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Production and Optimization of Exopolysaccharide from Thermophilic Bacteria

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Abstract

Exopolysaccharides (EPS) are the large molecular weight carbohydrate polymers extracted from higher plants, algae, fungi and bacteria. The thermophilic *Bacillus zhangzhounesis* 2CA and *Bacillus licheniformis* 2CS used in the present study were isolated from Çermik hot springs. The growth conditions of the strains designated as 2CA and 2CS in different basal media (M1, M2 and M3), different carbon sources and different concentrations of yeast extract (%w v^{-1}: 0.05, 0.1, 0.15 and 0.2) and the amount of EPS produced were investigated. In addition, the phenol-sulfuric acid method and the Lowry method were used to determine the amount of carbohydrates and proteins within the EPS produced by the bacteria, respectively. The highest total EPS dry weight for *B. licheniformis* 2CS was obtained as 121 mg in M3 medium (0.2% yeast extract + 1% sucrose), carbohydrate content in EPS was 333.28 µg mL^{-1} and protein content was 0.19 µg mL^{-1}. When these two bacteria were compared in terms of the amount of carbohydrates in the EPS produced, the highest amount of carbohydrates was found in EPS of *B. zhangzhounesis* 2CA (1087.03 µg mL^{-1}). The antibacterial effects of EPS were investigated against pathogenic microorganisms (*E. coli, S. aureus, K. pneumoniae* and *P. aeruginosa).* It was determined that the highest antibacterial activity against *E. coli* (with 16 mm zone diameter) was obtained with EPS produced by *B. licheniformis* 2CS bacteria in M3 medium (0.2% yeast extract + 1% sucrose).

Keywords: Antibacterial effect, exopolysaccharide production, thermophilic bacteria.

Termofilik Bakterilerden Ekzopolisakkarit Üretimi ve Optimizasyonu

Öz

Ekzopolisakkaritler (EPS), daha yüksek bitkilerden, alglerden, mantarlardan ve bakterilerden ekstrakte edilen büyük moleküler ağırlıklı karbonhidrat polimerleridir. Bu çalışmada kullanılan termofilik *Bacillus zhangzhounesis* 2CA ve *Bacillus licheniformis* 2CS, Çermik kaplıcalarından izole edilmiştir. Farklı bazal besiyerlerinde (M1, M2 ve M3), farklı karbon kaynaklarında (glikoz ve sükroz) ve farklı konsantrasyonlarda maya özütü (%w v^{-1}: 0.05,0.1, 0.15 ve 0.2) eklenen besiyerinde 2CA ve 2CS olarak isimlendirilen bakteri suşlarının çoğalma şartları ve üretilen EPS miktarı araştırılmıştır. Ayrıca, fenol-sülfürik asit yöntemi ve Lowry yöntemi, bakterilerin ürettiği EPS'deki karbonhidrat miktarını ve protein miktarını belirlemek için sırasıyla kullanıldı. *B. licheniformis* 2CS için en iyi toplam EPS kuru ağırlığı, M3 ortamında (%0.2 maya özütü + %1 sükroz) 121 mg, EPS'deki karbonhidrat miktarı 333.28 µg mL^{-1}, protein miktarı ise 0.19 µg mL^{-1} olarak elde edildi. Bu iki bakteri üretilen EPS'deki karbonhidrat miktarı bakımından karşılaştırıldığında, en yüksek karbonhidrat miktarının *B. zhangzhounesis* 2CA'daki EPS'de olduğu tespit edildi (1087.03 µg mL^{-1}). Test edilen mikroorganizmaların ürettiği EPS'nin patojenik mikroorganizmalara (*E. coli, S. aureus K. pneum*oniae ve *P. aeruginosa*) karşı antibakteriyel aktiviteleri araştırıldı. En iyi antibakteriyel etkinin, M3 ortamında (%0.2 maya özütü + %1 sükroz) *B. licheniformis* 2CS bakterisinin ürettiği EPS ile *E. coli*'ye karşı (16 mm zon çapı ile) olduğu belirlendi.

Anahtar Kelimeler: Antibakteriyel etki, ekzopolisakkarit üretimi, termofilik bakteriler

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INTRODUCTION

Bacterial exopolysaccharides (EPS) are macromolecular structures that can be found in the capsule structure tightly attached to the cell surface or in the mucous (slime) structure attached to the cell surface by loose attachment (Farag et al., 2020).

In addition, exopolysaccharides are also produced by plants, algae, yeast and fungi. EPS production by bacteria can take days, while three to six months are needed to be produced by plants. EPSs are branched and consist of repeating sugar units and non-carbohydrate components. Exopolysaccharides obtained as a result of polymerization of sugar units with similar or different structures are organic macromolecules. These sugar units can be glucose, galactose, rhamnose, fructose, mannose as well as some sugar derivatives such as N-acetylgalactosamine and N-acetylglucosamine. EPSs can contain proteins, DNA, phospholipids and non-carbohydrate structures such as pyruvate, sulphate, and phosphate also (Moretto et al., 2015; Saadat et al., 2019; Sethi et al., 2019; Angelin and Kavitha, 2020; Farag et al., 2020).

The identification of EPSs is carried out by analysing their carbohydrate content. If EPSs consist of a single type of monomeric structure, they are called homopolysaccharides. On the other hand, EPSs formed by the combination of two or more different monomers are called as heteropolysaccharides.

While most homopolysaccharides are neutral glucans, most heteropolysaccharides are polyanionic due to the uronic acid in their structure. Homopolysaccharides can be classified as α-D glucans and β-D glucans based on the bond structure and the position of the carbon (Llamas et al., 2012; Zannini et al., 2016).

EPSs protect bacteria from extreme conditions such as stress, temperature, light, pH, osmotic. If there is a lack of nutrients in the environment, EPS produced by microorganisms ensures the survival of the organism. It has been determined that some EPSs can have a virulence effect in different infectious diseases as well as have positive effects on human health (Llamas et al., 2012; Hidalgo-Cantabrana et al., 2014; Caggianiello et al., 2016).

There have been several studies on EPS production using bacterial strains such as; *B. subtilis* (Razack et al. 2013), *B. velezensis* (Moghannem et al. 2018), *B. alttitudinis* (Mohamed et al. 2018), *B. licheniformis* (Angel et al. 2018), *Geobacillus* (Wang et al. 2019) and *B. zhangzhounesis* (Miri et al. 2021).

Bacterial EPSs are also used in the food industries. Dextran, a type of EPS produced by bacteria, was the first commercially used bacterial exopolysaccharide. Fungal EPSs have antioxidant, antitumor and antibacterial effects. Microalgal EPSs can be found in a heteropolysaccharide structure consisting of a homopolymer of glucose or galactose or a combination of monomers of several different sugars (Freitas et al., 2017).

There are different ways to quantify EPSs obtained by microorganisms, these may be the measurement of dry mass of the polymer by lyophilized, Ludwig's anthron sulfuric acid method. In addition, carbohydrate determination of EPS can be made with the phenol-sulfuric acid method (Leroy and De Vuyst, 2016).

It is aimed to determine the presence of EPS in different media by using thermophilic and mesophilic bacteria and optimize the EPS production.

MATERIAL AND METHODS

Bacterial strains

In the study, thermophilic bacteria *Bacillus zhangzhounesis* 2CA (GenBank accession code: MT350124), *Bacillus licheniformis* 2CS (GenBank accession code: MT350130) isolated and identified from the Cermik hot water spring in Diyarbakır province (Matpan Bekler et al., 2020). Pathogenic microorganisms (*Escherichia coli* ATTC 25922, *Staphylococcus aureus* ATTC 25923, *Klebsiella pneumoniae and Pseudomonas aeruginosa*) were obtained from the Medical Microbiology laboratory of Dicle University Research Hospital for antibacterial test.

Bacteria production

The thermophilic *B. zhangzhounesis* 2CA and *B. licheniformis* 2CS Bacteria were cultivated in using Nutrient Broth (NB) liquid media in a shaker (120 rpm) for *B. zhangzhounesis* 2CA and *B. licheniformis* 2CS at 50 °C overnight (18-24 h), while pathogenic microorganisms were grown at 35 °C overnight (18-24 h).

Production of EPS using different basal media

Different basal media containing minimal amounts of chemicals were used to investigate EPS production. The basal media used were basal M1 medium (yeast extract 5 g L^{-1}, dipotassium hydrogen

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phosphate 1.5 g L^{-1} and magnesium sulphate heptahydrate 1 g L^{-1}) described by Ergene and Avcı (2018), basal M2 medium (K_2HPO_4 8 g L^{-1}, KH_2PO_4 2 g L^{-1}, $MgSO_4.7H_2O$ 0.5 g L^{-1}, $(NH_4)_2.SO_4$ 5 g L^{-1} and traces of yeast extract) described by Berekaa and Ezzeldin (2018) and finally basal M3 medium ($CaCl_2$ 0.05 g L^{-1}, $MgSO_4.7H_2O$ 0.05 g L^{-1}, KH_2PO_4 1 g L^{-1}, NaCl 2 g L^{-1}, $(NH_4)_2.SO_4$ 2 g L^{-1} with different concentrations of yeast extract) modified from Matpan Bekler et al. (2019). Bacteria were cultivated on the prepared media under optimum conditions. Its absorbance was adjusted at to a 0.5 McFarland turbidity standard (5×10^5 CFU mL^{-1}) using spectrophotometer. Bacteria were grown overnight and then optical density was measured by spectrophotometer (600 nm).

Production of EPS using different yeast extract concentrations

Basal M3 medium that showed the least growth for the control was selected and yeast extract was added to this medium at different concentrations (0.15% and 0.2%). Finally, the calculated amounts of yeast extract were added into the media and bacteria were grown under optimum conditions (pH 8.0 and 50 °C for the strain 2CA, pH 7.0 and 55 °C for the strain 2CS). The bacterial growth was measured by using a spectrophotometer at OD600.

Production of EPS using different carbon sources

In order to examine the effect of different carbon sources (glucose and sucrose) at 1% on bacterial EPS production, the basal medium was firstly autoclaved. Then carbon sources were dissolved in distilled water, passed through a sterile syringe filter (0.2 µm) and finally added to the autoclaved medium before bacterial cultivation.

Determination of EPS production by thermofilic bacteria on petri dishes

To detect EPS production from bacterial colonies on petri dishes, 10-fold diluted NB medium and basal M3 media containing 0.1% and 0.2% yeast concentrations were prepared. 1.5% agar was also added to the media for solid medium. Sucrose was added to the autoclaved media at a concentration of 1%. Discs were placed on solid media. *B. zhangzhounesis* 2CA and *B. licheniformis* 2CS strains grown overnight, in the NB medium were removed and washed twice. Different amount (20, 40 and 60 µL) of washed bacteria were taken and transferred onto the discs. Growth and EPS production conditions were observed in the media kept in incubator at 50 °C for 24 hours. It was observed whether slime structure was formed in petri dishes.

Isolation and extraction of EPS

The cultures were transferred into tubes and centrifuged at 9 000 rpm for 10 minutes. The supernatant part was taken and cold pure ethyl alcohol kept at 4 °C was added in the ratio of 1 (example): 2 (alcohol). The samples were kept at -18 °C for 1 day. The samples kept at -18 °C for one day were taken and centrifuged at 9000 rpm for 30 minutes. One to three mL of sterile hot distilled water was added to the pellet part and dissolved. Dissolved pellets were taken into dialysis tubes. Dialysis tubes were kept in distilled water in a shaking environment and distilled water was changed constantly for 48 hours. Dialyzed samples were taken into clean eppendorfs and dried in an incubator at 60 °C for 48 hours. The dried samples were used for the determination of protein and carbohydrate amount.

Determination of carbohydrates and protein amount in EPS

To determine the total amount of carbohydrates, phenol sulfuric acid method (Dubois et al. 1956) was applied. A stock glucose solution was prepared with a density of 1 mg mL^{-1} to create the standart curve . To calculate the EPS amount, 2-300 µg mL^{-1} glucose samples were prepared. Dried EPS samples were dissolved with 500 µL of distilled water. Samples were analysed using a spectrophotometer at a wavelength of 490 nm.

For protein quantification, Lowry method (Lowry et al. 1951) was used. The standard concentration (10 mg 1 mL^{-1} BSA) was prepared to generate the standard curve. Dried samples were dissolved in distilled water. 2 mL alkaline solution was added into glass tubes and mixed. It was kept in an incubator at 40 °C for 15 min. 500 µL of Folin–Ciocalteu reagent (FCR) diluted 1:1 was added to the samples . Samples were then analysed by using a spectrophotometer at a wavelength of 660 nm.

Determination of the antibacterial effect of EPS

Four pathogenic bacteria namely *E. coli*, *S. aureus*, *K. pneumoniae* and *P. aeruginosa* kept in the stock were taken and cultivated into NB liquid medium at 35 °C overnight (18-24 h) in a shaker. Bacteria was added to the media poured into Petri dishes and spreading was carried out with the help of a glass baguette. Blank discs were then placed on the medium. 10 µL of EPS samples was added onto these discs. The incubation was performed using an

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incubator at 35 °C. The zone diameters were measured one day later. Gentamicin (10 µg) was used as a reference antibiotic.

RESULTS AND DISCUSSION

Growth of thermophilic bacteria on different media

Thermophilic *B.zhangzhounesis* 2CA and *B.licheniformis* 2CS were cultivated on three different media. The absorbance of the grown cultures was measured at 600 nm wavelength in a spectrophotometer. Figure 1a clearly shows that M3 medium with the absence of yeast extract gives a better results in terms of lower growth compared to other two media. Berekaa (2014) used bacteria that are phylogenetically close to *B.licheniformis* and showed that there was an increase in EPS production by 1.9-2.8 times in the basal salt medium containing glucose or sucrose. Wang et al. (2019) used glucose as an energy source and found that *Geobacillus* WSUCF1 strain produced statistically higher EPS

Effect of yeast extract concentrations on EPS yield

Different concentrations of yeast extract (between 0.05-0.2%) were added to selected basal M3 medium. The highest growth was obtained for *B.zhangzhounesis* 2CA bacteria grown in the medium containing 0.2% yeast (Figure 1b). The growth increase was not observed in M3 medium containing 0.05% and 0.1% yeast extract for both thermophilic bacteria. Moghannem et al. (2018) evaluated several factors to explain the optimal growth medium composition for maximum EPS production with *B.velezensis* KY498625 strain. Different carbon and nitrogen sources were used, the result obtained from this experiment indicated that the best carbon source was molasses at 4% w v^{-1} with EPS production of 4.2 g L^{-1}, while the lowest EPS production (1.88 g L^{-1}) was with starch (4% w v^{-1}). The best nitrogen source 4.4 g L^{-1} was yeast extract at concentration 3 g L^{-1}.

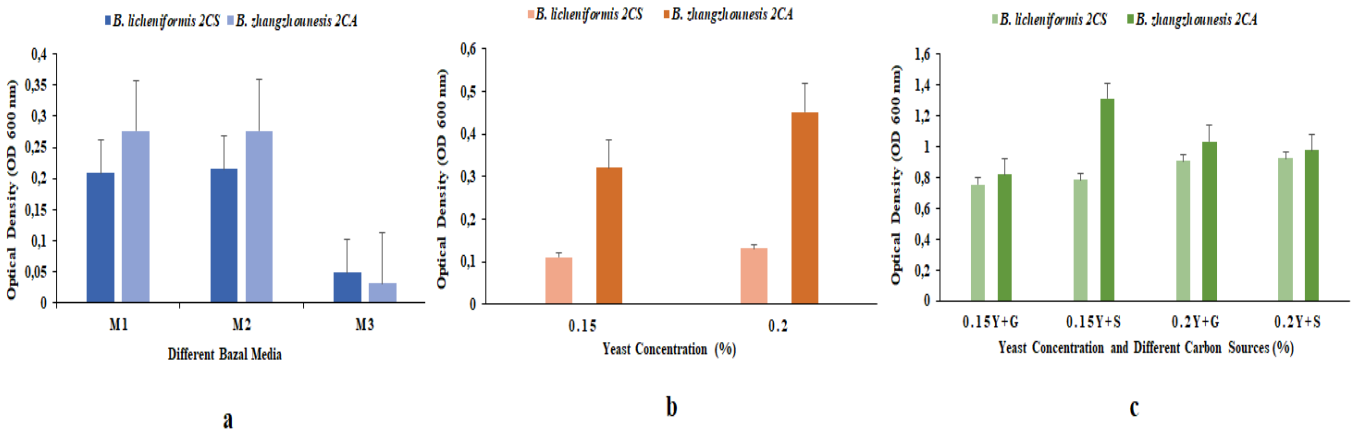

Figure 1.Growth graphs of *B.licheniformis* 2CS and *B. zhangzhounesis* 2CA a) on different basal media, b) yeast concentration c)yeast concentration and different carbon sources. Experiments were performed in three repetitions. The bars show the standard deviation.

Effect of different carbon sources on EPS yield

Sucrose and glucose (1% w/v) were added into M3 medium as carbon source seperately. Figure 1c shows that the best growth results were obtained from the basal medium M3 containing 0.2% yeast and 1% sucrose for *B.licheniformis* 2CS and 0.15% yeast and 1% sucrose for *B.zhangzhounesis* 2CA. Razack et al. (2013) tried to optimize a medium for the production of enhanced EPS from the soil isolate, *B. subtilis*, using the one factor at a time method. The highest yield was obtained with 2% of sugarcane molasses (4.86 g EPS L^{-1}), compared to the sucrose medium (2.98 g EPS L^{-1}).Angel et al. (2018) determined the optimum carbon source by examining the effect of different carbohydrates such as 12% glucose, sucrose, lactose, maltose, xylose and fructose on EPS production of *B. licheniformis* WSF-1 strain. They found that the highest carbon source wassucrose with 25% concentration and produced 2.9 g mL^{-1} exopolysaccharide.

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Determination of EPS production of thermophilic bacteria on petri dishes

As can be seen in Figure 2, two bacterial strains tested grow by forming a slime layer in sucrose-containing medium. This slime formation clearly shows that bacterial strains produce EPS. Paulo et al. (2012) also investigated the presence of EPS in lactic acid bacteria using discs in solid media. They investigated the EPS production by these bacteria under different conditions, different carbon sources, pH and varying temperatures, detecting the mucoid colonies on the discs.

Quantification of total EPS in thermophilic bacteria

For *B. licheniformis* 2CS and *B. zhangzhounesis* 2CA thermophilic bacteria, sucrose and glucose sugars were used for total EPS production in M3 medium.

Table 1 and Table 2 show EPS production by *B. licheniformis* 2CS and *B. zhangzhounesis* 2CA in 100 mL basal medium M3 containing two different yeast concentrations (0.15 and 0.2%) plus 1% carbon sources (glucose and sucrose). The best EPS production was observed in *B. licheniformis* 2CS as 121 mg mL^{-1} in the medium containing 0.2% yeast and 1% sucrose (Table 1), while the *B. zhangzhounesis* 2CA produced highest amount of 45 mg mL^{-1} EPS with 0.15% yeast and 1% sucrose (Table 2). Berekaa (2014) reported the amount of EPS produced by *B. licheniformis* QS5 in the presence of sucrose as 3.78 g mL^{-1}. Moreover, Ergene and Avcı (2018) tested various carbon sources of glucose, starch, lactose, whey, mannitol, sucrose, beet molasses for EPS production in *Bacillus* sp. ZBP4 strain and the best result producing total EPS was obtained using beet molasses as 1071 mg L^{-1}.

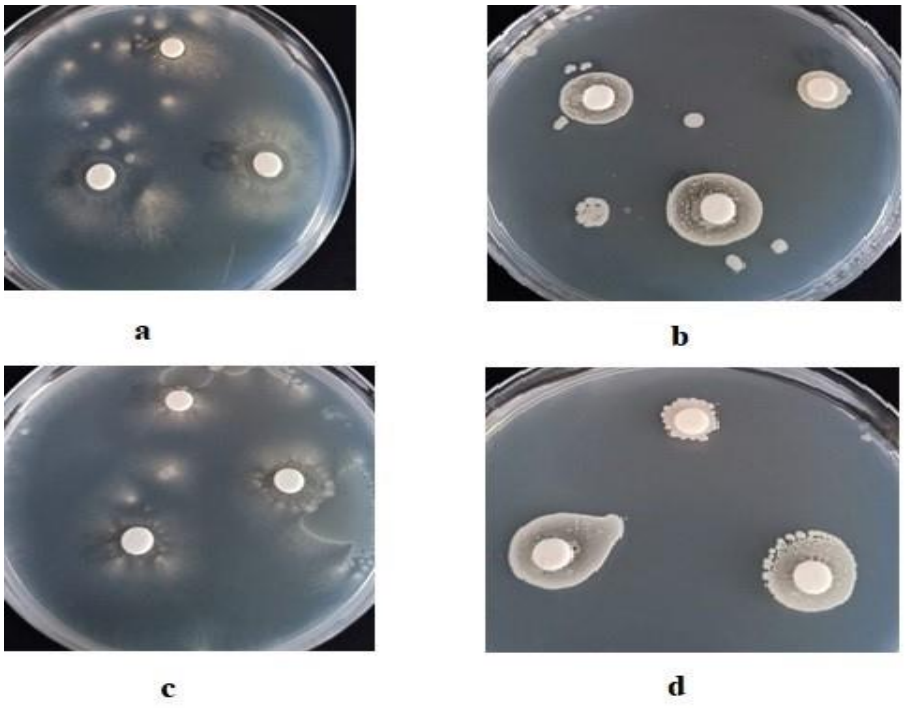

Figure 2. Detection of EPS production in *B. zhangzhounesis* 2CA on basal medium supplemented with sucrose (a; 0.1% yeast extract, control; b; 0.1% yeast extract+ 1% sucrose), *B. licheniformis* 2CS in basal medium supplemented with sucrose (c; 0.1% yeast extract, control; d;0.1% yeast extract+ 1% sucrose)

Determination of carbohydrate and protein amount in total EPS

As shown in Table 1 and Table 2, while the best carbon content result was 333.28 µg mL^{-1} for *B. licheniformis* 2CS, it was 1087.03 µg mL^{-1} for *B.zhangzhounesis* 2CA. Singh et al. (2011) found the amount of EPS produced by *B.licheniformis* as 576 mg L^{-1}, the amount of carbohydrates as 343.14 mg L^{-1}, and the amount of protein as 107.68 mg L^{-1}. Angel et al. (2018) determined the total carbohydrate

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amount as 58 ± 0.017 mg mL^{-1} and protein amount as 12 ± 0.021 mg mL^{-1} in EPS produced by *Bacillus licheniformis* WSF-1 strain.

As shown in Table 1 and Table 2, the highest protein amount was 1.29 µg mL^{-1} for *B.licheniformis* 2CS, while 1.38 µg mL^{-1} for *B.zhangzhounesis* 2CA.

Table 1. EPS dry weight, carbohydrate amount and protein amount of *B. licheniformis*

	EPS dry weight (mg 100 mL^{-1})				
	%0.15Y	%0.15Y+S	%0.15Y+G	%0.2Y	%0.2Y+S
	4.5	10.5	7.1	3.3	121
B. licheniformis 2CS	**Amount of carbohydrate (µg mL^{-1})**				
	133.74	272.46	216.56	54.63	333.28
	Amount of protein (µg mL^{-1})				
	0.25	0.52	1.29	0.03	0.19

Table 2. EPS dry weight, carbohydrate amount and protein amount of *B. zhangzhounesis*

	EPS dry weight (mg 100 mL^{-1})				
	%0.15Y	%0.15Y+S	%0.15Y+G	%0.2Y	%0.2Y+S
	6	45	7	5	30
B. zhangzhounesis 2CA	**Amount of carbohydrate (µg mL^{-1})**				
	135.45	985.57	403.39	628.48	1087.03
	Amount of protein (µg mL^{-1})				
	0.21	1.38	0.96	0.51	1.14

Determination of antibacterial effects of EPSs

Table 3 shows that EPS obtained from *B. licheniformis* 2CS in the medium containing 0.15% yeast with the presence of glucose showed antibacterial effect against *E. coli* with a zone diameter of 12 mm. EPS obtained from *B. licheniformis* 2CS in the medium containing 0.2% yeast with the presence of sucrose showed antibacterial effect against *E. coli* with a zone diameter of 16 mm.

Moreover, EPS obtained from *B. zhangzhounesis* 2CA in the medium containing 0.15% yeast with both presence of sucrose and glucose showed antibacterial effect against *S. aureus* with a zone diameter of 12 mm, while it did not show any antibacterial effect against *E. coli*, *K. pneumoniae* and *P. aeruginosa*. EPS obtained from *B. zhangzhounesis* 2CA in the medium containing 0.2% yeast with the presence of sucrose showed antibacterial effect against *E. coli* with a zone diameter of 10 mm. Mohamed et al. (2018) found that 75 µg $disc^{-1}$ concentration of EPS obtained from *B. alttitudinis* MSH2014 formed a 12.2 mm zone against *S. aureus*, 12.9 mm against *E. coli* and 7.7 mm against *P. aeruginosa*, while 100 µg $disc^{-1}$ concentration formed 15.1 mm zone diameter against *S. aureus*, 17.7 mm against *E. coli*, 10.6 mm against *P. aeruginosa*.

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Table 3. Antibacterial effects of EPSs (mm: zone of inhibition)

Bacteria	*E. coli*	*S. aureus*	*K. pneumoniae*	*P. aeruginosa*
0.15% -Y *B. licheniformis* 2CS	C; 10	C; 10	ND	C; 8
	G; 12	G; -		G; 8
	S; -	S; -		S; 8
0.2%-Y *B. licheniformis* 2CS	C; 8	C; 12	ND	C; 6
	S; 16	S; 12		S; 6
0.15%- Y *B.zhangzhounesis* 2CA	ND	C; 10	ND	ND
		G; 12		
		S; 12		
0.2%- Y *B.zhangzhounesis* 2CA	C; 6	ND	C; -	ND
	G; 6		G; 6	
	S; 10		S; -	

EPS obtained from medium containing yeast extract was used as control. Obtained after adding the carbon source to the medium the antibacterial effects of EPS were investigated (C, control; G, glucose; S, sucrose; Y, yeast; ND, not determined)

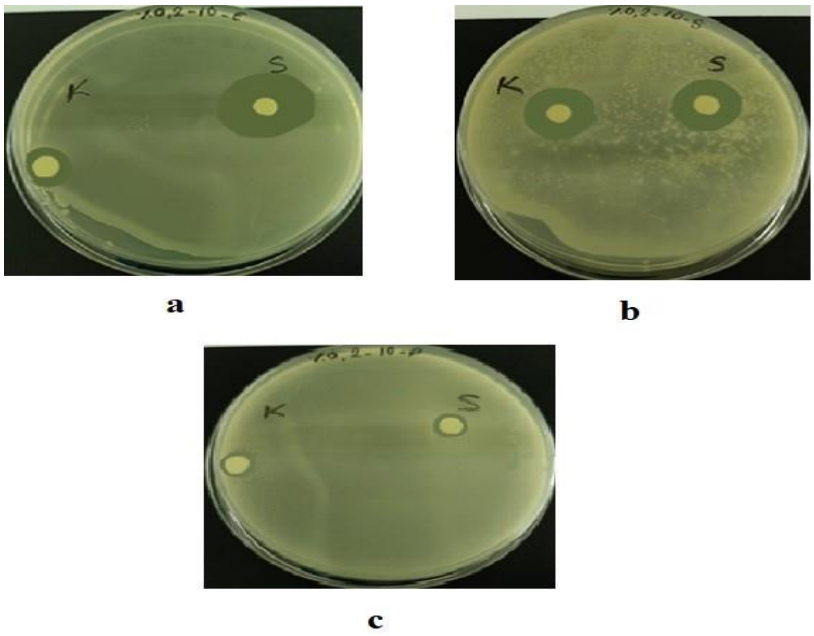

Figure 3. EPS in *B. licheniformis* 2CS a) Antibacterial effect against *E. coli* b) Antibacterial effect against *S. aureus* c) Antibacterial effect against *P. aeruginosa*

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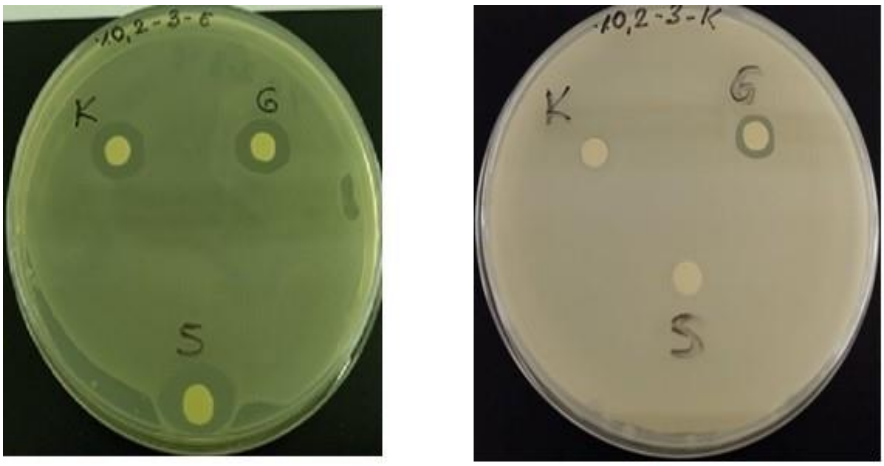

a b

Figure 4. EPS in *B. zhangzhounesis* 2CA a) Antibacterial effect against *E. coli* b) Antibacterial effect against *K. pneumoniae*

CONCLUSION

EPSs produced by bacteria enable other bacteria to survive in various extreme environments. EPSs, which can take place in many different industrial areas, are also used commercially. The present study investigates EPS production in thermophilic bacteria. For this, different media, different yeast extract concentration and different carbon sources were used. The best EPS production was obtained from *B. licheniformis* 2CS strain in the presence of sucrose, which is the carbon source, and yeast extract, which is the nitrogen source. The best total EPS dry weight of 121 mg was obtained from *B. licheniformis* 2CS. Protein and carbon determination methods were applied for the EPS obtained. The carbohydrate amount in EPS obtained from *B. licheniformis* 2CS was found to be 333.28 µg mL^{-1}, while the protein amount was found to be 0.19 µg mL^{-1}. The use of EPSs in different fields makes them important for future studies.

AUTHOR CONTRIBUTIONS

The study belongs to the master's thesis.
FRT: Conceptualization, data curation, formal analysis, visualization and writing-original draft; KG: funding acquisition, project administration, resources, data curation, formal analysis, investigation, methodology, writing-review and editing. FMB: analysed the data, formal analysis, article writing, NP: formal analysis All authors read and approved the final article.

CONFLICT OF INTEREST

The authors report no conflict of interest relevant to this article

RESEARCH AND PUBLICATION ETHICS STATEMENT

The authors declare that this study complies with research and publication ethics.

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