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Journal of Agricultural Sciences

Journal homepage:
www.agri.ankara.edu.tr/journal

Effects of Cultural Conditions on Exopolysaccharide Production by *Bacillus* sp. ZBP4

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ARTICLE INFO

Research Article

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Received: 20 February 2016, Received in Revised Form: 03 February 2017, Accepted: 08 February 2017

ABSTRACT

Microbial exopolysaccharides (EPSs) are of great interest for the application in various industries due to their gelling, stabilizing, emulsifying, and antioxidant properties. In the present study, EPS production of 12 *Bacillus* strains were investigated and the best producer, namely *Bacillus* sp. ZBP4, was selected for further studies in order to determine the effects of fermentation conditions on the biosynthesis of EPSs. Beet molasses was used as substrate in the experiments. The highest amount of EPS was obtained at 60 g L⁻¹ molasses concentration within 24 h. Optimum temperature and pH were determined as 45 °C and 5.0, respectively. Various carbon sources (glucose, starch, lactose, whey, mannitol, sucrose, beet molasses) have been tested for EPS production and beet molasses was found as the best. Using inorganic nitrogen source (ammonium sulfate) caused a decrease in the production of EPS. Tryptone gave the highest EPS yields amongst the organic nitrogen sources (yeast extract, peptone, tryptone) tested. Considerable increase in EPS production (1071 mg L⁻¹) has been observed when the experiment was conducted under the optimized conditions (using tryptone and 60 g L⁻¹ molasses at pH 5.0 and 45 °C in 24 h) which was 143 mg L⁻¹ before the optimization studies.

Keywords: *Bacillus*; Exopolysaccharide (EPS); Beet molasses

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1. Introduction

A group of microorganisms including the strains of bacteria, yeast and molds, are able to secrete high molecular weight polymers into their surroundings called as exopolysaccharide (EPS) (Koçberber & Dönmez 2008; Fang et al 2013). Microorganisms produce EPSs as a response to harsh conditions in order to prevent cell damage (Donot et al 2012). Main structures of EPSs are comprised of monosaccharides, particularly glucose, galactose and rhamnose (Welman et al 2003). The interest

in EPSs have increased considerably in the recent years, because of their physiological, chemical and rheological properties which make them suitable for a wide range of commercial applications in different fields, such as food, petroleum, cosmetics, textile, bioremediation and pharmaceuticals (Freitas et al 2011; Singh et al 2011; Öztürk et al 2014; Zhou et al 2014).

Even though numerous microorganisms can secrete EPSs, bacteria are considered as the best producers owing to the quality and quantity of EPSs

(Kumar 2012). During the recent decades several *Bacillus* strains reported to produce EPSs, such as *B. licheniformis* (Sing et al 2011), *B. subtilis* (Shih et al 2010) and *B. firmus* (Salehizadeh & Shojaosadati 2003). Levan is the best known EPS produced by *Bacillus* sp. which is mainly produced from sucrose. It is highly soluble in water, and has biological activity (e.g: anti-tumor and anti-inflammatory). It can be applied in food, feed, medicine, and cosmetics (Freitas et al 2011; Donot et al 2012).

The yield and the composition of EPSs produced by microorganisms are mainly dependent on the cultural conditions such as temperature, pH and medium composition (Tallon et al 2003; Çelik et al 2008). Sugars are mostly used for the production of EPSs as carbon source. However, there are researches concerning the utilization of cheaper carbon sources, generally agro-industrial wastes or by-products (Göksungur et al 2004; Freitas et al 2011). Molasses is a by-product of sugar industry either from the sugar cane or sugar beet. In Turkey, sugar industry relies on beet, hence beet molasses is readily available and the most preferred substrate in the fermentation industry. It has high sugar content (approximately 50%) that can be fermented by yeasts and bacteria. In addition, it contains nitrogen, vitamins and minerals which support fermentation (Yilmaz et al 2012; Abdul Razack et al 2013).

The aims of the current study were; a) to investigate the EPS production capabilities of some *Bacillus* strains that were isolated from various food and soil samples, b) after selecting the best EPS producing isolate, to produce EPS using molasses by this strain (*Bacillus* sp. ZBP4) and c) to determine effects of process conditions (temperature, pH and substrate concentration) and some nitrogen and carbon sources on the EPS production.

2. Material and Methods

2.1. Material

Molasses was obtained from Adapazarı Sugar Factory in the 2014 season. Nutrient agar, nutrient broth, trichloroacetic acid (TCA), phenol, yeast extract,

peptone, and tryptone were purchased from Merck (Darmstadt, Germany) and 3,5-dinitrosalicylic acid (DNS) was purchased from Sigma (USA).

2.2. Cultural conditions and selection of EPS producing microorganisms

The isolates which were maintained in nutrient broth containing 50% glycerol at -18 °C, were activated using nutrient agar plates at 35 °C for 24 h. Single colonies from the plates were inoculated into 30 mL nutrient broth in 100 mL Erlenmeyer flasks. Then, they were cultivated aerobically (120 rpm) at 35 °C for 24 h to be used as seed culture for the production of EPSs.

For the screening of EPS producing microorganisms, a medium containing (as g L⁻¹); glucose 10, yeast extract 5, K₂HPO₄ 1.5 and MgSO₄.7H₂O 1 was prepared. Unless otherwise mentioned, production experiments were conducted in 100 mL Erlenmeyer flasks containing 30 mL medium. The pH of the medium was adjusted to 7.0 prior to sterilization at 121 °C for 15 min. Then, the flasks were inoculated with 5% fresh cultures (having 2.0 optical density at 600 nm) and incubated at 35 °C on a shaking incubator for 24 h. EPSs produced by the isolates were extracted as described below and the amounts of total sugars were determined using phenol-sulfuric acid method (Dubois et al 1956). In screening experiments, *Bacillus* sp. ZBP4 isolate has produced the highest amount of EPS. Hence, it has been selected for further studies. This bacterium was previously isolated from a soil sample taken from the potato cultivation field and identified as *Bacillus subtilis* ZBP4 based on 16S rDNA sequence analysis. The sequence is available in GenBank (Acc No. KX811594) (Avci et al 2017).

2.3. Preparation of molasses

Raw molasses was diluted with deionized water to a ratio of 1:1 (w w⁻¹). After the adjustment of pH to 4.0 using 2 N HCl, it was boiled for the decantation of impurities. Upon cooling, it was first filtered through regular filter paper followed by centrifugation at 9000 rpm for 10 min. The resulting supernatant was used as substrate for EPS production.

2.4. Production of EPS from molasses

A basal medium was prepared which contained; molasses 40 g, yeast extract 5 g, K_2HPO_4 1.5 g and $MgSO_4 \cdot 7H_2O$ 1 g in 1000 mL of deionized water. Initial pH of the medium was adjusted to 7.0, except pH experiments. Five percent ($v v^{-1}$) fresh seed culture having optical density of 2.0 (at 600 nm) was used to inoculate flasks. The effect of temperature on the EPS production was determined by incubating the bacterium in the basal medium at varying temperatures ranging from 30 to 45 °C on a shaking incubator at 120 rpm for 24 h. In order to determine the effect of pH on the production of EPSs, initial pH of basal media were adjusted to different pH values between 4.0 and 9.0 by using either 2 N HCl or 2 N NaOH. Incubations for pH experiments were carried out at 35 °C on a shaking incubator at 120 rpm for 24 h. For the determination of the effect of substrate concentration, basal media were prepared using molasses at concentrations ranging from 10 to 60 g L^{-1} and the pH was adjusted to 7.0. The bacterium was grown in these media at 35 °C on a shaking incubator at 120 rpm for 24 h. The effect of nitrogen sources was studied by replacing yeast extract from the basal medium with ammonium sulfate, tryptone, and peptone (5 g L^{-1}) and one experiment was also performed without any nitrogen source. Selected carbon sources (starch, lactose, mannitol, glucose, whey) were also tested separately by replacing the molasses from the basal medium. The concentration of each carbon source was 30 g L^{-1} . In the experiments regarding the effects of nitrogen sources, the initial pHs of the media were adjusted to 5.0 by using 2N HCl, and all incubations were conducted with shaking at 120 rpm, 45 °C for 24 h.

Finally, an experiment was conducted at the optimized conditions where a medium containing; molasses 60 g, tryptone 5 g, K_2HPO_4 1.5 g and $MgSO_4 \cdot 7H_2O$ 1 g in 1000 mL of deionized water, was prepared with an initial pH of 5.0. The bacterium was incubated in this medium at 45 °C for 24 h on a shaking incubator at 120 rpm. Sample was taken at the end of the incubation and subjected to analysis of EPS.

2.5. EPS extraction

EPS extraction was done according to Koçberber & Dönmez (2008) with some modifications. Briefly, 5 mL sample was boiled for 15 min to inactivate enzymes in the fermentation broth. Upon cooling, TCA (4%; $w v^{-1}$) was added into the sample, and subsequently vortexed and centrifuged at 9000 rpm, 4 °C for 30 min to precipitate proteins. Supernatant was transferred into a clean tube and the same volume of 99.5% cold ethanol (at 4 °C) was added. The mixture was stored at 4 °C overnight. Then it was centrifuged at 9000 rpm, 4 °C for 30 min to precipitate the EPS. Supernatant was removed and pellets containing the EPSs were dissolved in 1 mL of deionized water and used as crude EPSs.

2.6. Determination of EPS amount

Total sugars were determined using phenol-sulfuric acid method described by Dubois et al (1956) using a glucose standard curve. Same samples were also subjected to reducing sugar analysis by 3,5-dinitrosalicylic acid (DNS) method (Miller 1959) to detect trace sugars in the samples and the results were subtracted from the total sugars determined by phenol-sulfuric acid method.

2.7. Statistical analysis

All the experiments were conducted at least in duplicate and values as mean \pm SD were reported. Duncan's Multiple Range Test was applied for the determination of the significance among the means ($P < 0.05$).

3. Results and Discussion

3.1. Screening of *Bacillus* strains for EPS production

Twelve *Bacillus* strains that were isolated from soil and some food samples were screened for their EPS productions. Two of the isolates did not produce EPSs and the rest of them produced varying amounts ranging from 13.4 to 143.1 mg L^{-1} (Table 1). EPS producing bacteria formed mucoid colonies on agar media that was also an indication of the formation of EPSs (Figure 1). The best EPS producer, *Bacillus* sp. ZBP4, was used for the production of EPSs from molasses.

Table 1- Production of exopolysaccharides by *Bacillus* strains isolated from various sources from glucose; the initial pHs of the media were adjusted to 7.0 and incubations were performed on a shaking incubator (120 rpm) at 35 °C for 24 h

<i>Bacteria</i>	<i>Amount of EPS (mg L⁻¹)</i>
<i>Bacillus</i> sp. BAST2	13.4
<i>Bacillus</i> sp. BMZE2	43.5
<i>Bacillus</i> sp. BMZE3	81.9
<i>Bacillus</i> sp. BMZE4	39.8
<i>Bacillus</i> sp. ZGT1	0.0
<i>Bacillus</i> sp. ZGT3	40.0
<i>Bacillus</i> sp. ZGT5	0.0
<i>Bacillus</i> sp. ZGT9	48.8
<i>Bacillus</i> sp. ZBP4	143.1
<i>Bacillus</i> sp. ZBP10	99.2
<i>Bacillus</i> sp. GİT2	48.5
<i>Bacillus</i> sp. BAT3	72.2

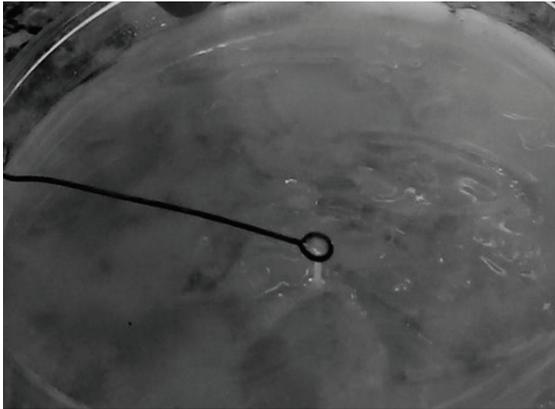


Figure 1- Mucoid appearance of exopolysaccharide (EPS) produced by *Bacillus* sp. ZBP4 on nutrient agar

3.2. Effect of growth parameters on EPS production

3.2.1. Effect of substrate concentration on EPS production

Optimization of the growth parameters effecting the EPS production have been investigated using beet molasses as carbon source which contained 46.3% (w w⁻¹) total sugars. Initially, optimal molasses concentration was determined using

10-60 g L⁻¹ molasses in the basal medium. The lowest EPS production has been detected at 10 g L⁻¹ concentration which was 68 mg L⁻¹. Significant increases in EPS production have been observed with increasing concentrations (P<0.05) and it reached its maximum (426 mg L⁻¹) at 60 g L⁻¹ molasses concentration which corresponds to 27.8 g L⁻¹ total sugars (Figure 2). It has been indicated that EPS production was stimulated by the excess of carbohydrate in the medium and limitation of carbon sources diminishes EPS synthesis (Van Geel-Schutten et al 1998; De Vuyst & Degeest 1999). A number of reported researches concerning the effect of substrate concentration suggest that optimum substrate concentration varies depending on the individual microorganism. For instance, Çelik et al (2008) tested the effect of substrate concentration on EPS production by *Pseudomonas aeruginosa* G1 and *Pseudomonas putida* G12 using xylose and found the maxima as 368 mg L⁻¹ and 262 mg L⁻¹ at 3% (w v⁻¹) and 2% concentrations, respectively. *Halomonas anticariensis* produced EPS better at 1% (w v⁻¹) glucose concentration (Mata et al 2006). *Bacillus licheniformis* produced maximum EPS (~600 mg L⁻¹) with 2% sucrose (w v⁻¹) (Larpin et al 2002). On the other hand, there are microorganisms requiring much higher substrate concentrations, such as *Rhizobium radiobacter* which produced 2834 mg L⁻¹ EPS on 10% whey (Zhou et al 2014).

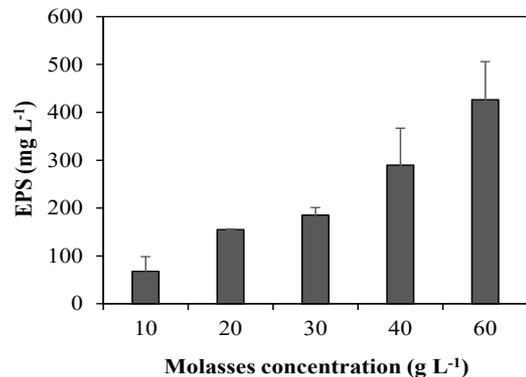


Figure 2- Production of exopolysaccharide by *Bacillus* sp. ZBP4 at varying molasses concentrations; the initial pHs of the media were adjusted to 7.0 and incubations were performed on a shaking incubator (120 rpm) at 35 °C for 24 h

Production of EPS was also determined during longer incubation periods and a drastic decrease (ca 30-40%) has been detected at 48 h and 72 h of the incubation compared with the production at 24 h (data not shown). The decrease in EPS yield after a certain incubation time is due to the activity of glycohydrolases secreted by the microorganisms into fermentation medium which catalyze the degradation of EPS (Li et al 2014).

3.2.2. Effect of temperature on EPS production

Temperature for EPS biosynthesis is crucial (Lui et al 2009). Thus, the impact of temperature on EPS production was determined by incubating the bacterium in the basal medium at temperatures ranging between 30 and 45 °C. Figure 3 depicts the EPS amounts produced at varied temperatures and growth of the bacterium which were given as optical density (OD_{600}) measured by using a spectrophotometer. Interestingly, temperatures beyond the optimum growth temperature (33-35 °C) of *Bacillus* sp. ZBP4 promoted the production of EPS, and the production became the lowest at temperatures 35 and 37 °C where the strain ZBP4 grows well. Means of the values determined at different temperatures were statistically different ($P < 0.05$). Various temperature dependencies were reported in the literature. For instance, Wang et

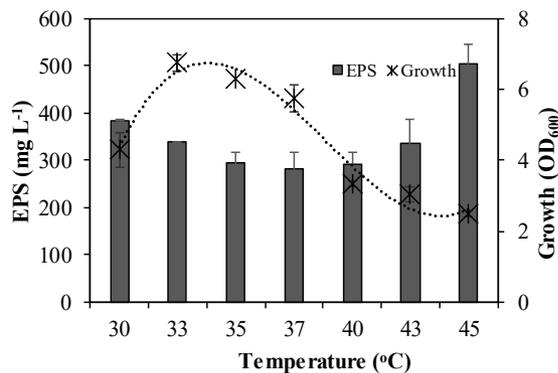


Figure 3- Growth and exopolysaccharide production by *Bacillus* sp. ZBP4 at different temperatures; the initial pH of the media were adjusted to 7.0 and incubations were performed on a shaking incubator (120 rpm) for 24 h

al (2011) obtained maximum amount of EPS with *Paenibacillus* sp. TKU023 at 37 °C while having lower yields at low temperatures which is not in accord with our findings. Lui et al (2009) reported the best EPS production temperature for *Paenibacillus polymyxa* as 24 °C. It is known that EPSs help the cell in protecting it from stress conditions such as temperature, pH, and light intensity, thus its production is a direct response to environmental conditions (Donot et al 2012). In our study, higher production rates of EPSs at lower or higher temperatures can be attributed to that phenomenon because *Bacillus* sp. ZBP4 produced the maximum amount of EPS at 45 °C, which is reasonably higher than the optimum growth temperature of the bacterium.

3.2.3. Effect of pH on EPS production

The pH of the medium is an important parameter affecting the cell membrane and structure thereby nutrient uptake and EPS production are also influenced (Liu et al 2009). In this regard, EPS production was performed at different pH values and the results showed that EPS biosynthesis has been significantly affected by the pH of the medium ($P < 0.05$) (Figure 4). The highest EPS production was achieved at pH 5.0 and it decreased with increasing pH values. pH 4.0 and pH 9.0 resulted in the lowest

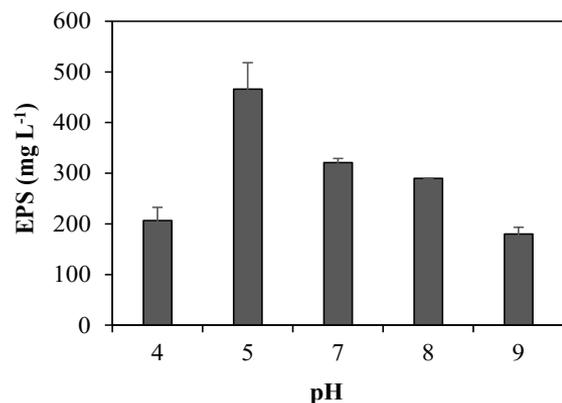


Figure 4- Effect of initial medium pH on the production of exopolysaccharide by *Bacillus* sp. ZBP4; incubations were performed on a shaking incubator (120 rpm) at 35 °C for 24 h

EPS productions. Levansucrase is an extracellular enzyme that catalyzes the production of levan from sucrose and it was reported that it is produced when the pH of the medium is acidic (Castillo & Lopez-Mungia 2004; Donot et al 2012). That can be possible explanation why our strain had produced EPS maximally at pH 5.0. Moreover, as mentioned above, harsh conditions forces microorganisms to secrete EPS for protection.

The medium composition affects the EPS secretion by microorganisms to a great extent (Çelik et al 2008). Carbon sources display different effect on catabolic repression and secondary metabolisms (Liu et al 2009). In this study, effects of different carbon sources on EPS production were tested by replacing molasses in the basal medium with glucose, lactose, starch, whey and mannitol. *Bacillus* sp. ZBP4 produced EPS on all the carbon sources tested in varying amounts. Yet, molasses was the best carbon source followed by whey and glucose which resulted in reasonably higher yields than the other carbon sources tested. Levan is synthesized by the enzyme levansucrase from sucrose (Donot et al 2012). Since the major sugar constituent of molasses is sucrose, most probably the stain ZBP4 is producing levan. However, when pure sucrose was used, the production of EPS decreased dramatically. It can be suggested that the amino acids found in molasses significantly promoted the production. The same case was also seen with whey which contains lactose and proteins. EPS production was doubled when whey was used compared with lactose. Similar results were obtained by Abdul Razack et al (2013) who produced EPS by *B. subtilis* from cane molasses and sucrose and they found 4.86 and 2.98 g L⁻¹ EPSs, respectively. As being economically viable and fermentable by many microorganisms, either cane or beet molasses were successfully used in a number of studies (Göksungur et al 2004; Abdel-Aziz et al 2012; Sirajunnisa et al 2012; Abdul Razack et al 2013). Mannitol gave the weakest yields followed by starch and lactose.

Several nitrogen sources (tryptone, peptone, yeast extract, ammonium sulfate) were tested in order to find out the best one for EPS production by *Bacillus*

sp. ZBP4 (Table 2). Ammonium sulfate that was used as inorganic nitrogen source led to decrease in EPS synthesis. Similarly, some researchers have also found decreased EPS yields with inorganic nitrogen compounds and it was proposed that some amino acids cannot be synthesized from inorganic nitrogen sources (Abdul Razack et al 2013). Amongst the organic nitrogen sources used, tryptone was found as an excellent ingredient with which 974±72 mg L⁻¹ EPS was obtained while it was 525±2 mg L⁻¹ when the yeast extract was used. Peptone seemed to be ineffective on EPS production which gave almost the same amount of EPS when there is no nitrogen supplement in the medium.

Table 2- Effects of carbon and nitrogen sources on the production of exopolysaccharides by *Bacillus* sp. ZBP4; the initial pH of the media were adjusted to 5.0 and all the incubations were carried out on a shaking incubator (120 rpm) at 45 °C for 24 h

Carbon sources	EPS (mg L ⁻¹)	Nitrogen sources	EPS (mg L ⁻¹)
Glucose	450 ^d ±10	Ammonium sulfate	388 ^a ±67
Starch	171 ^b ±42	Tryptone	974 ^c ±72
Lactose	220 ^{bc} ±9	Yeast extract	525 ^b ±2
Whey	452 ^d ±25	Peptone	448 ^{ab} ±42
Mannitol	103 ^a ±22	Without nitrogen**	432 ^{ab} ±19
Sucrose	243 ^{bc} ±21		
Molasses	505 ^d ±41		

*, means with different letters in each column are significantly different for each sample (P<0.05); **, only molasses was used in the medium without nitrogen supplementation

4. Conclusions

EPS production by *Bacillus* sp. ZBP4 was investigated using molasses as substrate. The effects of substrate concentration, pH, temperature, and carbon and nitrogen sources on the production of EPSs were determined. The strain produced the highest amount of EPS in the medium having an initial pH of 5.0, at 60 g L⁻¹ molasses concentration, at 45 °C in 24 h. Use of tryptone as nitrogen source has markedly enhanced the secretion of EPSs. The microorganism produced 1071 mg L⁻¹ EPS under the optimized conditions. The results showed that

the isolate *Bacillus* sp. ZBP4 can be good candidate for the production of EPSs and it was able to utilize molasses both as carbon and nitrogen sources. However, there is a need for more studies in this topic.

Acknowledgements

This work is part of the 2014-50-01-013 project financially supported by the Sakarya University Scientific Research Projects Units. We express our sincerest gratitude to them for their support Sakarya University Scientific Research Projects Units. The authors thank İnci CANTİK (Sakarya University, Department of Food Engineering) for performing statistical analysis.

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