production (Nicolaus *et al*. 2002, Doğan *et al*. 2015). In addition, bacterial EPS production showed differences when the bacteria were grown in the presence of various types of organic acids (Doğan *et al*. 2015).

Table 1. The effect of glucose and whey on EPS production by *Bacillus pseudomycoides* U10. The LB medium used for EPS production was supplemented with 1 g/L glucose or whey. The initial pH was 7.0 and temperature was 37 ^{o}C. LB was evaluated as control group and contained none of glucose and whey.

Time (h)	LB+Whey	LB+Glucose	LB (control)
24	116.85	97.15	37.26
36	101.99	158.80	46.06
48	152.30	93.93	66.98
60	199.65	69.95	92.74
72	316.46	113.73	62.69
84	115.42	98.28	62.36
96	51.02	61.15	91.86

3.2. Biochemical composition of EPS

The biochemical composition of EPS from U10 showed that carbohydrates and proteins were the major components of EPS. This result was in parallel with the findings of Guibaud *et al*. (2005) and Doğan *et al*. (2015). Table 2 has shown that the content of total carbohydrate decreased from 380 to 150 mg/g when whey was applied, while in the medium with glucose the total carbohydrate amount increased to 464 mg/g for dissolved EPS. On the other hand, the content of protein of EPS in LB+whey was higher than LB and LB+glucose. This result may be related to the composition of medium. A similar finding was also found for *Aeromonas hydrophila* (Castro *et al*. 2014). According to Castro *et al*. (2014) the key EPS components of *A. hydrophila* were proteins, indicating their importance for electron transfer reactions. In literature, the main component of EPS purified from *P. fluorescens* Biovar II was recorded to be uronic acid by Hung *et al*. (2005). But, uronic acid content of EPS of *B. pseudomycoides* U10 was low. Present result was similar to the content of the uronic acid of EPS purified from *B. licheniformis* B22 obtained by our previous study (Doğan *et al*. 2015). We considered the content of uronic acid of EPS of *Bacillus* species was low.

Table 2. Biochemical composition of purified EPSs (mg/g)

Growth Media	EPSs	Total Carbohydrate	Total protein	Uronic acid
LB	Dissolved	380	211	12
	Particulate	206	256	9
LB+whey	Dissolved	150	182	16
	Particulate	106	308	3
LB+glucose	Dissolved	464	122	5
	Particulate	176	340	5

3.3. Thermogravimetric analysis (TGA) of EPS

TGA results of EPSs obtained in different growth media from U10 are shown in Figure 1. There was two-stage weight loss together with increasing temperature. The first stage, about ten percent of total EPS weight loss, was observed between 20 and 200°C. This stage corresponds to loss of bonding water molecules and degradation of the carboxyl group related to (Kumar *et al.,* 2004). Second stage of degradation, causes maximum weight loss of 60-70 %, was observed at 200-400 ºC that corresponds to pyrolysis of EPS. Although TGA curves of four samples were shown similarities, total weight loss of EPSs in LB (K1 and K2) show somewhat lower than whey added LB (K5 and K6). Relatively lower weight loss values of EPSs were correspond to EPS particles with higher *d*-values, indicating particle size affect the thermogravimetric data.

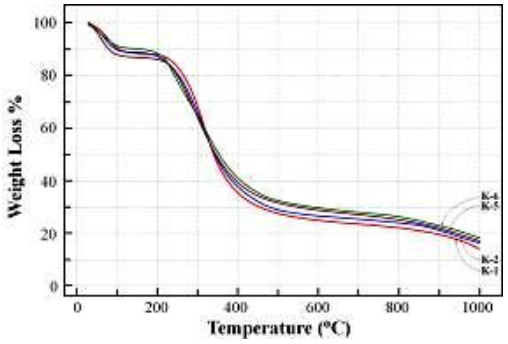

Figure 1. TGA curves of EPS from different media (K1: dissolved EPS in LB, K2: particulate EPS in LB medium, K5: dissolved EPS in LB+whey, K6: particulate EPS in LB+whey.

3.4. X-Ray Diffraction (XRD) analysis of EPS

The XRD patterns of EPS produced by *B. pseudomycoides* U10 bacterium under different growth environments (the media of LB, TSB, NB, whey and glucose) exhibits an extremely broad and asymmetric peak near 2θ = ~ 22º (d_{hkl} = ~ 4 Å), which indicates mainly of amorphous like nature, i.e., poorly developed ordering of EPS structure (Fig. 2). After decomposition of asymmetrical peak (fitting by WINFIT program), at least two peaks were determined as EPS_1 (d = 4.1 to 4.4 Å) and EPS_2 (d = 3.7 to 4.0 Å). However, it is not known that these two groups of peaks belong to which material; it may be evaluated as first peak (EPS_1) associates with chitin and/or chitosan, whereas second peak (EPS_2) is related to proteins (Doğan *et al*., 2015). However, XRD patterns of EPSs don't show distinct differences for different media conditions, *d*-values of EPSs in LB medium (K1 and K2) exhibit somewhat higher than media of whey+LB and whey+LB (K5 and K6). These differences should be related to increasing EPS particles structure, i.e., core radius and/or shell thicknesses that caused from whey addition.

3.5. Structural characterization of EPS using Small Angle X-Ray Scattering (SAXS)

The SAXS is a powerful method to investigate nano-aggregations formed by the self-assembly of polymers in melted, solution, or crystal state (Hamley and Castelletto 2004). Dogsa *et al*. (2005) have investigated EPS of slime-producing marine bacterial isolate. According to the results of SAXS, they have modelled the structure of EPS as a network of randomly coiled polymeric chains with denser domains of polymeric chains. In our study, the EPS structures extracted from U10 cells, including the size, shape, and bilayer thickness were characterized using SAXS. The results indicate that more stable EPS structure can be achieved within aqueous (0.5% w/v) at room temperature. Model-independent approximations using Guinier, Porod and Kratky plots can extract morphology characteristics on the basis of which a suitable model shape may be chosen to fit the SAXS profiles (Guinier 1939; Guinier and Fournet 1955; Glatter and Kratky 1982). The SAXS data for EPS of U10 cells can be fitted reasonably well using a lamellar with prolate core shell model, as shown in Figure 3. The EPSs have a lamellar and prolate core shell with a major core radius of 1001.52 ± 5.01 Å, a major shell thickness of 1201.46 ± 2.40 Å, and a bilayer thickness of 14.07 ± 0.72 Å in the water solution with grown in LB. In the LB+whey, the EPS solutions have a lamellar and prolate core shell with a major core radius of 845.75± 5.01 Å, a major shell thickness of 998.94± 2.40 Å, and a bilayer thickness of 12.04± 0.72 Å. For the addition of glucose as a water solvent with LB, the EPS solutions have a lamellar and prolate core shell with a major core radius of 845.75 ± 3.22 Å, a major shell thickness of 998.94 ± 3.14 Å, and a bilayer thickness of 12.04 ± 0.81 Å (Table 3). As a result, the radius of a major core, shell thickness and bilayer thickness obtained for the EPS decrease from 1001.52 ± 5.01 to 845.75 ± 3.22 Å, 1201.46 ± 2.40 to of 998.94 ± 3.14 Å and 14.07 ± 0.72 to 12.04 ± 0.81 Å with the addition of glucose as a solvent in LB. The proposed prolate structural model derived from these considerations is schematically illustrated in Figure 4.

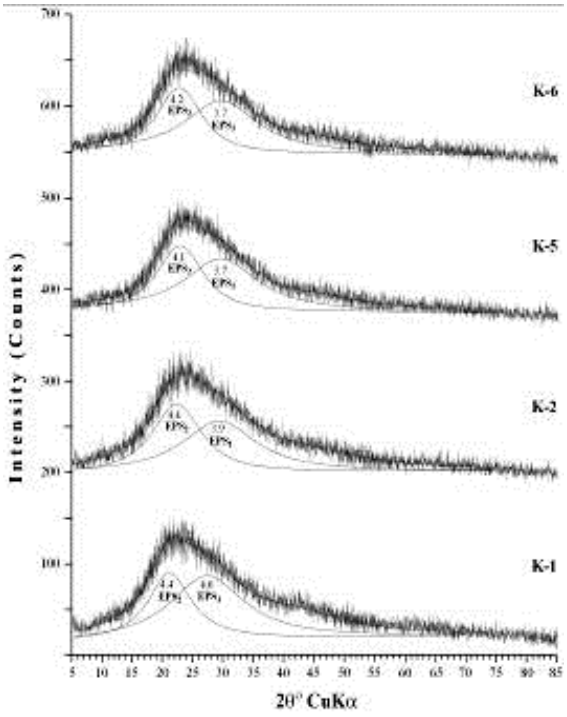

Figure 2. XRD patterns of EPS from different media. (K1: dissolved EPS in LB medium, K2: particulate EPS in LB medium, K5: dissolved EPS in LB+whey, K6: particulate EPS in LB+whey).

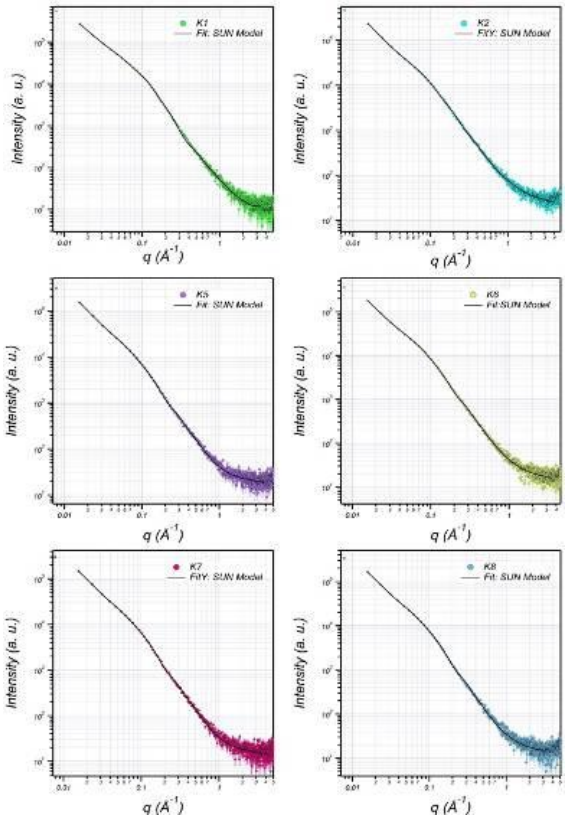

Figure 3. The SAXS data are fitted (solid curves) using lamellar + prolate core shell model (K1: dissolved EPS in LB medium; K2: particulate EPS in LB, K5: dissolved EPS in LB+whey, K6: particulate EPS in LB+whey, K7: dissolved EPS in LB+Glukoz, K8: particulate EPS in LB+Glukoz)

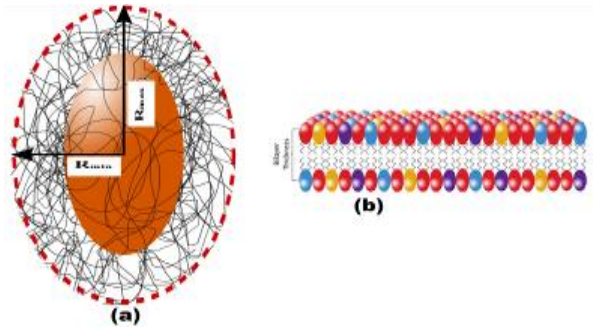

Figure 5. (a) In the schematic illustration of the model, Rmin and Rmax are the minor and major radii. (b) A proposed prolate structural model for the EPS of *B. pseudomycoides* U10 with the bilayer thickness of the membrane presentation, respectively.

Conclusion

The XRD data of EPS produced by *B. pseudomycoides* U10 bacterium under different growth environments indicate amorphous (i.e. poorly developed ordering) structure. Two different EPSs peaks (EPS_1 = 4.1-4.4 Å and EPS_2 = 3.7-4.0 Å) were detected probably belonging to chitin/chitosan and proteins, respectively. Whey addition was caused to an increase for *d*-values of EPS particles. TGA curves of EPSs exhibits two-stage (20-200°C and 200-400°C) weight loss together with increasing temperature. The first stage has 10% weight loss and corresponds to loss of bonding water molecules (or moisture) and degradation of the carboxyl groups. The second stage has maximum weight loss (60-70 %) and resulted from pyrolysis of EPS. Whey addition to LB decreased weight loss values of EPSs. As a conclusion, whey addition was changed EPS structures, such as, higher d-values, lower weight losses and more filamentous structures, which seems to be related to increasing durability and/or stability. Thus, whey added media was more suggestible for EPS production by *B. pseudomycoides* U10.

Acknowledgements

This study is a part of MSc of Kubra Betul SOLMAZ. The authors would like to thank the scientific research council of Pamukkale University, Turkey, for research grant 2014FBE036.

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