



RESEARCH ARTICLE

THE EFFECT OF DNASE I, RNASE A, AND PROTEINASE K ON FACULTATIVE  
THERMOPHILE *BREVIBACILLUS AGRİ D505B* BIOFILMS

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ABSTRACT

Thermophilic bacteria have been isolated from man-made thermal habitats and natural thermal habitats. *Brevibacillus agri* D505b was isolated from the geothermal region in Turkey. Thermophilic bacilli can form biofilm in areas such as dairy manufacturing plants, water systems, paper-machine, and can create serious problem due to their spore-forming. Therefore, determining the biofilm-forming properties of these bacteria is very significant for the areas. The aim of this study was to determine the effect of environmental conditions on planktonic growth and biofilm formation, the concentrations of protein, carbohydrate, and extracellular DNA (eDNA) from extracellular polymeric substances (EPSs), and the effects of DNase I, RNase A and proteinase K on eDNA in the biofilm matrix of the isolate. As a result, optimal values of the isolate for planktonic growth and biofilm formation were determined as pH 7.0, 1% NaCl, 50°C, and pH 9.0, 0% NaCl, 45°C, respectively. Genomic DNA (gDNA) and eDNA were isolated, then were treated with DNase I, RNase A and proteinase K. The gDNA was only all degraded by DNase I. However, eDNA was not affected by DNase I, RNase A and proteinase K. Moreover, eDNA was determined to be resistant to all the enzymes tested in this study. The eDNA might be protected by EPS components and/or extracellular membrane vesicles (EVs) structures. In addition, the molecular weights of the gDNA and eDNA were calculated larger than 20 kb. Thus, the presence of eDNA in the biofilm matrix of *B. agri* was confirmed with agarose gel imaging and spectrophotometric analysis.

**Keywords:** *Brevibacillus agri* D505b, Biofilm formation, Extracellular DNA.

1. INTRODUCTION

The Gram-positive spore-forming genus *Bacillus* occurs in a wide range of environments, from soil to food and dairy processing surfaces [1]. *Bacillus* spp. spores are often present in raw milk, playing a significant role in the bacterial impairment of milk and milk products. Several spore-forming species have been isolated on dairy farms so far [2]. Zhao et al. [3] identified thermophilic spore-forming bacteria such as *Brevibacillus* spp., *Anoxybacillus* spp., and *Geobacillus* spp. in the dairy industry. In another study, Lücking et al. [4] identified spore-forming bacteria in dairy products, including

*Brevibacillus agri*, *Bacillus pumilus*, and *Anoxybacillus flavithermus*. Moreover, *Brevibacillus* sp. was isolated from soil by Vivas et al. [5].

The contamination of industrial plants and products with spore-forming bacteria is a common problem [6]. Spore-forming bacteria have been shown to sporulate within single- and multiple-species biofilms and to release these immensely stress-resistant spores, thus increasing the risk of cross-contamination of food [7]. Paperboard and food-packaging paper primarily contain spore-forming bacteria belonging to the genera *Bacillus*, *Paenibacillus*, and *Brevibacillus* as contaminants [8]. In addition, Pereira and Sant'Ana [9] investigated spore-forming bacteria in raw materials such as sugar, cocoa, starch, and milk powder. These raw materials are contaminated by spore-forming bacteria and are significant for the quality of final products. Furthermore, spores of *Bacillus* species prevalently contaminate the food. Dried foods such as cereal and milk powders are frequently contaminated with spores and when water is present, these spores can germinate, leading to spoilage or food poisoning [10]. Moreover, aerobic spore-forming bacteria have serious impacts on food quality and safety, so the bacteria are a potential cause of disease [11]. *B. agri* has been associated with human infections [12]. Ogarkov et al. [13] showed that in the final late stages of chronic tuberculosis, samples obtained from patients contained strains of various species of *Bacillus* spp. and *Brevibacillus* spp. In addition, *B. agri* was isolated in association with an outbreak of waterborne illness. In a study, Logan et al. [14] identified *B. agri* from clinical, dairy, and industrial specimens (gelatin processing plant, antibiotic fermenter, sterilized milk), and 3 strains were associated with the outbreak of waterborne illness.

In our previous studies, we carried out the experiments including biofilm formation on six abiotic surfaces (stainless steel, glass, polyvinyl chloride, polypropylene, polystyrene, and polycarbonate) used in industry and biofilm control with sanitation agents of *B. agri* D505b. We determined that the isolate was a strong biofilm producer [15] and [16]. The objective of this work was to determine the effect of environmental conditions on planktonic growth and biofilm formation, the concentrations of protein, carbohydrate, and eDNA from EPSs and the effects of DNase I, RNase A and proteinase K enzymes on *B. agri* D505b biofilms. eDNA is a major structural component of many bacteria. Enzymatic deterioration of eDNA can weaken the biofilm structure and release microbial cells from the surface. DNases could evidence a potent strategy for biofilm control [17].

## 2. MATERIAL AND METHODS

### 2.1. Culture Conditions of the Isolate

The endospore-forming, aerobic and facultative thermophilic *B. agri* D505b was isolated from sediment sample in the hot spring (Dikili, Izmir, Turkey). We used the isolate, which was previously isolated in our lab, to study its biofilm properties. The 16S rRNA gene of the isolate was registered with GenBank Accession Number FJ430048 [18]. The isolate was first cultured in Tryptic Soy Agar (TSA, Merck, Germany) at 55°C for 18 h and was subsequently incubated in Tryptic Soy Broth (TSB, Merck, Germany) for 18 h at 55°C in a shaking incubator (170 rpm). The culture was again incubated in TSB at 55°C for 6 h under shaking. All biofilm assays were carried out with a culture that was 6 h old in the mid-exponential growth phase.

### 2.2. The Effects of Environmental Conditions on Planktonic Growth and Biofilm Formation

The effects of pH (4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0), salinity (0.0, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0%), and temperature (37, 40, 45, 50°C) on planktonic growth and biofilm formation were determined in 96-well polystyrene microtiter plates (LP, Italy) spectrophotometrically (OD 595 nm) for 0, 6, 18, 24,

and 48 h in TSB. In addition, the crystal violet (CV) staining method was applied for optimal biofilm growth assay at the end of 48 h of incubation. For the pH adjust, NaOH and HCl solutions were used. In this study, we first-time determined optimal biofilm values, and the values were used in all experiments.

### **2.3. Quantification of Biofilm Formation with Crystal Violet in Microtiter Plates**

This assay was carried out using the CV staining assay described by Woodward et al. [19] and Stepanović et al. [20] with a few changes. To the wells of 96-well polystyrene microtiter plates were added 90  $\mu$ L of TSB without NaCl and 10  $\mu$ L of bacterium culture. The plates were washed two times with physiological saline at the end of 48 h of incubation; thus, planktonic cells were removed. The remaining adhered cells were fixed with 95% methanol (Merck, Germany) and were incubated at 22°C temperature for 15 min. Then the wells were stained with 1% crystal violet dye (Merck, Germany) at 22°C for 30 min. Then, the plates were washed under running tap water to remove residues of stain and were air-dried. Afterward, the dye bound to the biofilm cells was dissolved with ethanol:acetone (Merck, Germany), and the amount of biofilm was measured at an optical density (OD) of 595 nm using a microplate reader (BioTek Elisa reader,  $\mu$ Quant, Biotek Inc., USA). The negative controls contained only TSB.

### **2.4. The Determination of Concentrations of Protein, Carbohydrate, and eDNA**

The D505b isolate was grown at its optimal conditions on TSA plates for 18 h and 0.1 g of bacterium biomass was collected with sterile plastic loops. The biomass was entirely disintegrated by vortexing at maximum speed for 2 min in 2 mL of physiological saline, containing glass bead (diameter 3 mm). After vortexing, the suspension was centrifuged at 32.000  $\times g$  for 7 min. The pellet was used for gDNA isolation. The supernatant was primarily filtered through a 0.22  $\mu$ m membrane filter (Sartorius, France) and used for protein, carbohydrate, and eDNA assays. Polysaccharide concentration was quantified by the phenol-sulfuric acid method [21]. Glucose was used as a standard for the determination of the calibration curve. The Lowry method was applied for the determination of the protein concentration. Bovine serum albumin (BSA) was used as a standard [22]. eDNA isolation was conducted partially by the method described by Wilson [23]. The filtered supernatant was treated with chloroform and isoamyl alcohol (volume 24:1) for eDNA isolation. Afterward, the solution was centrifuged and treated with phenol-chloroform-isoamyl alcohol (volume 25:24:1). The solution was again centrifuged. Isopropanol was added to the supernatant and was waited for 20 min at -20°C. The solution was centrifuged and was added cold ethanol (70%) on precipitate. The solution was centrifuged, and the supernatant was poured. Tris-EDTA buffer was added on eDNA and was solved. The gDNA extraction was performed using a genomic DNA purification kit (Fermentas K0512, Thermo Fisher Scientific Inc., USA). Finally, gDNA and eDNA qualities were measured with the absorbance values at 260 nm/280 nm with a NanoDrop Spectrophotometer (Thermo Scientific NanoDrop Lite, USA). The DNA samples were subjected to 1.5% agarose gel electrophoresis at 120 V for 45 min. Then, the products were visualized with a Quantum ST4 Gel Documentation System (Vilber Lourmat, France). The molecular weights of DNA samples were determined via the Quantum-Capp software system (Vilber Lourmat).

### **2.5. The Treatment of gDNA and eDNA with DNase I, RNase A, and Proteinase K Enzymes**

In this assay, gDNA and eDNA were first isolated according to section 2.4. Afterward, 10  $\mu$ L of gDNA (295.5 ng/ $\mu$ L) or eDNA (604.6 ng/ $\mu$ L) samples were treated with DNase I (1.45, 1.7, 2.5, and 3.0 mg/mL) (Sigma-Aldrich, DN25, USA), RNase A (0.90 mg/mL) (Sigma-Aldrich, R6513, USA), and proteinase K (0.85 mg/mL) (Sigma-Aldrich, P2308, USA) enzymes at 37°C for 1 h in 96-well polystyrene microtiter plates. Finally, agarose gel electrophoresis (1.5%) was applied at 120 V for 45 min. The products were visualized with the Vilber Lourmat Quantum ST4 Gel Documentation System.

The molecular weights of DNA samples were determined with the Quantum-Capp software system. As negative controls were used samples without enzyme treatment.

### 2.6. The Effect of DNase I on Mature Biofilm

This assay was conducted as defined previously by Grande et al. [24] with a few modifications. The purpose of the assay was to determine whether the eDNA was sensitive or resistant to DNase I. First, 95  $\mu\text{L}$  of TSB and 5  $\mu\text{L}$  of bacterium culture were added to a 96-well polystyrene microtiter plates and were incubated for 40 h (mature biofilm) at 45°C. The biofilms were then treated with 100  $\mu\text{L}$  of DNase I (100  $\mu\text{g}/\text{mL}$ ) (Sigma-Aldrich, DN25) for 2, 4, 8, and 12 h at 37°C. The plate wells were washed with physiological saline. Subsequently, the CV staining assay was applied to the plates. The biofilm samples were treated with physiological saline for positive controls.

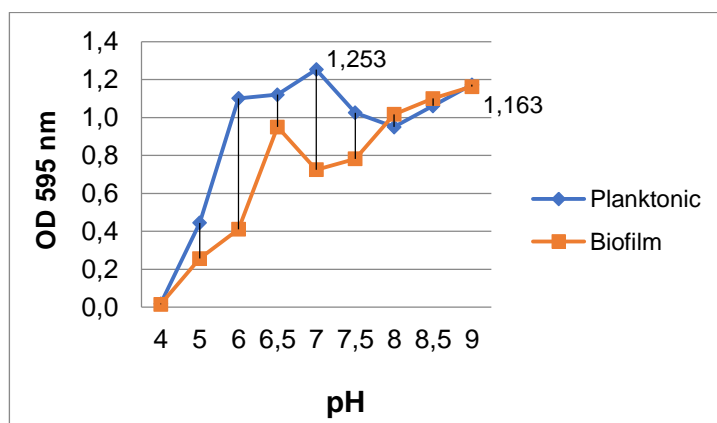
### 2.7. Statistical Data Analyses

All the experiments were conducted in three replicates on three independent days. All statistical analyses were performed using SPSS 17.0 statistical program (SPSS Inc., Chicago, IL, USA). The one-way analysis of variance (ANOVA) was applied to determine whether there are any statistically significant differences between the means of independent groups. Probability levels of  $p < 0.05$  were considered statistically significant.

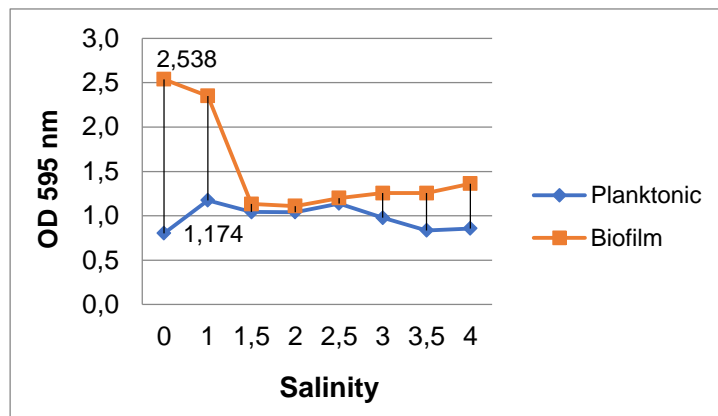
## 3. RESULTS

### 3.1. Optimization Assays

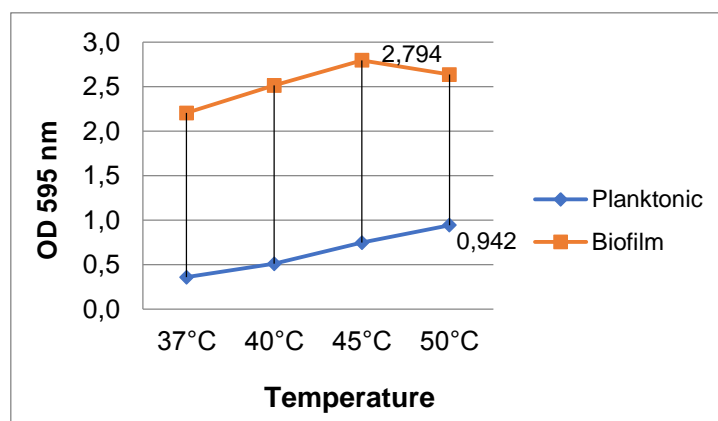
The optimal values of the isolate for planktonic growth were determined as pH 7.0 ( $\text{OD}_{595\text{nm}} 1.253 \pm 0.003$ ), 1% NaCl ( $\text{OD}_{595\text{nm}} 1.174 \pm 0.04$ ), and 50°C ( $\text{OD}_{595\text{nm}} 0.942 \pm 0.17$ ), whereas optimal values for biofilm formation were measured as pH 9.0 ( $\text{OD}_{595\text{nm}} 1.163 \pm 0.3$ ), 0% NaCl ( $\text{OD}_{595\text{nm}} 2.538 \pm 0.2$ ), and 45°C ( $\text{OD}_{595\text{nm}} 2.794 \pm 0.3$ ) in 96-well microtiter plates (Figs. 1, 2, and 3). All parameters were found different for the optimal planktonic growth and the biofilm formation of the isolate. The pH value for biofilm formation was much higher than that for optimal planktonic growth. In addition, the isolate preferred an alkaline and salt-free environment for optimal biofilm production. The biofilm formation decreased with the increased salt amount.



**Figure 1.** The effects of pH on the planktonic growth and biofilm formation of the isolate.



**Figure 2.** The effects of salinity (NaCl) on the planktonic growth and biofilm formation of the isolate.



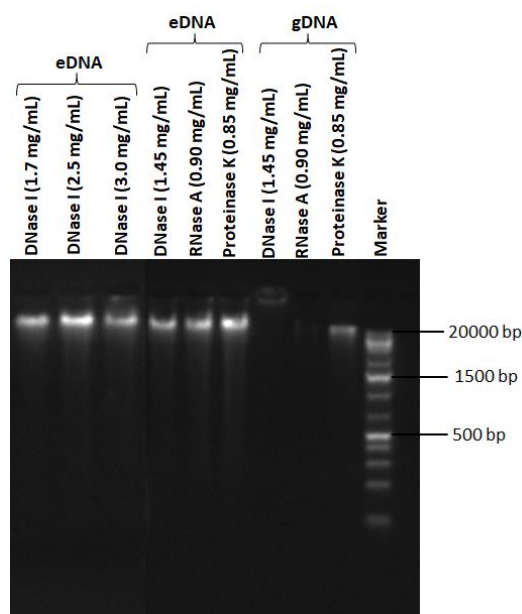
**Figure 3.** The effects of temperature on the planktonic growth and biofilm formation of the isolate.

### 3.2. Protein, Carbohydrate, and eDNA Analysis

The concentrations of total protein and carbohydrate were quantified as 600  $\mu\text{g}/\text{mL}$  and 18.4  $\mu\text{g}/\text{mL}$ , respectively. The amount of eDNA was measured as 604.6  $\text{ng}/\mu\text{L}$ , whereas the gDNA was quantified as 295.5  $\text{ng}/\mu\text{L}$ . Furthermore, the molecular weights of the gDNA and eDNA were calculated larger than 20 kb (Fig. 4). Thus, the presence of eDNA in the biofilm matrix of *B. agri* was confirmed for the first time with agarose gel imaging and spectrophotometric analysis.

### 3.3. The Treatment with DNase I, RNase A, and Proteinase K of gDNA and eDNA

The gDNA was only all degraded by DNase I. On the other hand, eDNA was not affected by any of the enzymes. To confirm the resistance, eDNA was treated with higher concentrations of DNase I (1.7, 2.5, and 3.0  $\text{mg}/\text{mL}$ ). But the results did not change (Fig. 4). Our results showed that the strong biofilm producer *B. agri* D505b was very resistant to DNase I, RNase A, and proteinase K.



**Figure 4.** Agarose gel electrophoresis image of the eDNA and gDNA of D505b; different DNase I concentrations applied to eDNA (1.7, 2.5, and 3.0 mg/mL); DNase I (1.45 mg/mL), RNase A (0.90 mg/mL), and proteinase K (0.85 mg/mL) treatment of the eDNA and gDNA. Marker (Fermentas Gene Ruler 1 kb Plus DNA Ladder, 75-20000 bp).

### 3.4. The Effects of DNase I on Mature Biofilm

DNase I enzyme (100 µg/ mL) was applied to the mature biofilm (40 h) for 2, 4, 8, and 12 h in 96-well polystyrene microtiter plates. DNase I had no significant effect on the biofilm biomass of *B. agri* D505b. According to the results, the residual biofilm amounts measured after the DNase I treatment were as 16.56% for 2 h and 20.04% for 12 h.

## 4. DISCUSSION

The optimal values the isolate for planktonic growth were determined as pH 7.0, 1% NaCl, and 50°C, whereas optimal biofilm formation was measured as pH 9.0, 0% NaCl, and 45°C in 96-well polystyrene microtiter plates (Figs 1, 2, and 3). It was seen that all parameters were different for the planktonic growth and biofilm formation of the isolate. The pH value for biofilm formation was much higher than that for optimal growth. Furthermore, the isolate preferred an alkaline and salt-free environment for optimal biofilm production. The biofilm formation decreased as the salt amount increased. High salt concentration in the growth medium resulted in lower environmental water activity ( $a_w$ ), which is destructive to cellular activities. Moreover, osmotic drift influences the response of the tested *Bacillus* species' biofilm growth on polystyrene [25].

Extracellular DNA (eDNA) of microbial origin is common in natural terrestrial and aquatic environments [26]. Catlin and Cunningham [27] obtained eDNA from *Staphylococcus*, *Pseudomonas* and *Alcaligenes faecalis*. The presence of eDNA was indicated for the facultative thermophile *Brevibacillus agri* for the first time in our study. Several studies demonstrated that the eDNA of biofilm

cells could be sensitive to the DNase I of eDNA. Nijland et al. [28] demonstrated that DNase I (above 15 ng/mL) caused the dispersal of biofilm of *Bacillus licheniformis*. We determined that the DNase I activity had limited effect at a concentration of 100 µg/mL within 12 h on the mature biofilm of the isolate (20.04%). On the other hand, our results showed that DNase I, RNase A and proteinase K enzymes had no effect on eDNA in the biofilm matrix of *B. agri* D505b (Fig. 4). Apparently, the eDNA of *B. agri* D505b could be protected from these enzymes. This could be explained by the rigid pellicle structure and strong biofilm structure of the isolate. We detected in our previous study that *B. agri* D505b was a strong biofilm producer (OD595 nm: 3.365) [16]. Moreover, the resistance to DNase I may possibly result from the conserved folded structure of eDNA after purification. The purified eDNA which has lost its carbohydrate and protein interactions might become rearranged after chemical treatments, and gain a more compact and condense structure with additional folding [29]. Similarly, DNase I had no effect on *Helicobacter pylori* eDNA. The eDNA might be protected by other EPS and/or EV-like structures [30]. The eDNA-containing EVs plays a part in biofilm production, bacterial colonization, and subsequent resistance to removal techniques [31]. EVs in biofilms interact with eDNA to strengthen structural integrity [32]. Rivera et al. [33] reported the isolation of EVs from Gram-positive, spore-forming *Bacillus anthracis*. The first hint of the presence of EVs in Firmicutes came from the studies on *Bacillus cereus* and *Bacillus subtilis* [32]. Soler et al. [34] observed that vesicles and eDNA were produced by hyperthermophilic archaea. In addition, DNA in vesicle preparation was found immensely resistant to DNase treatment. Their observations suggest that eDNA could be less or more strongly associated with the vesicles. Vesicles could be a significant factor determining the DNA stability and protecting it from degradation in natural environments. It is unclear how stable eDNA could exist in hydrothermal environments. However, their results demonstrated that eDNA could be preserved in high-temperature environments via its association with vesicles produced by hyperthermophilic archaea. Blesa and Berenguer [35] determined the long-term protection of thermophilic *Thermus thermophilus* eDNA from DNase associated with EVs generated by cell lysis. Vesicle-protected eDNA can withstand nuclease activity, which allows the movement of eDNA over long distances. EVs are frequently generated by bacteria during the growth phase or upon integration within a biofilm [36].

The characterization of the eDNA of bacterial biofilms revealed that it consists of fragments of high molecular weight (about 30 kb) [37]. We detected that the molecular weight of eDNA was larger than 20 kb with agarose gel electrophoresis (Fig. 4). On the other hand, Ali Mohammed et al. [38] reported that the size of the eDNA of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* was detected to be about 100 bp. The size is generally larger than the one defined for other biofilms. Moreover, the size of eDNA has been reported to range from less than 100 bp to 10 kb [38]. In conclusion, this paper determined that changing environmental conditions could increase the biofilm formation abilities of the isolate. In addition, the presence of eDNA in the biofilm matrix of *B. agri* was confirmed with electrophoresis and spectrophotometric methods. It was seen in this study that DNase I, RNase A and proteinase K enzymes had no effect on the *B. agri* D505b biofilms. Therefore, our data suggest that the effect of different enzyme combinations and concentrations should be tested for the biofilm control of *B. agri* in future studies. The synergistic effect of enzymatic sanitation agents can be determined for biofilm removal. In addition, revealing the association between eDNA and EVs for *Brevibacillus agri* is significant. Any enzyme which can degrade eDNA holds a potential to be used along with antibiotics as a co-treatment agent.

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