Int. J. Environ. Res., 9(3):1107-1116, Summer 2015
ISSN: 1735-6865

Characterization of Extracellular Polysaccharides (EPS) Produced by Thermal *Bacillus* and Determination of Environmental Conditions Affecting Exopolysaccharide Production

Dogan, N.M.[1], Doganli, G.A.[2*], Dogan, G.[1] and Bozkaya, O.[3]

[1]Pamukkale University, Arts and Science Faculty, Biology Department, 20070 Denizli, Turkey
[2]Pamukkale University, Technology Faculty, Department of Biomedical Engineering, 20070 Denizli, Turkey
[3]Pamukkale University, Engineering Faculty, Department of Geological Engineering, 20070 Denizli, Turkey

Received 28 Aug. 2014; Revised 19 Jan. 2015; Accepted 21 Jan. 2015

ABSTRACT: In this study, the neutral monosaccharide composition of the Extracellular Polysaccharide (EPS), extracted from thermal *Bacillus,* was determined by Liquid Chromatography-Mass Spectrometry and High Performance Liquid Chromatography. Our analysis indicated that the EPS consisted of rhamnose, mannose, galactose, glucose, fructose, arabinose and xylose. In addition to the neutral sugars in the EPS, it also contained 230 mg protein/g EPS and 17.18 mg uronic acid /g EPS. The X-ray diffraction data indicated mainly of amorphous nature (80 %) and the presence of chitin, chitosan, protein and calcite. Thermogravimetric analysis curve showed that degradation of EPS takes place in three steps (13.38% at < 180 °C. 45.62% at 180-500 °C, 25.55% at > 500 °C) indicating moisture content and high content of carboxyl group, pyrolysis temperature and decomposition of calcite crystals, respectively. Additionally, laboratory batch experiments were performed characterize the effects of different natural organic acids, pH levels, temperatures and Cr(VI) concentrations on microbial EPS production by *Bacillus licheniformis* B22. Our results indicate that organic acids caused enhanced EPS release. Alginic acid was the most efficient organic acid at EPS production in *B. licheniformis* B22. The optimum pH level was 6.0-7.0 and the highest EPS production was observed at 50 ºC for *B. licheniformis* B22. In addition, EPS production increased with increased chromium in the growth medium due to the toxic effect of Cr(VI) on cells. Maximum EPS production was observed when 150 mg/L Cr(VI) was added to the medium.

Key words: Chromium, EPS, Thermal bacterium, XRD, TGA

INTRODUCTION

Extracellular Polysaccharides (EPS) is a product of cell lysis and adsorbed organic substance in wastewater (Sheng *et al.,* 2010). EPS, which is the essential part of in microbial aggregates, aid in keeping these aggregates together in a three-dimensional matrix (Sheng *et al.,* 2010). The EPS that released by bacteria have been shown to serve multiple functions including the promotion of the initial attachment of cells to solid surfaces. The formation and maintenance of microcolonies and mature biofilm structures; the durable biofilm structure; water retention; the absorption of exogenous organic compounds for the accumulation of nutrients from the environment; and enhanced biofilm resistance to environmental stresses and disinfectants (Laspidou, & Rittmann, 2002; Czaczyk, & Myszka, 2007). EPS also has an important function in the flocculation of bacteria and it can be used as carbon and energy sources in food shortages by microorganisms (Sutherland, 2001; Zhang, & Bishop, 2003; Sheng *et al.,* 2005).

*Corresponding author E-mail: gulumseracar@pau.edu.tr

The major components of EPS are carbohydrates and proteins (Sheng *et al.,* 2010). Also, some inorganic compounds, nucleic acids, uronic acids and lipids have been found in EPS (Frolund *et al.,* 1996; Dignac *et al.,* 1998; D'Abzac *et al.,* 2010a; D'Abzac *et al.,* 2010b). The respective concentrations of these components are significantly affected from extraction methods and sludge origin of EPS (Sheng *et al.,* 2010). It is reported that the composition of the EPS obtained from different microbial aggregates is heterogeneous (Wingender *et al.,* 1999). This situation is related to many factors, such as extraction method, culture conditions, processing parameters, analytical tool used, growth phase and bioreactor type (Sheng *et al.,* 2010; Nielsen, & Jahn, 1999).

Additionally, microbial EPSs include carboxylic, phosphate, amine and hydroxylic functional groups that are useful for interact with metal ions, e.g., chromium (Guibaud *et al.,* 2008). Proton and metal binding ability of EPS varies with its composition (Priester *et al.,* 2006). Some researchers reported that metal ion binding ability of EPS was related with phosphate and carboxyl functional groups (Guibaud *et al.,* 2005; Tourney *et al.,* 2009; Kantar *et al.,* 2011). For example, EPSs play an important role in Cr(VI) tolerance and may also enhance Cr(VI) removal according to a study (Ozturk *et al.,* 2009).

In previously studies, different species of microorganisms have been assessed in terms of their ability to produce EPS (Nicolaus *et al.,* 2002; Leppard *et al.,* 2003; Guibaud *et al.,* 2005; Nandal *et al.,* 2005). These studies showed that EPS formation is significantly influenced by environmental factors such as pH level, oxygen concentration, temperature, medium composition, salt ion concentration or heavy metal concentration. Some work in the literature also showed that fungi, algae or bacteria cultures may remove toxic heavy metals through their EPS molecules (Ozdemir *et al.,* 2003; Iyer *et al.,* 2004; Guibaud *et al.,* 2005). For example, Priester *et al.* (Priester *et al.,* 2006) showed that chromium exposure to *P. putida* resulted in elevated extracellular carbohydrates, protein, DNA, and EPS sugars that were relatively enriched in N-acetyl-glucosamine, rhamnose, glucose, and mannose. Their results also provide evidence on Cr toxicity-mediated cell lysis that is responsible for enhanced EPS production. In a study with the cyanobacteria strain *Synechocystis* sp. BASO672, Ozturk *et al.* (2009) determined that toxic elements such as hexavalent chromium induced the microbial EPS production. Kazy *et al.* (Kazy *et al.,* 2002) found that a Cu(II) resistant *Pseudomonas aeruginosa* strain produced four times the amount of EPS when Cu(II) was in the growth media. In our previous work (Acar *et al.,* 2011), we showed that organic acids (e.g., alginic acid, galacturonic, glucuronic acid) enhanced the microbial Cr(VI) reduction rates by *Bacillus licheniformis* B22 bacterium. Despite the overwhelming evidence about the positive effect of organic acids on bacterial Cr(VI) reduction, little is known regarding the role of these compounds on microbial EPS production and following microbial Cr(VI) reduction.

This study was aimed to better understand the composition of bacterial EPS and the effect of environmental conditions such as different organic acids, temperature, pH level and Cr(VI) concentration on the production of EPS by thermal *Bacillus*. We also investigated the total amount of uronic acid, which plays an important role in metal binding. However, to the best of our knowledge, there is not sufficient information in the literature regarding the use of thermophilic and/or thermotolerant strains for EPS production. To date, it has not been clear whether these organisms are likely to be useful sources of polymers. Consequently, the main subject of our study was to determine the structure of EPS and relationship between Cr(VI) removal and EPS production in the tested thermal bacterium.

MATERIALS & METHODS

All chemicals used in the study were reagent grade or better. Water was supplied from a Human Power-Pure water system in all experiments. Firstly, 2.829 g K_2Cr_2O7 (294.19 g/mol) (Merck) was dissolved in 1 L UV-water for preparation of Cr(VI) stock solution. Secondly, chromium solution was filtered separately and added to the media before experiments. D(+)-glucuronic acid sodium salt monohydrate ($C_6H_9NaO_7$ H_2O) (Merck), D(+) galacturonic acid monohydrate ($C_6H_{10}O_7$ H_2O) (Sigma-Aldrich), ($C_6H_8O_7$ H_2O) (Merck) and alginic acid sodium monohydrate (Sigma-Aldrich) were used in the experiments as organic acids (ligands).

The *B. licheniformis* B22 strain was isolated from the Pamukkale thermal region (Denizli), Turkey and was previously identified and reported as a Cr(VI) resistant bacterium (Acar *et al.,* 2011). The bacterial culture was inoculated in growth media Tryptic Soy Broth (TSB) consisting of peptone from casein (17.0 g/L), peptone from soymeal (3.0 g/L), glucose (2.5 g/L), NaCl (5.0 g/L) and dipotassium hydrogen phosphate (2.5 g/L). The culture was aerobically incubated at 40 ºC, 45 ºC or 50 ºC with constant shaking at 125 rpm. Culture growth monitored by measuring the optical density (OD) at 600 nm. The culture suspension was prepared and adjusted by comparing against 0.5 McFarland turbidity standard tubes ($1.5x10^8$ cfu/mL) for all tests.

The purification of EPS from the bacteria was performed according to the method of Hung *et al.* (2005). %2 bacteria (*B. licheniformis* B22) were inoculated into 1 L of TSB. After incubation, the bacterial culture was centrifuged at 3500 rpm for 30 min. The extracellular polysaccharides obtained from supernatant and pellet is defined as 'dissolved EPS' and 'particulate EPS' respectively. Finally, dissolved and particulate EPS fractions obtained from B22 were combined and freeze-dried for storage and analysis. A part of freeze dried EPS was used for determination of total protein, uronic acid, phosphorus and total carbohydrate content and another portion of EPS were used for High Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry (LC-MS) analyses.

The total carbohydrate content of the freeze-dried EPS was determined by the phenol sulfuric acid

method. Glucose was used as standard for total carbohydrate measuring (Southgate, 1976). The amount of total protein was measured using a modified Lowry method (Hartree, 2004). Total uronic acid concentration in EPS was quantified by a spectrophotometric method (Hung, & Santschi, 2001). The phosphorus (P) content of the EPS was analyzed via ICP-MS (Agilent 7500ce) at the Izmir Institute of Technology. The monosaccharide composition of the freeze-dried EPS was detected via LC-MS and HPLC. LC-MS analysis of EPS was carried out at the Molecular Biology Laboratory, Ege University. HPLC analysis of EPS was carried out at the Central Laboratory, Molecular Biology and Biotechnology R&D Centre, Middle East Technical University.

X-ray diffraction (XRD) uses a fundamental method in the development of many scientific fields for determining various materials and allows measurement spacings between layers of atoms or atomic planes (d_{hkl}) and determination of crystal structure of an unknown material. In addition to inorganic materials, this method also revealed the structure and function of many biological molecules, including vitamins, drugs, proteins and nucleic acids such as DNA. X-ray diffraction (XRD) studies were performed on the Bruker D8 Advance model XRD with Ni filtered CuKα radiation ($\lambda = 1.54056$ 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 Istanbul Technical University, Turkey. Diffraction peaks were plotted as 2θ value and diffracted X-rays were calculated with Bragg's law $d = \lambda / 2 \sin\theta$. Crystallinity index [$CI_{XRD}$ = peak areas of crystals / (peak areas of crystals + peak area of amorphous peak)] can estimate by means of areas of crystalline and amorphous peaks (e.g., Alexander, 1969; Ricou, Pinel, & Juhasz, 2005).

Thermogravimetric analyses (TGA) were carried out on a Perkin Elmer SII-Diamond TG-DTA Instruments thermal analysis system in dinitrogen atmospheres, applying a heating rate of 10 ºC min"1 in a temperature range of 0–1000 ºC at Pamukkale University, Denizli, Turkey. TGA is a method of thermal analysis in which changes in physical and chemical properties of materials measure as a function of increasing temperature with constant heating rate. This method is commonly used to determine selected characteristics of materials that exhibit either mass loss or gain due to decomposition, oxidation, or loss of volatiles (such as moisture), and useful technique for the study of polymeric materials.

Kinetic experiments were performed to determine the EPS production rates by *B. licheniformis* B22, in the growth medium with and without organic acid. The 250 mL flasks containing 100 mL growth medium with the certain organic acid concentration (1 g/L) were inoculated with 2 mL cultures at the logarithmic phase. All media were autoclaved at 121 ºC for 15 min before use in EPS production experiments. Then cultures were aerobically incubated at 40 ºC, 45 ºC and 50 ºC with constant shaking at 125 rpm. Beginning immediately after inoculation, samples were drawn at regular time intervals (every 12/6 h), and analyzed for their total carbohydrate content. We investigated the effects of Cr(VI) on EPS production by the microorganism in the absence or presence of organic acids (galactronic acid, glucuronic acid and alginic acid) in a series of batch kinetic experiments. The experiments were carried out in a similar fashion as explained above, but contained 100 mg/L Cr(VI). Also, the growth of cells was routinely monitored by spectrophotometer at 600 nm. The experiments were carried out in duplicate. Additional kinetic experiments were also performed to determine the role of different pH (6.0, 7.0, 8.0), temperatures (40, 45 and 50 ºC) and initial Cr(VI) concentrations (100 and 150 mg/L) on EPS production by the B22 strain in systems containing or not containing organic acids. To determine the effect of pH values on EPS production, the pH of the medium was set with 6 N HCl and 6 N NaOH.

EPS was isolated by ethanol precipitation as described in Frengova *et al.* (2000). Samples (1 mL) were withdrawn (every 6 or 12 hours) and were first boiled at 100 ºC for 10 min. After cooling, the samples were treated with 17% (v/v) of 85% trichloracetic acid solution and centrifuged. After removing the cells and protein by centrifugation, the EPS was precipitated by ethanol. The EPS was then recovered by centrifugation at 14 000 rpm for 20 min at 4 ºC. The total carbohydrate concentration (expressed as mg/L) was determined in each sample by the phenol-sulfuric acid method (Dubois *et al.,* 1956). Glucose was used as the standard.

RESULTS & DISCUSSION

In the environment, organisms such as algae, cyanobacteria and bacteria excrete extracellular acid polysaccharides such as uronic acids for response to high metal concentrations or food shortages (Hung, & Santschi, 2001). Moreover, these polysaccharides play an important role in heavy metal detoxification (Hung, & Santschi, 2001). Due to their extensive heavy metal binding capacities, EPSs are recommended as surface-active decontamination agents (Pagnanelli *et al.,* 2000). The content and amount of EPS have been shown to vary in several studies based on the particular bacterial strain and type of metal exposure (Priester *et al.,* 2006; Aquino, & Stuckey, 2004). In general, proteins, polysaccharides, lipids, nucleic acids and humic substances are mainly found in EPS composition and EPS is known to contain ionizable functional groups,

Table 1. Major composition of EPS produced by *B. licheniformis* B22.

	Parameter[a]			
Isolate	**TCHO**	**Protein**	**URA**	**Phosphorus**
B22	252	230	17.18	21.77

Table 2. Monosaccharide composition of EPS produced by *B. licheniformis* B22.

Monosaccharide composition of EPS									
Determined by LC-MS (µg/mL)							Determined by HPLC (µg/mL)		
Mannose	Rhamnose	N-acetyl Glucose amine	Glucose	N-acetyl Galactose amine	Galactose	Arabinose +Xylose	Glucose+ Maltitol	Fructose	Sorbitol+ Ksilitol
5.89	2.73	6.4	13.54	1.15	12.45	0.64	22.8	21.7	20.3

such as hydroxylic, phosphoric, amino and carboxylic groups (Liu, & Fang, 2002). In the present study, bacterial EPS was extracted and characterized from thermal isolate *B. licheniformis* B22, and EPS production was investigated during its growth in batch culture to understand its role in metal removal.

Our analysis of the biochemical composition of EPS shows that carbohydrates and protein are the major components of EPS with a concentration of 252 mg/g and 230 mg/g, respectively. Despite this finding, Phosphorus (P) and Uronic Acid (URA) content was relatively low (21.17 and 17.18 mg/g, respectively; Table 1). Previous studies have also shown that carbohydrate and protein were the essential components of the EPS (Guibaud *et al.,* 2011). On the other hand, the main component of EPS purified from *P. fluorescens* Biovar II was found to be uronic acid (Hung *et al.,* 2005).

The monosaccharide composition of the bacterial EPS extracted from B22 isolate was characterized and quantified by LC-MS and HPLC; these results are given in Table 2. Despite the use of freeze-dried EPS in both analyses, the results were slightly different (Table 2). The presence of glucose in the EPS sample was determined with both HPLC and LC-MS analysis. Mannose, rhamnose, galactose, arabinose and xylose could not be detected with HPLC analysis but they could be determined by LC- MS. Ribose was not detected with either type of analysis. On the other hand, EPS of *B. licheniformis* B22 was composed mainly of glucose (31.63%) and small amounts of arabinose and xylose (1.49%).

Nicolaus et al. (Nicolaus *et al.,* 2002) reported that EPS produced by aerobic thermophiles contains 81% carbohydrate, 3% protein and 2% nucleic acid. In the

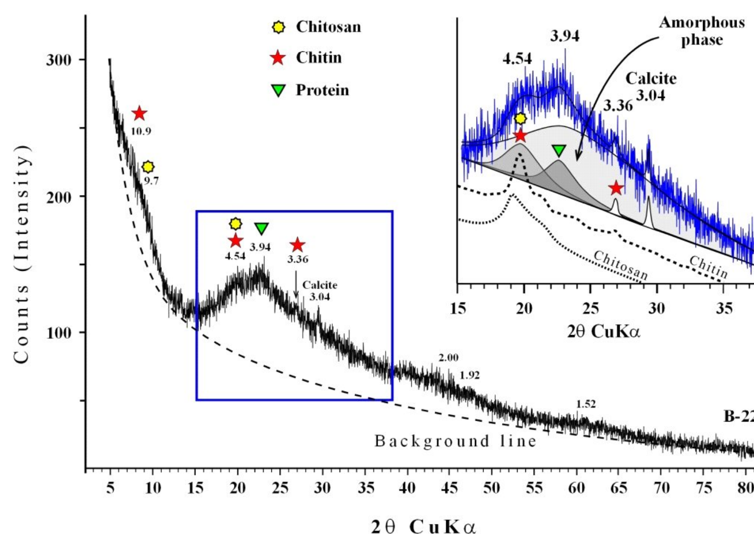

Fig. 1. XRD pattern of EPS. Peak fitting analysis and peak areas of crystalline and amorphous phases were calculated with WINFIT software program. XRD traces of chitin and chitosan were modified from Ifuku *et al.* (2003) and Muzzarelli *et al.* (2004), respectively.

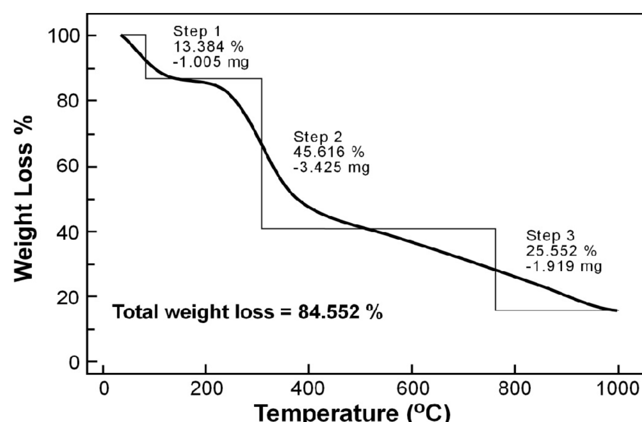

Fig.2. TGA curve of EPS with three degradation stages.

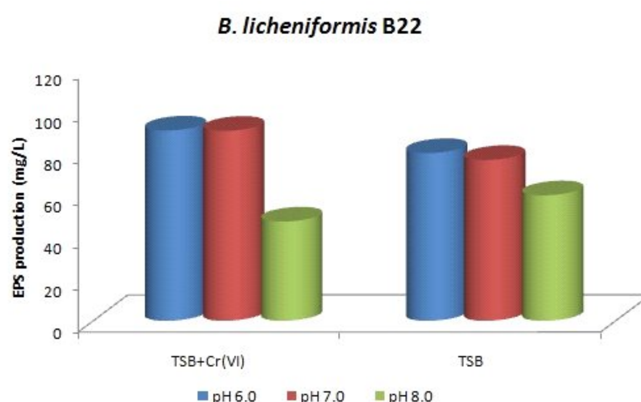

Fig. 3. Effect of different pH levels on EPS production by *B. licheniformis* B22 with and without 100 mg/L Cr(VI) in TSB media at 60 hours of incubation (T: 40°C).

same study, it was determined that the EPS was composed of mannose, glucose, galactose, and mannose-amine monomers by GC-MS analysis. The XRD pattern of EPS produced by *B. licheniformis* B22 bacterium exhibits an extremely broad peak that indicates mainly of amorphous nature. The detailed decomposition of broad peak by using peak fitting program (WINFIT by Krumm (1996)) show that a few characteristic diffraction peaks indicating the presence of chitin (10.91, 4.54, 3.36 Å) chitosan (9.72, 4.54 Å) and protein (3.94 Å) as poorly crystalline (or semi-crystalline) phases in addition to amorphous phase (Fig. 1). Relatively narrow peak at $2\theta = 29.36°$ (3.04 Å) with low intensity refers to minor amounts of calcite ($CaCO_3$). Crystallinity index (CI_{XRD}) value of EPS was determined as 0.20 (20 %) that indicates semi-crystalline and amorphous phases are dominant (80 %).

TGA curve showed that degradation of EPS obtained from *B. licheniformis* B22 takes place in three steps. Initially 13.38% loss in the weight of EPS was observed at approximately < 180 ºC, and then 45.62% and 25.55% of degradation was observed between 180-500 ºC and > 500 ºC in second and third stages respectively (Fig. 2). First stage, sixteen percent of total EPS weight loss from 20 to 180 ºC, was recorded due to moisture content and high content of carboxyl group which increase the degradation level at first stage related to bonding more water molecules (Kumar *et al.,* 2004). Second stage of degradation (71.2%) was observed with maximum loss at 300 ºC correspond to pyrolysis temperature of EPS. The total weight loss of EPS occurred after 400 ºC. The third stage at high temperatures (>500 ºC) should be correspond to decomposition of calcite crystals as stated by XRD data.

There have been a number of studies on EPS production by *Bacillus* species (Larpin *et al.,* 2002; Binupriyaa *et al.,* 2010; Liu *et al.,* 2010; Chowdhury *et al.,* 2011). Carbon sources and growth conditions such a temperature, pH, incubation time and the composition of medium have been shown to affect EPS production in bacteria (Larpin *et al.,* 2002; Kýlýç, & Dönmez, 2008). Fig. 3 shows the influence of the different pH values of the medium to the bacterial EPS production containing 100 mg/L initial Cr(VI) and those not containing any Cr(VI) at the 60th hours of incubation.

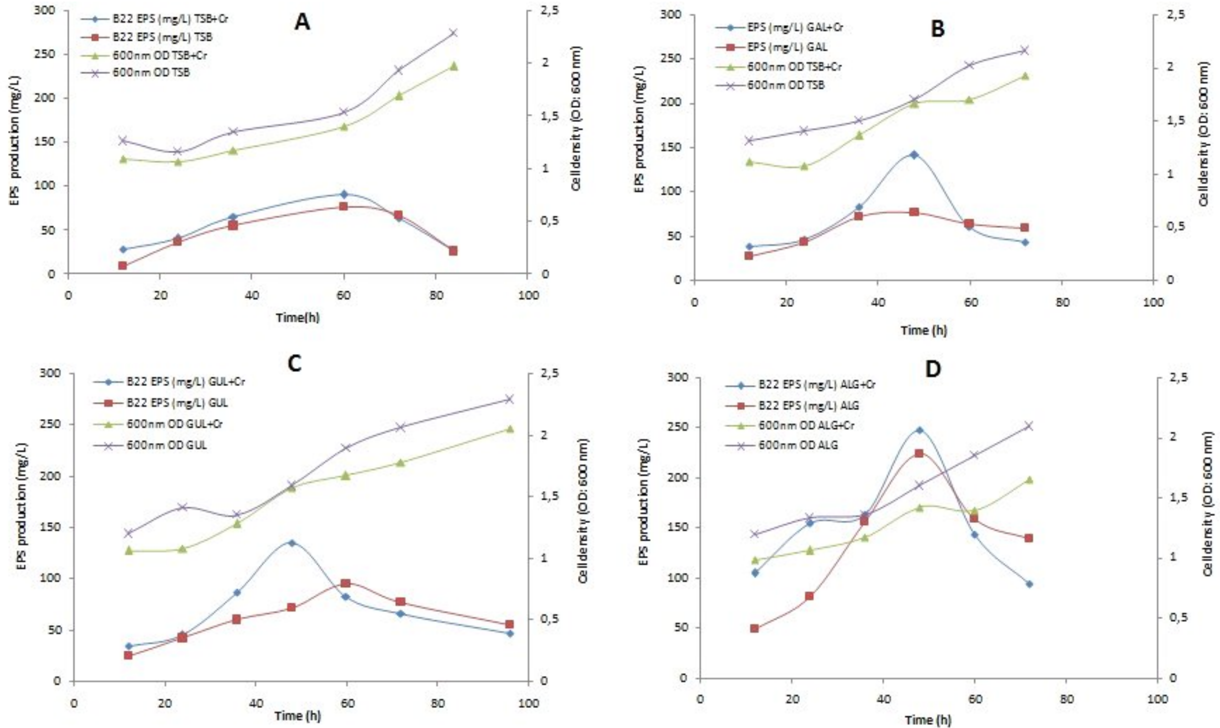

Fig. 4. Effect of different organic acids (1 g/L) on EPS production by *B. licheniformis* B22 and cell growth curves with and without 100 mg/L Cr(VI) at 40æ%C, pH 7.0. A; TSB media B; Galactronic acid (GAL) containing media, C; Glucuronic acid (GUL) containing media, D; Alginic acid (ALG) containing media.

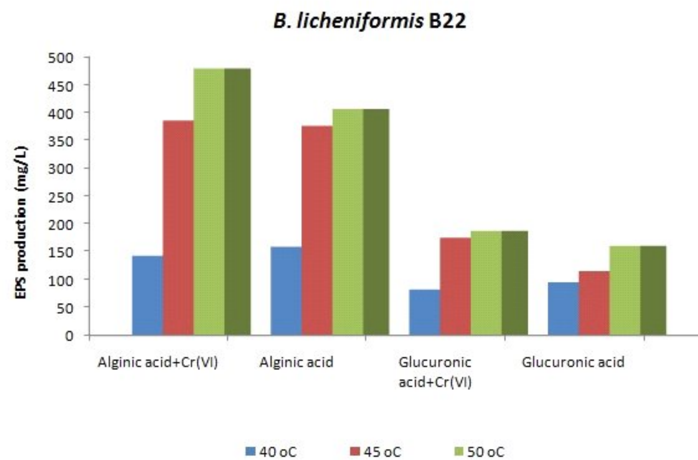

Fig. 5. Effect of different temperatures on EPS production by *B. licheniformis* B22 with and without 100 mg/L Cr(VI) in the presence of 1 g/L alginic acid or glucuronic acid at 60 hours of incubation (pH 7.0).

To determine a suitable pH value for the maximum EPS production by live *B. licheniformis* B22 cells, pH 6.0, 7.0 and 8.0 were initially tested. The maximum EPS production was obtained at pH 6.0 (90.43 mg/mL) and 7.0 (90.26 mg/mL) and decreased towards more alkaline (pH 8.0; 47.16 mg/mL) conditions in the growth media with and without Cr(VI). In addition, it seems that EPS production was higher in Cr(VI) containing medium than in medium without Cr(VI) at all pH values. Also, in other strains it has been seen that EPS production depends on pH (Kýlýç, & Dönmez, 2008). For instance, Torres *et al.* (2012) stated that *Enterobacter* A47 strain produced the most EPS at pH 7.0. Similarly, it has been observed that the suitable pH level is 7.0 to obtain a maximum EPS yield by *P. aeruginosa* (Kýlýç, & Dönmez, 2008). In another study, the highest EPS production by the *Streptococcus phocae* PI80 isolate was recorded at pH 6.5 (Kanmani *et al.*, 2011).

The effects of organic acids such as galacturonic acid, glucuronic acid and alginic acid on EPS production by *B. licheniformis* B22 were assessed; results are shown in Fig. 4. These experiments were carried out at pH 7.0 for B22 bacteria based on the pH data given above. The samples were taken at different incubation times and their total EPS contents were analyzed. Bacterial EPS production enhanced significantly by adding organic acids compared to the culture system containing only the growth medium. We also found that the type of organic acid used in the study, considerable effect the EPS production rate of the bacteria. While the greatest EPS production in the control (without organic acids) occurred at 60 hours both with and without Cr(VI), the greatest EPS production occurred at 48 hours in the presence of the organic acids (Fig. 4(A-D)). In general, we also found higher EPS production in Cr(VI) containing media compared media lacking Cr(VI). However, this does not show that the EPS has better property. The composition of produced EPS could be different under different culture conditions. It is known that the production of EPS takes place because of cellular stress. Therefore, as expected, adding Cr(VI) to the growth media increased bacterial EPS production. This result was clearly observed in galactronic acid containing medium (Fig. 4(B)). When comparing the effects of the different organic acids on EPS production by B22 bacteria, the maximum EPS production (at 48 hours) was observed in bacteria grown in alginic acid containing medium (with Cr(VI): 247.07 mg/L; without Cr(VI): 223.71 mg/L). The lowest EPS production was observed in glucuronic acid containing medium (with Cr(VI): 134.74 mg/L; without Cr(VI): 95.11 mg/L) (Fig. 4(C)).

In general, bacterial EPS production showed differences when the bacteria were grown in the presence of various types of organic acids due to the structural differences between the organic acids. The inclusion of alginic acid in the culture medium may have been stressful for the cells, thereby resulting in increased EPS production. After adding alginic acid into growth medium, the EPS production by the B22 strain increased more than 100%. In one previous study of the exopolysaccaride production potential of the thermophilic bacteria, while carbon sources such as trehalose, galactose and glucose were found to stimulate EPS production of the 4008 strain, the addition of trehalose as sole carbon source increased EPS production in the 4009 strain by almost 1000-fold (Nicolaus *et al.,* 2002). With the aim of determining the effect of temperature and chromium concentration on EPS production, B22 bacteria were inoculated into the TSB medium containing alginic acid or glucuronic acid in the absence or presence of Cr(VI). As shown in Fig. 5, EPS production by *B. licheniformis* B22 generally increased with an increase in temperature in both alginic acid and glucuronic acid containing media. EPS production at 50 ºC was higher than that at 40 ºC and 45 ºC. In alginic acid-containing growth medium (with 100 mg/L Cr(VI)), EPS production by the B22 bacteria increased by a rate of 169% at 45 ºC and 235% at 50 ºC compared to 40 ºC. In glucuronic acid containing medium (with 100 mg/L Cr(VI)), EPS production by B22 bacterium increased at 45 ºC and 50 ºC by the rate of 112% and 127% respectively, compared to 40 ºC. These results indicate that EPS production at 50 ºC was distinctly higher than that at 40 ºC and 45 ºC by *B. licheniformis* B22 bacterium. These findings could explain why the colonies at 50 ºC were more mucoidal. However, it was reported that Enterobacter A47 had produced the maximum EPS at 30 ºC (Torres *et al.,* 2012) and by *Streptococcus phocae* PI80 at 35 ºC (Kanmani *et al.,* 2011). In addition, we found in previous studies that the greatest rate of Cr(VI) reduction by B22 bacteria occurred at 50 ºC (Acar *et al.,* 2011). In the present study, the greatest Cr(VI) reduction and enhanced EPS release was corrected each other at the high temperature. As a result, we can conclude that under stress conditions, the bacteria increase EPS production, and the EPS molecules produced by the bacteria help reduce the Cr(VI) concentration.

Also, Chromium (VI) has been shown to be a toxic substance that may lead to the production of microbial exudates, e.g., EPS. The formation of EPS protects the cells from the toxic effect of chromium (Aquino, & Stuckey, 2004; Sheng *et al.,* 2005; Kýlýc, & Dönmez, 2008). All of our studies on the effects of organic acids, temperature and pH on EPS production were conducted in growth media with and without Cr(VI). We found that EPS production was higher in growth media containing Cr(VI). Fig. 6 shows the effects of the initial Cr(VI) concentration on microbial EPS production by *B. licheniformis* B22 in the absence or presence of alginic acid or glucuronic acid, which had the greatest and the lowest effect on EPS production of B22 strain, respectively. According to Fig. 6, EPS production was similar for bacteria grown in media with 100 mg/L Cr(VI) and without Cr(VI). However, we observed that Cr(VI) clearly increased the EPS production of B22 bacteria in growth medium containing 150 mg/L Cr(VI) and alginic acid (366.33 mg/L). In TSB medium not containing any organic acids, 150 mg/L Cr(VI) increased EPS production 363%. Similar results were observed in alginic acid and glucuronic acid containing media (130% and 125%, respectively). As a result, the EPS production of *B. licheniformis* B22 cells increased approximately 2- to 3-fold when exposed to 150 mg/L Cr(VI). Similar results have been reported in literature (Fang *et al.,* 2002; Aquino, & Stuckey, 2004).

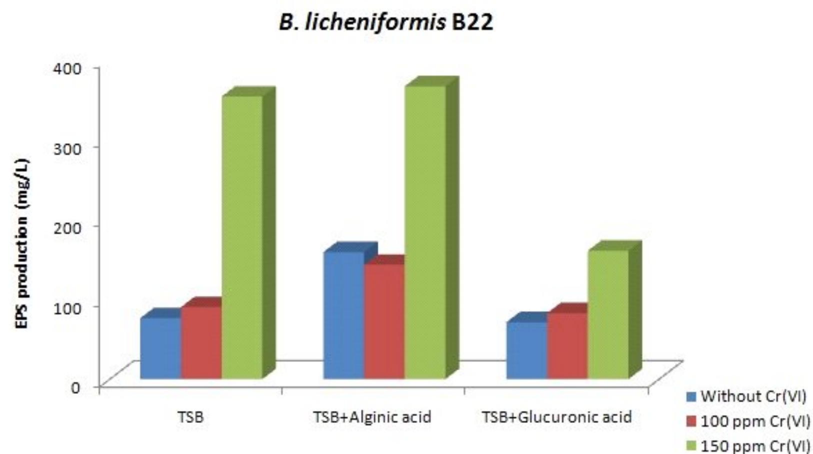

Fig. 6. Effect of initial chromium concentration on EPS production by *B. licheniformis* B22 in the absence and presence of 1 g/L alginic acid and glucuronic acid at 60 hours of incubation (T: 40 °C, pH 7.0).

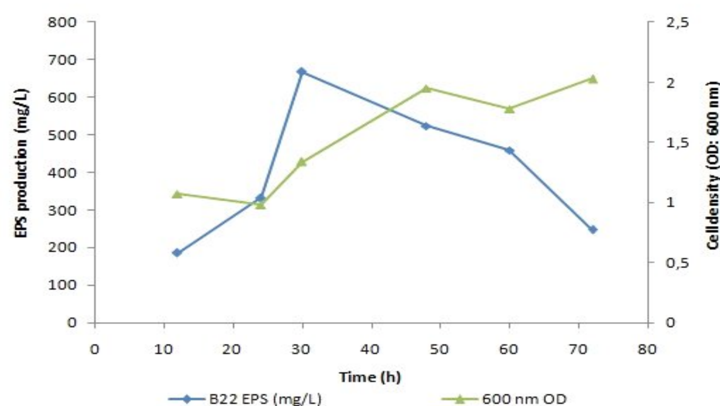

Fig. 7. EPS production by *B. licheniformis* B22 at optimum conditions (T: 50 °C, pH 7.0, 150 mg/L Cr(VI) and 1 g/L alginic acid.

Additionally, in order to obtain maximum EPS production yield, finally a set of experiment was established at optimal conditions. The optimal conditions at which *B. licheniformis* B22 bacterium produced maximum EPS were determined as 50 °C, pH 7.0 and alginic acid containing media with 150 mg/L Cr(VI). Under these conditions, maximum EPS production was performed at 30th hours of incubation and 667.83 mg/L EPS was produced by the bacterium (Fig. 7).

CONCLUSIONS

The use of thermophilic bacteria for EPS production is not to be deeply investigated. We have demonstrated EPS production of thermal isolate *B. licheniformis* B22 comprehensively in terms of monomer composition, characterization and the effect of environmental conditions. The EPS produced by thermal isolate have mainly composed of glucose. In addition, the XRD and TGA data showed EPS was mainly of amorphous nature and contained chitin, chitosan, protein and calcite. For the highest EPS production the optimum pH level was 6.0-7.0 and it was highly affected by the increasing temperatures, Cr(VI) concentrations and present of organic acids. A positive correlation was determined between Cr(VI) toxicity and EPS production.

ACKNOWLEDGEMENTS

The authors would like to thank the scientific research council of Pamukkale University, Turkey, for research grant 2010FBE026. The last author thanks to Dr. O. Isik ECE (Istanbul Technical University) and Dr. Emin KARAPINAR (Pamukkale University) for providing XRD and TGA experiments.

REFERENCES

Acar, G., Dogan, N.M., Evgen, E. and Dogan, G. (2011). Cr(VI) Reduction by *Bacillus licheniformis* B22 Isolated From Pamukkale Thermal Region. Currunt Opinion in Biotechnology (Abstracts), **22S,** S15-S152, 69-70.

Alexander, L.E. (1969). X-ray diffraction methods in polymer science (New York Wiley Interscience).

Aquino, S.F. and Stuckey, D.C. (2004). Soluble microbial products formation in anaerobic chemostats in the presence of toxic compounds. Water Research, **38,** 255–266.

Binupriyaa, A.R., Sathishkumar, M., Kuc, C.S. and Yun, S. (2010). Sequestration of Reactive Blue 4 by free and immobilized *Bacillus subtilis* cells and its extracellular polysaccharides. Colloids and Surfaces B: Biointerfaces, **76,** 179–185.

Chowdhury, S.R., Basak, R.K., Sen, R. and Adhikari, B. (2011). Production of extracellular polysaccharide by *Bacillus megaterium* RB-05 using jute as substrate. Bioresource Technology, **102,** 6629–6632.

Czaczyk, K. and Myszka, K. (2007). Biosynthesis of Extracellular Polymeric Substances (EPS) and Its Role in Microbial Biofilm Formation. Polish Journal of Environmental Studies, **16 (6),** 799-806.

D'Abzac, P., Bordas, F., Joussein, E., van Hullebusch, E., Lens, P.N.L. and Guibaud, G. (2010a). Characterization of the mineral fraction associated to extracellular polymeric substances (EPS) in anaerobic granular sludge. Environ Science and Technology, **44,** 412–8.

D'Abzac, P., Bordas, P.F., van Hullebusch, E., Lens, P.N.L. and Guibaud, G. (2010b). Extraction of extracellular polymeric substances (EPS) from anaerobic granular sludges: comparison of chemical and physical extraction protocols. Applied Microbiology and Biotechnology, **85,** 1589-99.

Dignac, M.F., Urbain,V., Rybacki, D., Bruchet, A., Snidaro, D. and Scribe, P. (1998). Chemical description of extracellular polymeric substances: implication on activated sludge floc structure. Water Science and Technology, **38(8–9),** 45–53.

Dogan, N.M., Kantar, C., Gulcan, S., Dodge, C.J., Yilmaz, B.C., Mazmanci, M.A. (2011). Chromium(VI) Bioremoval by *Pseudomonas* Bacteria: Role of Microbial Exudates for Natural Attenuation and Biotreatment of Cr(VI) Contamination. Environmental Science and Technology, **45(6),** 2278–2285.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Analytical Chemistry, **28,** 350-356.

Fang, H.H.P., Xu, L.C. and Chan, K.Y. (2002). Effects of Toxic Metals and Chemicals on Biofilm and Biocorrosion. Water Research, **36,** 4709-4716.

Frengova, G.I., Simova, E.D., Beshkova, D.M. and Simov, Z.I. (2000). Production and monomer composition of exopolysaccharides by yogurt starter cultures. Canadian Journal of Microbiology, **46,** 1123–1127.

Frolund, B., Palmgren, R., Keiding, K. and Nielsen, P.H. (1996). Extraction of extracellular polymers fromactivated sludge using a cation exchange resin. Water Research, **30**, 1749-58.

Guibaud, G., Comte, S., Bordas, F., Dupuy, S. and Baudu, M. (2005). Comparison of the complexation potential of extracellular polymeric substances (EPS), extracted from activated sludges and produced by pure bacteria strains, for cadmium, lead and nickel. Chemosphere, **59,** 629-638.

Guibaud, G., Bordas, F., Saaid, A., D'abzac, P. and van Hullebusch, E. (2008). Effect of cadmium and lead binding by extracellular polymeric substances (EPS) extracted from environmental bacterial strains. Colloids Surf B Biointerfaces, **63,** 48-54.

Hartree, E.F. (2004). Determination of protein: a modification of the lowry method that gives a linear photometric response, Agricultural Research Council, Unit of Reproductive Physiology and Biochemistry, 307.

Hung, C.C. and Santschi, P.H. (2001). Spectrophotometric determination of total uronic acids in seawater using cation-exchange separation and pre-concentration by lyophilization. Analytica Chimica Acta, **427,** 111–117.

Hung, C.C., Santschi, P.H. and Gillow, J.B. (2005). Isolation and characterization of extracellular polysaccharides produced by *Pseudomonas fluorescens* Biovar II, Carbohydrate Polymers, **6,** 141–147.

Ifuku, S., Shervani, Z. and Hiroyuki Saimoto, H. (2013). Chitin Nanofibers, Preparations and Applications, Advances in Nanofibers, Russel Maguire (Ed.), ISBN: 978-953-51-1209-9, InTech, DOI: 10.5772/57095. Available from: http://www.intechopen.com/books/advances-in-nanofibers/chitin-nanofibers-preparations-and-applications.

Iyer, A., Mody, K. and Jha, B. (2004). Accumulation of hexavalent chromium by an exopolysaccharide producing marine *Enterobacter cloaceae*. Marine Pollution Bulletin, **49,** 974–977.

Kanmani, P., Satish K.R., Yuvaraj, N., Paari, K.A., Pattukumar, V. and Arul, V. (2011). Production and purification of a novel exopolysaccharide from lactic acid bacterium *Streptococcus phocae* PI80 and its functional characteristics activity in vitro. Bioresource Technology, **102,** 4827–4833.

Kantar, C., Demiray, H. and Dogan, N.M. (2011). Role of microbial exopolymeric substances (EPS) on chromium sorption and transport in heterogeneous subsurface soil. I. Cr(III) complexation with EPS in aqueous solution. Chemosphere, **82**, 1489-1495.

Kazy, S.K., Sar, P., Singh, S.P., Sen, A.K. and D'Souza, S.F. (2002). Extracellular polysaccharides of a copper-sensitive and a copper-resistant *Pseudomonas aeruginosa* strain: synthesis, chemical nature and copper binding, World Journal of Microbiology and Biotechnology, **18(6),** 583-588.

Kilic, N.K. and Donmez, G. (2008). Environmental conditions affecting exopolysaccharide production by *Pseudomonas aeruginosa, Micrococcus* sp., and *Ochrobactrum* sp., Journal of Hazardous Materials, **154,** 1019–1024.

Krumm, S., WINFIT 1.2: version of November 1996 (The Erlangen geological and mineralogical software collection) of WINFIT 1.0: a public domain program for interactive profile-analysis under WINDOWS. XIII Conference on Clay mineralogy and Petrology, Praha, 1994. Acta Universitatis Carolinae, Geologica, **38**, 253-261.

Kumar, C.G., John, H.S., Choi, J.W., Koo, Y.M. and Changa, C.S. (2004). Purification and characterization of an extracellular polysaccharide from haloalkalophilic *Bacillus* sp. I-450. Enzyme and Microbial Technology, **34,** 673-681.

Larpin, S., Sauvageot, N., Pichereau, V., Laplace, J.M., Auffray, Y. (2002). Biosynthesis of exopolysaccharide by a *Bacillus licheniformis* strain isolated from ropy cider. International Journal of Food Microbiology, **77,** 1-9.

Laspidou, C.S. and Rittmann, B.E. (2002). A Unified Theory for Extracellular Polymeric Substances, Soluble Microbial Products and Active and Inert Biomass. Water Research, **36,** 2711-2720.

Leppard, G.G., Droppo, I.G., West, M.M. and Liss, S.N. (2003). Compartmentalization of metals within the diverse collodial matrices comprising activated sludge microbial flocs. Journal of Environmental Quality, **32,** 2100–2108.

Liu, H. and Fang, H.H.P. (2002). Characterization of electrostatic binding sites of extracellular polymers by linear programming analysis of titration data. Biotechnology and Bioengineering, **80,** 806–811.

Liu, C., Lu, J., Lu, L., Liu, Y., Wanga, F. and Xiao, M. (2010). Isolation, structural characterization and immunological activity of an exopolysaccharide produced by *Bacillus licheniformis* 8-37-0-1. Bioresource Technology, **101,** 5528–5533.

Muzzarelli, C., Stanic, V., Gobbi, L., Tosi, G. and Muzzarelli, R.A.A. (2004). Spray-drying of solutions containing chitosan together with polyuronans and characterisation of the microspheres. Carbonhydrate Ploymers, **57,** 73-82.

Nandal, K., Sehrawat, A.R., Yadav, A.S., Vashishat, R.K. and Boora, K.S. (2005). High temperature-induced changes in exopolysaccharides, lipopolysaccharides and protein profile of heat-resistant mutants of *Rhizobium* sp. (Cajanus). Microbiologycal Research, **160,** 367–373.

Nicolaus, B., Lama, L., Panico, A., Schianomoriello, V., Romano, I. and Gambacorta, A. (2002). Production and Characterization of Exopolysaccharides Excreted by Thermophilic Bacteria from Shallow, Marine Hydrothermal Vents of Flegrean Ares (Italy). System Applied Microbiology, **25,** 319–325.

Nielsen, P.H. and Jahn, A. (1999). Extraction of EPS. In: Wingender, J., Neu, T.R. and Flemming, H.C. editors. Microbial extracellular polymeric substances: characterization, structure and function. Berlin Heidelberg: Springer-Verlag; p: 49–72. Chapter 3.

Ozdemir, G., Ozturk, T., Ceyhan, N., Isler, R. and Cosar, T. (2003). Heavy metal biosorption by biomass of *Ochrobactrum antropi* producing exopolysaccharide in activated sludge. Bioresource Technology, **90,** 71–74.

Ozturk, S., Aslim, B. and Suludere, Z. (2009). Evaluation of chromium(VI) removal behaviour by two isolates of *Synechocystis* sp. in terms of exopolysaccharide (EPS) production and monomer composition. Bioresource Technology, **100,** 5588–5593.

Pagnanelli, F., Petrangeli P.M., Toro, L., Trifoni, M. and Vegli, U.F. (2000). Biosorption of metal ions on Arthrobacter sp.: biomass characterization and biosorption modelling. Environmental Science and Technology, **34,** 2773–2778.

Priester, J.H., Olson, S.G., Webb, S.M., Neu, M.P., Hersman, L.E. and Holden, P.A. (2006). Enhanced Exopolymer Production and Chromium stabilization in *Pseudomonas putida* unsaturated biofilms. Applied and Environmental Microbiology, 1988–1996.

Ricou, P., Pinel, E. and Juhasz, N. (2005). Temperature experiments for improved accuracy in the calculation of polyamide-11 crystallinity by X-ray diffraction, in: Advances in X-ray Analysis, International Centre for Diffraction Data, USA, **48,** 170-175.

Sheng, G-P., Yu, H-Q. and Yue, Z-B. (2005). Production of Extracellular Polymeric Substances from *Rhodopseudomonas acidophila* in the Presence of Toxic Substances. Applied Microbiology and Biotechnology, **69,** 216-222.

Sheng, G-P., Yu, H-Q. and Li, X-Y. (2010). Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. Biotechnology Advances, **28,** 882–894.

Southgate, D.A.T. (1976). Determination of Food Carbohydrates. (England: Applied Science Publishers Ltd.)

Sutherland, I.W. (2001). Biofilm exopolysaccharides: a strong and sticky framework. Microbiology- SGM , **147,** 3–9.

Torres, C.A.V., Antunes, S., Ricardo, A.R., Grandfils, C., Alves, V.D., Freitas, F. and Reis, M.A.M. (2012). Study of the interactive effect of temperature and pH on exopolysaccharide production by *Enterobacter* A47 using multivariate statistical analysis. Bioresource Technology, **119,** 148–156.

Tourney, J., Ngwenya, B.T., Mosselmans, J.W.F. and Magennis, M. (2009). Physical and chemical effects of extracellular polymers (EPS) on Zn adsorption to *Bacillus licheniformis* S-86. Journal of Colloid and Interface Science, **337,** 381-389.

Wingender, J., Neu, T.R. and Flemming, H.C. (1999). What are bacterial extracellular polymeric substances? In: Wingender, J., Neu, T.R. and Flemming, H.C. editors. Microbial extracellular polymeric substances: characterization, structures and function. Berlin Heidelberg: Springer-Verlag; p: 1-18. Chapter 1.

Zhang, X.Q. and Bishop, P.L. (2003). Biodegradability of biofilm extracellular polymeric substances. Chemosphere, **50,** 63–9.