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BIOFILM FORMATION AND CONTROL OF FACULTATIVE THERMOPHILE *BREVIBACILLUS AGRI* D505B

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ABSTRACT. *Brevibacillus agri* D505b is an aerobic, non-pathogenic, endospore-forming facultative thermophilic bacillus. Six abiotic surfaces (stainless steel, glass, polyvinyl chloride, polypropylene, polystyrene, and polycarbonate) were compared with viable cell enumerations. According to results, D505b cells could able to attach all these surfaces. Stainless steel (6.10 log CFU/cm^2) was found to be the most effective surface for biofilm formation. Polycarbonate (6.03 log CFU/cm^2) was found as the second best surface. Furthermore, the D505b biofilm was treated with 15 different sanitation agents and trichloroacetic acid (TCA) was determined to be the most effective one (80.3% removal). Our results showed that the strong biofilm producer *B. agri* D505b was very resistant to various sanitation agents. However, TCA significantly inhibited biofilm formation for the isolate.

1. INTRODUCTION

Biofilm coined by Bill Costerton in 1978, which is a heterogeneous structures comprising different populations of microorganisms surrounded by a matrix (mostly of exopolysaccharides) that allows their attachment to inert (e.g., glass, plastic) or organic (e.g., skin, mucosa) surfaces [1]. Furthermore, biofilms are formed over a surface, mostly industrial surfaces, including pipelines and membrane systems that come in direct contact with a flowing product [2,3]. Surfaces of food processing equipment, including closed systems such as pipes, valves and pumps or open systems such as conveyors, are regularly found to be contaminated by microorganisms [4]. Moreover, Bacilli can form biofilms on surfaces during dairy

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processes [5]. Aerobic spore-forming bacteria, such as *Bacillus*, *Brevibacillus*, and *Geobacillus*, are able to survive industrial pasteurization and form biofilm within pipes and stainless steel equipment [6]. Stainless steel is widely used in industry and has an established record of biofilm formation responsible for deterioration and corrosion [7]. Thermophilic *Bacillus* species easily attach and grow on stainless steel surfaces. Furthermore, thermophilic bacilli grow and adhere to surfaces in the evaporators of milk powder manufacturing plants [8].

Biofilms cause many problems in food-processing industry, which are energy losses, blockage, cleaning, hygiene, corrosion, and material deterioration in equipment, sensors, detectors [9]. Furthermore, bacterial biofilms may both cross-contamination dairy products and may cause corrossion of the metal surfaces [3]. Bacterial contamination and product spoilage because of biofilm formation are recurring problems [10]. Thermophilic bacilli are potential contaminants in a variety of industries such as paper mills, canning, juice pasteurization, sugar refining, gelatin production, dehydrated vegetable manufacture and dairy product manufacture [11]. Bacterial biofilms are more difficult to eliminate from within a system than free-living cells. Adherent microorganisms become highly resistant to cleaning procedures and disinfection [3,4]. Cleaning and disinfection studies have focused on eliminating food-borne pathogens such as *Listeria* and have neglected other contaminating organisms such as thermophilic bacilli [10]. Enzymes are considered green countermeasures against biofilm formation in the food industry owing to their biodegradability and low toxicity [12]. Various sanitation agents for biofilm control are widely used. For example, the lytic action of lysozyme on bacteria can be ascribed simply to resolution of the rigid cell-wall structures [13]. Tsiaprazi-Stamou et al. (2019) determined that formulation A, containing amylase-protease-lipase was the most effective in biofilm cleaning [12]. Nisin is also useful for the inhibition of cell-wall synthesis [14], and it is effective against important Gram-positive foodborne pathogens and spoilage agents [15]. Takao et al. (2016) examined the effects of disinfectants such as Mazak P and benzalkonium chloride on a natural biofilm model of a dental unit waterline [16]. However, *B. agri* was found within the surviving bacteria. Biofilm control agents and regimes may not provide any effect for some bacteria. New strategies should be developed in this case. Similarly, Faille et al. (2002) found that the resistance of both *Bacillus cereus* and *Bacillus subtilis* spores to a cleaning procedure [4].

In our preliminary studies on biofilms, a facultative thermophilic isolate, *B. agri* D505b, was detected as a strong biofilm producer ($OD_{595\ nm}$: 3.365) [17]. Little is known about control of thermophilic bacilli biofilms. The aim of this study was to

detail the biofilm formation on abiotic surfaces of *B. agri* D505b isolate and its biofilm control. To the best of our knowledge, this is the first paper with regard to biofilm formation, and control of the facultative thermophile *B. agri*.

2. Materials and Methods

2.1. Bacterial Strain and Culture Conditions

The endospore-forming facultative thermophilic and aerobic bacillus *B. agri* D505b was isolated from sediment samples in the Dikili district of İzmir, Turkey. The 16S rRNA gene of the D505b isolate was registered with GenBank Accession Number FJ430048 [18]. The isolate was first cultured in tryptic soy agar (TSA) at 55 ^{o}C for 18 h and was subsequently incubated in tryptic soy broth (TSB) for 18 h at 55 ^{o}C in a shaking incubator. The culture was again incubated in TSB at 55 ^{o}C for 6 h under shaking. All biofilm assays were carried out with culture that was 6 h old in the mid-exponential growth phase.

2.2. Biofilm Formation on Surfaces

The bead vortexing method of Giaouris and Nychas (2006) with a few modifications was applied for cell viability assay on abiotic surfaces [19]. First of all, stainless steel (grade 316L), polypropylene, polystyrene, polyvinyl chloride, polycarbonate coupons (R: 14 mm), and glass slides (20 mm x 26 mm x 1 mm) were treated with isopropanol overnight and were washed for 30 min in a detergent solution. The coupons and glass slide were washed under running tap water and with deionized water, respectively. Afterwards, the surfaces were air-dried and autoclaved. The surfaces were placed into 6-well polystyrene microtiter plates containing TSB. Subsequently, bacterial culture was inoculated onto the plates and was incubated for 48 h. The surfaces were then removed with sterile forceps were rinsed with 4.5 mL of physiological saline to eliminate planktonic cells. Then, the surfaces were scratched. The surfaces and the suspensions were cited to tubes containing only glass beads and then were vortexed for 2 min. Viable cell numbers were calculated with the drop plate method [20]. Surfaces in TSB were used as negative controls. The results were converted to the logarithmic base (log CFU/cm^2). All assays were performed in duplicate.

TABLE 1. Sanitation agents for biofilm control and their effects.

Effect	Agents	Concentration	Temperature	Time	References
Protein	AP	0.16 U/g	37°C	60 min	[10]
	Protease	0.16 U/g	37°C	60 min	[10]
	Subtilisin	1%	37°C	30 min	[10]
	Trypsin	3%	37°C	3 h	[8]
	SDS	3%	100°C	10 min	[8]
Polysaccharide	α-Amylase	1%	37°C	30 min	[11]
	Cellulase	1.66%	37°C	30 min	[11]
	SM	100 mM	22°C	60 min	[8]
	Lysozyme	2%	37°C	60 min	[8]
	TCA	10%	100°C	15 min	[8]
Antimicrobial	Nisin	2 mg/mL	37°C	24 h	[8]
Pro-oxidant	PM	2 mg/mL	22°C	30 min	[8]
	ST	10 mg/mL	22°C	5 min	
Quorum sensing	Furanone	1 mg/mL	22°C	60 min	[22]
	Triclosan	2 mg/mL	22°C	60 min	[23]

2.3. Biofilm Control with Sanitation Agents

Protease, Alkaline protease (AP), lysozyme, α-amylase, cellulase, subtilisin, trypsin, nisin, furanone, triclosan, sodium metaperiodate (SM), potassium monopersulfate (PM) and sodium thiosulfate (ST) combination, sodium dodecyl sulfate (SDS), and trichloroacetic acid (TCA) were used in this assay. Bacteria culture (5 μL) and TSB (95 μL) were added to the polystyrene microtiter plates. The plates were incubated for 24 h at 45°C. Then the wells were emptied and rinsed with physiological saline. The plate wells were filled with 15 different sanitation agents under the suitable conditions with a few reference modifications (Table 1). Finally, the CV staining assay was applied to wells. The wells containing only the appropriate solvent without its cleaning agent were used as positive controls. The results were calculated using the formula of Pitts et al. (2003) [21].

2.4. Statistical Data Analyses

All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA and Tukey and Dunnett tests were applied. Probability levels of < 0.05 were considered statistically significant.

3. Results

3.1. Biofilm Formation on Abiotic Surfaces

In this assay, scraping and bead vortexing were applied to cells. Six abiotic surfaces were compared with viable cell enumerations with the plate counting method such surfaces included stainless steel, polypropylene, glass, polyvinyl chloride, polystyrene, and polycarbonate. According to results, D505b cells could attach to all surfaces. The viable cell numbers were calculated based on all surface areas and varied from 4.47 to 6.10 log CFU/cm^2 for abiotic substrates. Grade 316 L stainless steel (6.10 log CFU/cm^2) was found to be the most effective surface for biofilm formation (Figure 1).

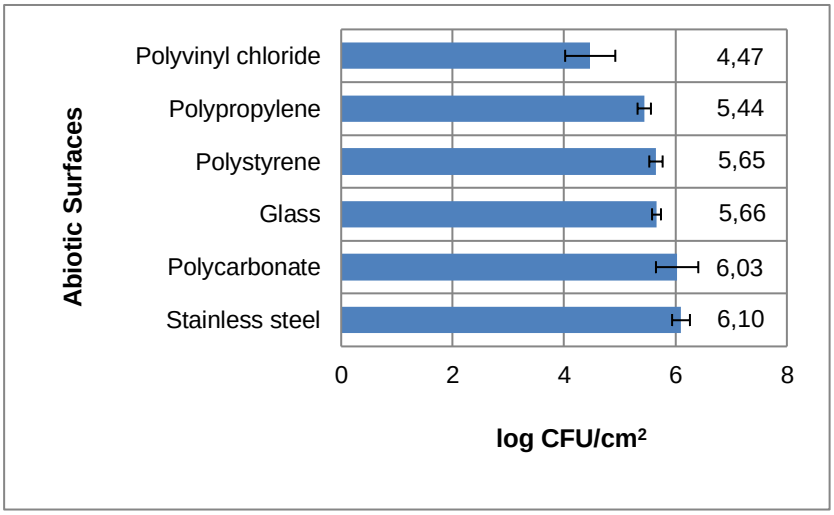

Figure 1. The biofilm formation of D505b cells on abiotic surfaces.

3.2. Biofilm Control with Various Agents

Fiftheen different sanitation agents were used for biofilm control. TCA (80.3%) provided the best biofilm removal among these agents. According to our results, other sanitation agents did not show sufficient effects for biofilm control (< 50%). It seems that TCA may have effect for *B. agri* biofilm control. Only TCA agent had an effect on the breakdown of surface polysaccharide in extracellular polymeric substance (EPS) matrix. Five different agents (AP, protease, subtilisin, trypsin, SDS) were used for degrade of surface proteins in EPS matrix. However, biofilm structure of the isolate was not affected by sanitation agents (Figure 2).

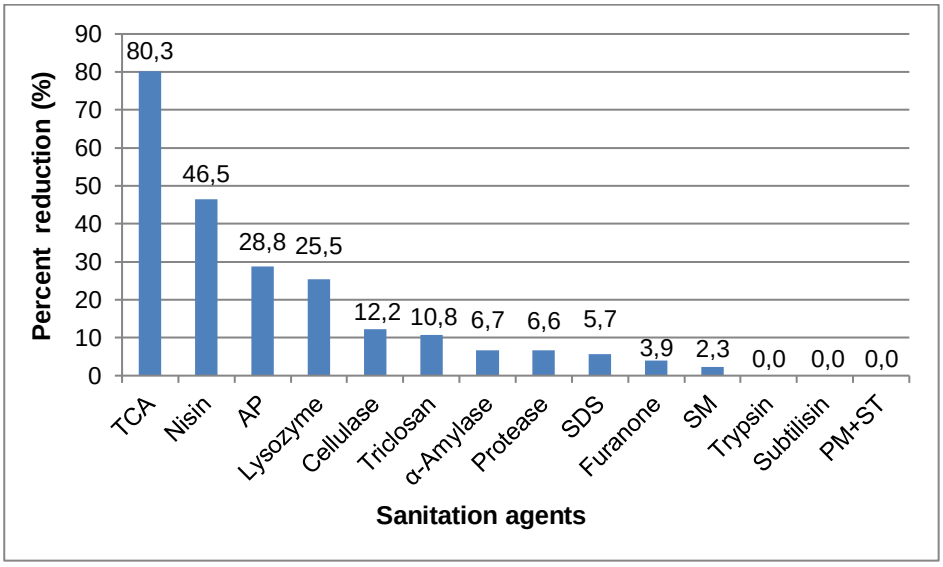

FIGURE 2. Biofilm control of the D505b isolate with different sanitation agents.

4. DISCUSSION

Bacterial spores are strongly hydrophobic. The spores have generally tended to adhere more to both hydrophobic and hydrophilic surfaces than to vegetative cells [24]. Food-related *Bacillus* species formed biofilms on polystyrene surfaces.

Polystyrene has been widely used in food packaging. Closed systems such as pipes, pumps, and valves or open systems such as conveyors are found to be contaminated by *Bacillus* spp., *Escherichia coli*, and *Listeria monocytogenes* [25]. In general, it is assumed that glass and stainless steel are hydrophilic materials, while plastics are hydrophobic materials [26]. Bacteria are able to attach to a wide variety of different materials, including glass, 304 and 316 stainless steel, plastics, rubber, polytetrafluoroethylene, and various organic polymers, which are used in modern processing equipment [21,28]. We tested both hydrophilic and hydrophobic materials for the biofilm formation of *B. agri* D505b. Our results indicated that D505b isolate formed biofilm on both hydrophobic and hydrophilic surfaces. The most suitable surface for biofilm production by D505b was grade 316L stainless steel (6.10 log CFU/cm^2). Its second choice was also detected as polycarbonate surface (6.03 log CFU/cm^2) (Figure 1). In a similar study, Mafu et al. (1990) showed that *L. monocytogenes* cells could attach to stainless steel, glass, and polypropylene surfaces [29]. In another study, Song et al. (2012) demonstrated that *E. coli* yielded differing amounts of biomass on stainless steel (1.7x10^7 CFU/coupon) and polycarbonate (4.1x10^7 CFU/coupon) [30]. A strain of *Yersinia ruckeri* form biofilms on solid supports such as fiberglass and polyvinyl chloride (PVC) [28]. The hydrophobic properties of endospores and their resistance to heat, disinfectants, and desiccation allow them to survive cleaning procedures [31]. Endospores of some bacterial species are known to have high thermal resistance and can survive disinfection and heat sterilization [24]. *L. monocytogenes* in biofilm was more resistant than single cells to sanitizers and heat [32]. Enzymes like protease and a-amylase, have gained attention as alternative agents that could demolish the EPS matrix and attack bacterial cells [12]. However, our study demonstrated that TCA was the most effective agent among 15 different sanitation agents for *B. agri* D505b biofilm control (80.3%). Similarly, Parkar et al. (2003) showed that TCA caused a 100% loss of viability of *Bacillus flavithermus* strain B12-C^m [8]. Nisin was determined as the second most effective agent (46.45%). The other agents were not able to be successful on biofilm removal. Our result showed that lysozyme (2%) reduced biofilm formation of *B. agri* D505b by 25.5% (Figure 2). Eladawy et al. (2020) reported that the highest reduction (19%) was seen in lysozyme concentration of 30 μg/mL for *Pseudomonas aeruginosa* [33].

5. Conclusion

Brevibacillus is one of the most widespread genera of Gram-positive bacteria, which recorded from the diverse environmental habitats [34]. Therefore, it is essential to

provide biofilm control of this bacterium. Our studies showed that *B. agri* could form biofilms on stainless steel, glass, polyvinyl chloride, polypropylene, polystyrene, and polycarbonate surfaces. To biofilm control may require investigation in a different time and temperature intervals on these surfaces. Current methods for controlling thermophilic bacilli and their biofilm growth in dairy manufacturing plants include increasing the cleaning frequency, the use of disinfectants, altering temperatures, reducing the surface area and the use of dual equipment [35]. Our data suggest that different new sanitation regimes should be tested for the biofilm control of *B. agri*. Besides, the synergistic effect of enzymatic detergents can be determined to biofilm control on different abiotic surfaces.

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