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PRODUCTIVE BIOFILMS FROM MESOPHILIC TO THERMOPHILIC ENDOSPORE-FORMING BACILLI FOR INDUSTRIAL APPLICATIONS

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ABSTRACT

The basic biofilm characteristics and extracellular polymeric substances (EPSs) production of *Bacillus licheniformis* E114 (mesophile), *Bacillus pumilus* D75b, *Brevibacillus thermoruber* B93 (facultative thermophile) and *Anoxybacillus caldiproteolyticus* D621 (thermophile) isolates were evaluated in this study. They were initially investigated in terms of their pellicle formation at the air-liquid interface, complex carbohydrate production in TSA (Tryptic Soy Agar) medium supplemented with calcofluor dye, and also colony morphologies in TSA medium supplemented with Congo Red dye. Their biofilm productions on abiotic surfaces such as polystyrene and stainless steel were also quantified by crystal violet binding assay and viable cell enumerations. The ideal way for the mechanical removal of the adherent cells from the steel surface such as bead vortexing or scraping were also tested for each isolate. The optimal growth and biofilm production parameters of E114, D75b, B93, and D621 in terms of pH, NaCl, and temperature were determined. Under optimal conditions, their EPSs components such as carbohydrate, protein, and eDNA were also measured quantitatively. The isolates evaluated in this study presented colony morphotypes in different characteristics on Congo Red agar that display also unique biofilm forming capacities for each isolate. Also, useful optimization approaches were determined that might be adapted to potential industrial applications for the biofilm and EPSs producers. According to these findings, four different endospore-forming bacilli exhibiting mesophilic to thermophilic growth, and biofilm production, possessing various EPSs contents which may be novel were elucidated that could be important in many industrial processes.

Keywords: Biofilm production, endospore-forming bacilli, EPSs, facultative thermophilic, mesophilic, thermophilic

INTRODUCTION

Biofilm is defined as aggregated cells embedded in slime like extracellular polymeric substances (EPSs) which adhere to each other or to a surface. Therefore, biofilm generation on a surface is directly related with the EPSs production which constructs the complex three dimensional structures of biofilm **(Teh *et al.*, 2015)**. During the last decade, many improvements have been made in discovering new microbial extracellular substances that possess novel industrial importance. These biological polymers have importance especially in medicine, pharmacology, agriculture, and packaging as well as in waste management **(Vert *et al.*, 2012)**.

Members from *Bacillaceae* family are very divergent in the worldwide and comprise many species which display various characteristics in terms of growth parameters such as temperature, pH, and salinity requirements. Moreover, *Bacillaceae* family members are able to produce endospores that are resistant to unfavourable life conditions, and they can survive under extreme environmental conditions. These endospore-forming bacilli were classified into 3 groups according to their temperature requirements; the mesophiles (T_{opt}: 30-40°C), the facultative thermophiles (T_{opt}: 40-50°C), and the thermopiles (T_{opt}: 50- ≥70°C) **(Nazina *et al.*, 2001; Cihan *et al.*, 2012)**.

The diversity of *Bacillaceae* family members throughout the world is not only due to their endospore-forming capabilities, but also depends on their biofilm and EPSs production abilities in their natural habitats. Despite their spectacular diversity, only some of the specific species belonging to *Bacillaceae* members from genus *Bacillus* **(Parkar *et al.*, 2001; Doroshenko *et al.*, 2001; Ronimus *et al.*, 2003; Lazarevic *et al.*, 2005; Veening *et al.*, 2006; Coorevits *et al.*, 2008)**, *Brevibacillus* **(Bihari *et al.*, 2010; Radchenkova *et al.*, 2011; Yasar Yildiz *et al.*, 2015)**, *Paenibacillus* **(Ben-Jacob and Cohen, 1998; Scheldeman *et al.*, 2005; Burgess *et al.*, 2009)**, *Anoxybacillus* **(Parkar *et al.*, 2001; Burgess *et al.*, 2009; Palmer *et al.*, 2010; Dai *et al.*, 2011)**, *Geobacillus* **(Parkar *et al.*, 2001; Flint *et al.*, 2001; Arena *et al.*, 2009; Kambourova *et al.*, 2009)**, *Aeribacillus* **(Burgess *et al.*, 2009; Yasawong *et al.*, 2011; Radchenkova *et al.*, 2013)**, *Lysinibacillus*, *Oceanobacillus*, and *Ureibacillus* **(Coorevits *et al.*, 2008; Burgess *et al.*, 2009)** were focused either because of their biofilm characteristics or their EPSs components and the presence of their spores in some dairy products. However, there is limited information in terms of their differences in biofilm formation and the effect of temperature on this process. Hence, four different biofilm producer species from *Bacillaceae* family which display mesophilic (*B. pumilus* D75b), facultative thermophilic (*B. licheniformis* E114, *B. thermoruber* B93) and thermophilic (*A. caldiproteolyticus* D621) growth were evaluated in detailed for their basic biofilm formation characteristics. Thus, this study is the first which compares the endospore-forming bacilli displaying mesophilic to thermophilic growth in terms of their biofilm characterizations as well as the relationship between their biofilms and some surfaces/interfaces. Consequently, all these findings showed that our bacterial isolates have strong biofilm producers with their EPSs contents which might be promising for many industrial applications.

MATERIALS AND METHODS

Bacterial isolates

Biofilm producing and endospore-forming bacilli of E114 (a water sample of Kozakli hot spring, Nevsehir), D75b (a sediment sample of Balcova geothermal power plant, Izmir), B93 (a soil sample of Turgutlu hot spring, Manisa) and D621 (a soil sample from Dikili-Kaynarca hot spring, Izmir) isolates were obtained from different geothermal regions of Turkey previously **(Coleri *et al.*, 2009)**. The isolates were stored at -86°C in Medium I (MI) broth cultures supplemented with 20% glycerol (**Suzuki *et al.* 1996**). The taxonomic data of these endospore-forming bacilli were previously presented in detail according to their phenotypic characteristics, 16S rRNA gene sequences and genomic fingerprinting profiles **(Cihan *et al.*, 2011; Cihan *et al.*, 2012**; **Koc *et al.*, 2015)**.

In these studies, the 16S rRNA gene sequence analyses revealed that E114, D75b, B93 and D621 all belong to *Bacillaceae* family from *B. licheniformis* DSM13^T, *B. pumilus* DSM 27^T, *B. thermoruber* DSM 7064^T and *A. caldiproteolyticus* DSM 15730^T **(Cihan *et al*., 2011; Cihan *et al*., 2012)**. The isolates were categorized as mesophilic (E114, optimum growth at 35°C), facultative thermophilic (D75b: optimum growth at 50°C, and B93: optimum growth at 50°C) and thermophilic (D621, optimum growth at 55°C) according to their temperature requirements. The isolates of D75b and B93 were also found to be halotolerant. It was revealed that, these four isolates are able to produce a great amount of pellicle in the air-liquid interface of MI broth. Since the presence of a pellicle formation is a hint for biofilm production, these isolates were considered to be studied for their capability of biofilm formation and EPSs production for further studies.

Biofilm growth conditions

Prior to the biofilm assays, a series of culture enrichment was carried out. An overnight bacterial culture on Tryptic Soy Agar (TSA), (Merck, Germany) was inoculated into Tryptic Soy Broth (TSB), (Merck, Germany) and incubated at 37°C (for mesophilic E114), 45ºC (for facultative thermophiles of D75b and B93) and 55-65°C (for thermophilic D621) for 18 h at 170 rpm. This turbid culture was again transferred into a new TSB and grown at appropriate temperature for additional 6 h. The 6 h-old final cultures in the logarithmic growth phase were used for entire biofilm assays.

Determination of pellicle formation

Pellicle development assay was performed in 5 mL of TSB without NaCl after inoculated with 100 µl of the enriched culture. The incubation was carried out at 37°C, 45°C or 55°-65°C for 4 days under static conditions. The test tubes were visually examined every day for the ring and pellicle formation at the liquid-air interface **(Kobayashi, 2007)**.

Determination of the complex carbohydrates on calcofluor containing medium and bacterial motility assay

The level of fluorescent brightener calcofluor binding to β(1-3)-D- or β(1-4)-D-linkages was assessed for the determination of the presence of carbohydrates such as cellulose or its derivate in the EPSs matrix. After culture enrichment, 50 µL of the bacterial isolate was dropped onto TSA without NaCl containing 50 mg/L calcofluor (Sigma-Aldrich, USA) and incubated at appropriate temperature for 48 h. Calcofluor binding was detected under UV light lamp. The results were photographed and presented as positive or negative according to the presence of fluorescence. As negative control *Salmonella infantis* DMC12 was used **(Zogaj *et al*., 2001)**. The flagella staining were performed by using BD Flagella Stain Droppers Kit (USA).

Determination of biofilm morphotype on Congo Red Agar (CR)

Congo red was used to observe the colony morphotypes and colour as well as to detect the amyloid fibres like curli **(Romero *et al*., 2010)**. Biofilm morphotypes were identified on the TSA without NaCl containing 40 mg/L Congo red (Sigma-Aldrich, USA). The enriched bacterial culture was dropped onto the same plate with 20, 30, 40 and 50 µL of culture volumes and incubated for 4 days under static conditions. The changes in the diameter, morphology and colour of the colonies were observed. In addition, at the second and fourth days of incubation, the colonies were also photographed by using stereomicroscope with a 0.63X magnification **(Römling and Rohde, 1999; Milanov *et al*., 2015a)**.

Microtiter plate assay

The crystal violet binding assay was performed based on a modified protocol described previously **(Stepanović *et al*., 2000)**. After the culture enrichment, a portion of 10 µL was transferred to each well of U-bottom polystyrene microtiter plates having 96-wells (LP, Italy) containing 190 µL of TSB without NaCl and plates were incubated statically for 48-96 h, at appropriate temperatures. After incubation, all the planktonic cells were removed by gently washing the wells with serum *physiologic* (SP). Adherent cells were fixed with 200 µL of 95% methanol, incubated at room temperature for 15 min, and then emptied and dried at room temperature for additional 15 min. Each well was filled with 1% (w/v) crystal violet solution (200 µL), (Merck, Germany) and incubated for 30 min at room temperature. Finally, the plates were rinsed under running tap water. The dye bounded to adherent cells was resolubilized with ethanol:acetone (70:30 w:w), (Sigma-Aldrich, USA), and OD of each well was measured at 595 nm by using Elisa reader (BioTek Elisa reader, µQuant, Switzerland). Eight replicates were used for each analysis. The cut-off OD (OD_C) values of biofilm quantities for the microtiter plate assay were calculated with the following formula: average OD of negative control (medium only) + 3x standard deviation (SD) of negative control. The isolates were categorized as follows: OD ≤ ODc; non-producer, ODc<OD ≤ 2 × ODc; weak, 2×ODc <OD ≤ 4 × ODc; moderate, 4 × ODc < OD; strong **(Saxena *et al*., 2014)**.

Determination of biofilm formation on stainless steel surface

The number of biofilm cells were enumerated by using the bead vortexing method described previously with a few modifications **(Giaouris and Nychas, 2006)**. Rectangle stainless steel (grade 316 L, in 2.5 x 0.8 x 0.1 cm in dimensions) coupons were used as abiotic substratum in this study. The coupons were treated with isopropanol (Sigma-Aldrich, USA) over night and agitated with 10% sodium dodecyl sulphate (SDS), (Merck, Germany) for 30 min before rinsing with deionized water. The previously autoclaved sterile coupons were placed aseptically into 3.5 mL of TSB containing tubes before inoculation with 1 mL of the enriched bacterial culture. The tubes were incubated at 37°C, 45°C or 55-65°C depending on the isolates during 48 h. At the end of incubation period, the coupons were removed and rinsed with SP for the removal of the planktonic cells. After washing, one of the coupons was taken into a new tube containing 4.5 mL of SP and the attached biofilm cells were mechanically agitated by scraping with loops. The second coupon was directly placed into a different tube containing sterile 3.5 mL of SP including glass beads (diameter 3 mm) and, then agitated by vortexing for 2 min (IKA, Germany), at maximum speed **(Parkar *et al*., 2001; Giaouris and Nychas, 2006)**.
Colony forming unit was enumerated by drop plate method to evaluate the attached viable biofilm cells on these coupons **(Herigstad *et al*., 2001)**. 100 µL of the agitated suspension was used to prepare serial dilutions in sterile 900 µL of SP for each sample. Bacterial counts were done on MI agar plates after incubation for 24 h at appropriate temperatures. Only coupon and sterile TSB containing test tubes were used as negative controls. The results were calculated as colony forming units per unit area (CFU/cm^2) and then converted to logarithmic base (logCFU/cm^2). The data obtained from mechanical agitating methods of scraping and bead vortexing were used to assess the best method for the removal of attached cells from the surface. For each coupon, all experiments were performed in duplicate.

Determination of optimum growth and biofilm formation conditions

The optimal conditions for bacterial growth were evaluated by spectrophotometrically measuring the OD600 nm at the incubation times of 0, 6, 18, 24, and 48 h for various temperatures, pH values, and salinities in 96-well microtiter plates containing TSB medium. The temperature for optimal growth was determined by incubating the strains at temperatures ranging from 30 to 65ºC. The effect of pH and salinity was checked in TSB media which have various pH values ranging from 4.0 to 11.0, or were supplemented with different NaCl concentrations (0 to 5%). After 48 h incubation, crystal violet binding assay was carried out as described previously for the determination of biofilm production in these environmental parameters.

Extraction and quantification of protein, carbohydrate, and eDNA (extracellular DNA) of EPSs

The isolates were grown at their optimal biofilm producing conditions on TSA plates for 18 h and 0.5 g of the bacterial biomass were collected from the plates before partial purification of their EPSs. The biomass were suspended and subjected to agitation procedure in 2 mL of SP and centrifuged at 13.000 rpm for 7 min. After centrifugation, the removed supernatant was filtered through a 0.22 µm membrane filter (Sartorious, France) and used for the quantitative measurement of protein, carbohydrate, and eDNA contents.
For the quantification of carbohydrate content, phenol-sulphuric acid method was applied **(Dubois *et al*., 1956)**. Calibration curve was determined by using glucose standard. One mL of the filtered supernatant was mixed with 0.5 mL of phenol (Sigma-Aldrich, USA) and 5 mL of concentrated sulphuric acid (Merck, Germany) in this assay. The results were measured spectrophotometrically at 490 nm. Protein concentration of EPSs was also measured at 660 nm based on the method described by Lowry **(Lowry *et al*., 1951)**, and 0.1 to 10 mg/mL concentrations of BSA (Sigma-Aldrich, USA) were used as protein standard.
Genomic DNA was extracted from the bacterial pellet by using genomic DNA purification kit (Fermentas K0512, USA). Approximately a 500 µL portion of the filtered supernatant was also used for eDNA purification procedure using phenol/chlororoform/isoamylalcohol method which was slightly modified **(Wilson, 2001)**. After precipitation with 70% ethanol (Sigma-Aldrich, USA), the eDNA was air-dried and then dissolved in 30 µL of TE buffer (Tris-EDTA,

pH 8.0). Both the purity and quantity of genomic and eDNA were checked by evaluating the absorbance values at 260 and 280 nm wavelengths by nanodrop (Nanodrop 2000, Thermo Fisher Scientific, USA). DNA samples were also visualized after subjected to 1.5% agarose gel electrophoresis at 120 V for 1 h.

Statistical analysis

Statistical analyses were performed to all the data using SPSS 17.0 software (IBM, USA) by performing one-way ANOVA, Tukey's and Dunnett's tests. Probability levels of <0.05 were considered as statistically significant.

RESULTS

Pellicle formation of the isolates

According to the findings, biofilm formation and EPSs characteristics of the E114, D75b, B93, and D621 isolates were summarized in Table 1. In the case of pellicle formation, bacterial cultures were visualized during 96 h growth in TSB for assessing their ability of forming a ring like structure at the liquid-air interface. All the isolates initially produced a ring structure at the interface by attaching to the glass surface after 6 h of growth at applied temperatures. The pellicles increased gradually by the accumulation of EPSs from 24 to 96 h and, the floating pellicle at the air-liquid interface completely covered the surface of the broth cultures (Fig 1a). Despite rigidly shaking, the pellicles of E114, D75b, and B93 could not be dispersed. By contrast, thermophilic D621 isolate was able to form a fragile pellicle both at 55°C and 65°C that could easily be dispersed by slightly shaking.

Determination of complex exopolysaccharides **in EPSs and bacterial motility assay**

E114, D75b, and B93 isolates, having rigid pellicles could become fluorescently visible on TSA plates without NaCl, supplemented with calcofluor that binds to complex exopolysaccharides such as cellulose or its derivate in the EPSs matrix (Fig 1b). On the other hand, the exopolysaccharides, produced by D621 isolate forming fragile pellicles, could able to bind to calcofluor by generating lesser bright fluorescence under UV-light. In addition, E114, B93, D621, and D75 cells were all found to be motile both on solid and on air-liquid surfaces. While E114, B93, and D621 were found to harbour peritrichous flagella, the D75 isolate was observed to have lophotrichous flagellum (Fig 1c).

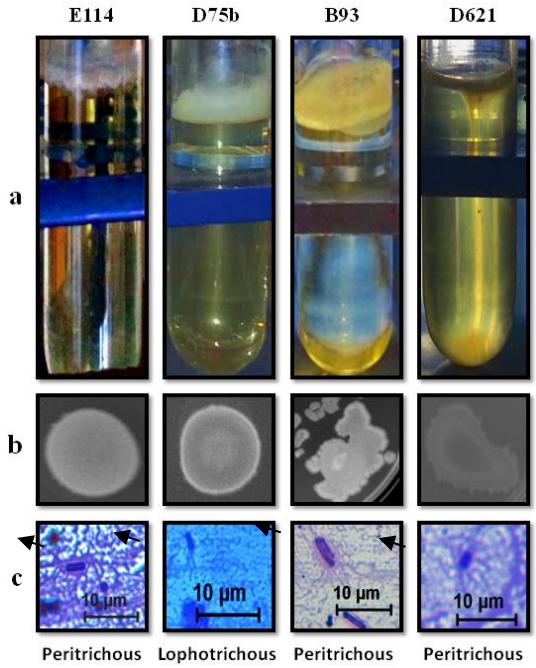

Figure 1 Some phenotypic characteristics of E114, D75b, B93, and D621. Isolates were incubated at different temperature values at 37°C (E114), 45°C (D75b, B93) and 55-65°C (D621) according to the bacterial growth conditions (a). Pellicle formation in the liquid-air interface during 96 h of growth under static conditions (b). Fluorescence of the colonies under UV light growth on calcofluor supplemented plates after 24 h incubation, and (c) Light microscope micrograph showing the flagella staining of the 4 h-old cultures (Arrows indicate the flagella positions).

Colony morphotypes of the isolates on Congo Red Agar (CR)

The changes in the colony morphologies of E114, D75b, B93, and D621 isolates on CR plates at the days 2 and 4 (left), and their related stereo micrographs (right) were given in Fig. 2. E114, D75b, and B93 isolates had opaque and non-pigmented cream coloured colonies, while thermophilic D621 had pale yellow colour and transparent colonies when incubated on TSA plates supplemented with CR. On the other hand, *B. licheniformis* E114 displayed a circular, raised, a mucoid colony morhotype with undulate edges, rough and dull surface, having red colour at the end of 96 h-incubation period on CR plates. Although the entire colony colour was turned into red, the ring like structure in the middle, containing bundles had a darker red colour. In the case of *B. thermoruber* B93, its colonies were circular, mucoid, and raised, having orange coloured, undulate edges and smooth surface with nested ring formations which contained bundles in the middle. The increase in the colony diameters were measured as 0.9 cm. The diameters of these nested ring formations, which turned into dark orange in the centre, were increased gradually during the incubation period. The circular, flat, smooth, dull, non-mucoid and red colonies of *B. pumilus* D75b isolate had lobate margins at the end of 96 h-incubation period. The size of the colonies was measured with a 0.4 cm increase. There was a darker red ring having bundle formation only in edges in contrast to E114 and B93 isolates which had bundles in the centre of the colonies. Finally, the general colony morphotype was quite different in *A. caldiproteolyticus* D621, when compared with its mesophilic and facultative thermophilic counterparts. The orange colonies that are darker in their edges were punctiform, flat, non-mucoid, rough and shiny with lobately margins. Also a ring formation was observed in the dark orange coloured lobate margins. The change in its colony diameters was measured as 0.45 cm.

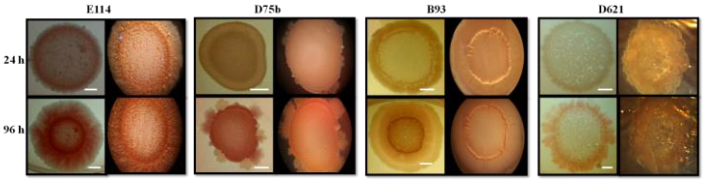

Figure 2 Dye binding properties and colony morhotypes of E114, D75b, B93 and D621 isolates on TSB without NaCl plate containing Congo Red and 3% agar during 4 days of incubation. On the left sides of the figures, general colony view and their right sides, the stereo microscope micrographs with a magnification of 0.63 x were presented. Bar indicates 5 mm.

Microtiter plate assay

E114, D75b, B93, and D621 isolates were found to be strong biofilm producers according to the cut off values after 48 h incubation. The crystal violet binding assay displayed that *B. licheniformis* E114 produces a great amount of biomass with an $OD_{595\ nm}$ value of 3.661 (SD ± 0.290) at the end of 96 h-growth in TSB (without NaCl at 37°C, pH 7.3). The amount of biofilms for D75b, and B93 isolates were measured as 3.319 (SD ± 0.359) and 2.379 (SD ± 0.337) at 45°C respectively. The thermophilic D621 isolate was also found to produce strong biofilms at 55°C ($OD_{595\ nm}$: 0.293, SD ± 0.105) and 65°C ($OD_{595\ nm}$: 0.475, SD ± 0.073).

Biofilm formation of the isolates on stainless steel surface

Stainless steel was used in order to determine the adherence capacities of the isolates. *B. licheniformis* E114, *B. pumilus* D75b and *B. thermoruber* B93 could easily attached to the surface of the coupons during a 48-h incubation period. In contrast to these findings, the viable cell numbers of *A. caldiproteolyticus* D621 on stainless steel were low as in the case of its lower biomass formation on polystyrene plates. Of those mechanical agitating methods, bead vortexing was found to be superior to scraping for D75b, B93 and D621 ($logCFU/cm^2$ values for vortexing: 4.44, 4.88, 1.85; scraping: 4.28, 4.61, 1.75, respectively), when they were applied to remove the attached cells from the surface of the stainless steel. By contrary, the viable cell enumerations of *B. licheniformis* E114 was found to be 5.79 $logCFU/cm^2$ by scraping, whereas it was found as 5.7 $logCFU/cm^2$ in bead vortexing. The $logCFU/cm^2$ values between scraping and bead vortexing assays were found to be statistically different ($p < 0.05$; Tukey's test). Therefore, from now on according to these comparative cell removal studies, bead vortexing was preferred for dispersion of D75b, B93 and D621 isolates, while for removal of E114 cells scraping was employed.

Optimal growth and biofilm formation conditions of the isolates

B. licheniformis E114 was found to grow and form biofilms in a wide range of pH values (pH 5.0 to 11.0), salinities (0 to 5.0%) and temperatures (30-45°C), whereas the isolate could only form stronger biofilms at higher pH (8.0) and salinity (4.0%) values compared to its optimal growth (pH 6.5, 1.5% NaCl) parameters. In the case of *B. pumilus* D75b, the isolate was able to grow and form biofilms between pH 7.0 and 10.0. A more alkaline pH (pH 8.5) was required for optimal biofilm formation of D75b when compared with its optimal growth at pH 6.0. However, both the salinity (at 0% NaCl) and temperature (at 45°C) requirements for its biofilm formation were found to be lower than in its optimal growth conditions. Though the isolate was halotolerant and facultative thermophilic, its biofilm generation was restricted by the increased salinity and temperature values. In the case of *B. thermoruber* B93, the isolate was able to grow between wide pH values ranging from 6.0 to 11.0 and said to be halotolerant (0-5.0%), but it could produce stronger biofilms between pH 6.0 to 9.5 only in the absence of NaCl at 50°C. Therefore, NaCl and pH changes were found to be restrictive parameters on biofilm generation of B93. Thermophilic *A. caldiproteolyticus* D621 isolate could grow at pH 6.0 to 8.0, at temperatures in between 50°C and 65°C, but was not able to grow and form biofilm in the presence of NaCl. Its optimal biofilm formation was observed at a lower pH value (at pH 6.0), but at a higher temperature (at 55°C), when compared with its optimal growth conditions (pH 8.0, 50°C). Therefore, low pH and high temperature and absolutely the absence of NaCl increased the biofilm formation of D621 isolate. Dunnett's test was performed for the evaluation of optimal environmental conditions for biofilm forming and growth and biofilm formation capacities of all isolates could able to be increased by optimizing processes (Dunnett's test $p < 0.05$), (Fig. 3).

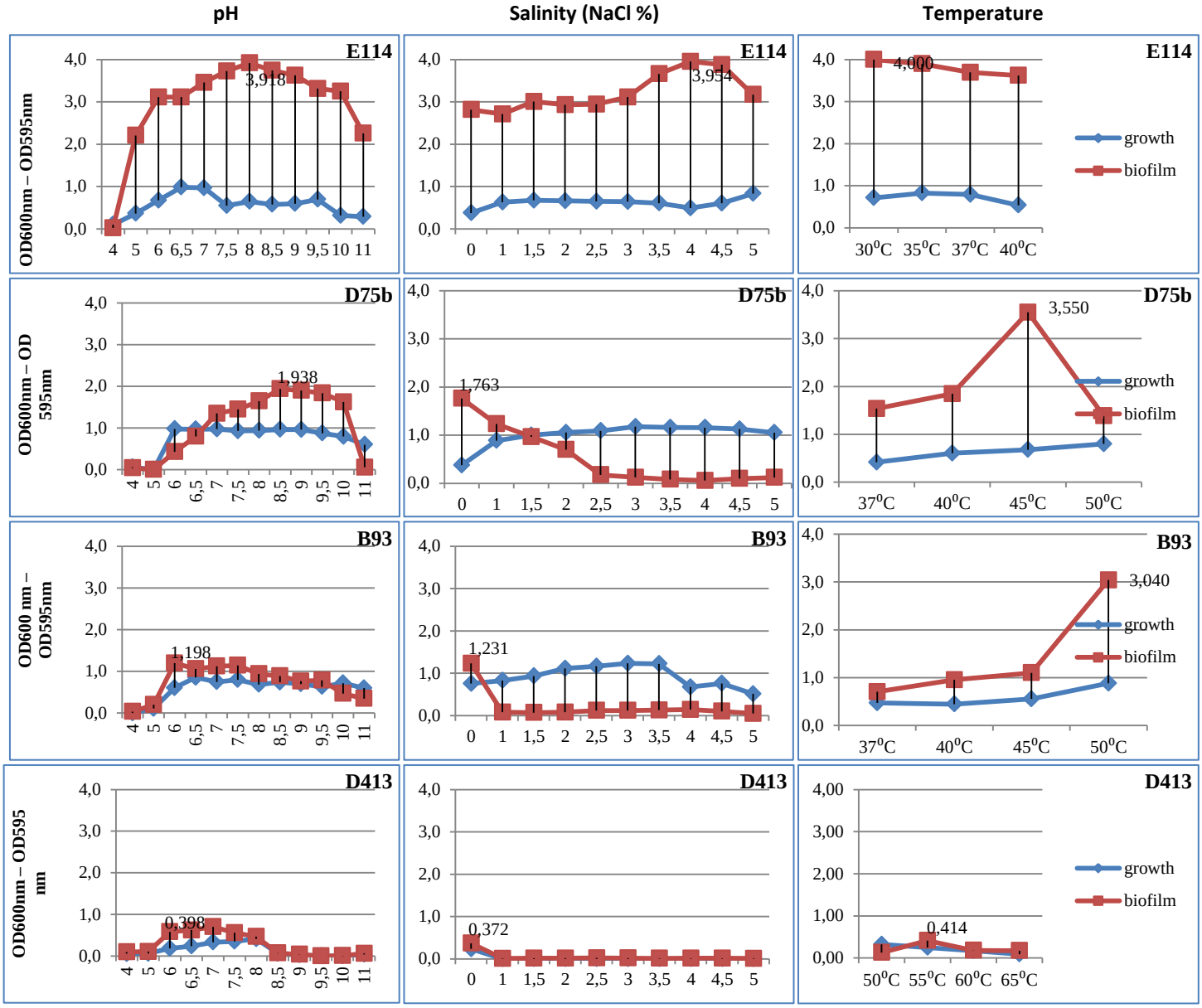

Figure 3 The influence of pH, salinity and temperature on the amount of biofilm (OD_{595nm}) and also on the bacterial growth (OD_{600nm}) for E114, D75b, B93 and D621 isolates after 48 h growth.

Effects of optimal conditions on biofilms formed on polystyrene and stainless steel surfaces

When the biofilm production on stainless steel and polystyrene surfaces were compared before and after optimization studies, an increase in the production of biofilm was achieved for all of the isolates (Fig. 4). At their individual optimal conditions, the amount of the biomass on polystyrene surfaces were increased up to 41%, 28%, 9%, and 7% for D621, B93, E114 and D75b, isolates after 48 h incubation, respectively. Moreover, 35%, 13%, 9%, and 2% increase could be achieved in the viable cell numbers of D621, D75b, B93, and E114 isolates on the surface of the stainless steel respectively. The most surprising data were obtained from the mesophilic *B. licheniformis* E114 and thermophilic *A. caldiproteolyticus* D621 isolates. At optimal conditions, *B. licheniformis* E114 was found to be the superior in regards to the biomass formed on polystyrene surface, whereas the lowest increments in the cell numbers was also counted for this isolate, although it reached to the highest (5.91 logCFU/cm^2) cell numbers on the stainless steel coupon compared to the other three bacilli. Surprisingly, thermophilic D621 isolate was the one which produced the lowest biomass and viable cell numbers (2.49 logCFU/cm^2) for both of the abiotic surfaces used, but

the highest increments in cell counting assays on stainless steel were achieved on D621 isolate after applying optimal conditions.

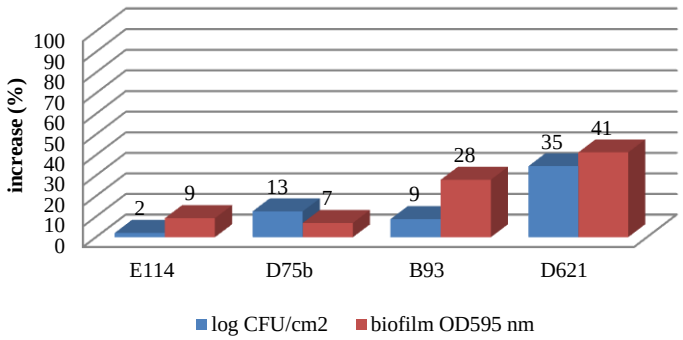

Figure 4 The amount of viable cell numbers and biomass before and after optimization of biofilm formation assays (In the viable cell enumerations, the agitating methods of bead vortexing results were compared for D75b, B93, and D621 isolates, whereas data after scraping was used for E114).

Quantification of protein, carbohydrate and eDNA contents of EPSs

General EPSs contents of E114, D765b, B93 and D621 isolates were presented in Table 1. Among the isolates, the highest carbohydrate content was measured as 82 µg/mL in *B. thermoruber* B93. The protein contents of E114, D75b, B93 and D621 were quantified as 831, 1103, 2343, and 3235 µg/mL (at 30°C, 45°C, 50°C, 55°C), respectively. There was a correlation between the thermostability and the protein content of EPSs matrix. The protein content in *A. caldiproteolyticus* D621 was nearly 4-fold higher, when compared with its mesophilic *B. licheniformis* E114 counterpart. The molecular weights of eDNA and genomic DNA in the biofilm differed from each other. A 22.1, 22.6, 22.2 and 23.5 kb genomic DNA were observed for the biofilm associated sessile cells of E114, D75b, B93 and D621 isolates respectively, which differed from their related eDNA contents having smaller molecular weights (20.1, 17.7, 18.4 and 21.4 kb, respectively), (Fig. 5). The amount of eDNA contents E114 and D621 isolates were found to be quite high.

Table 1 General biofilm and EPSs characteristics of E114, D765b, B93 and D621 isolates.

Parameters			***B. licheniformis* E114**	***B. pumilus* D75b**	***B. thermoruber* B93**	***A. caldiproteolyticus* D621**
Optimal conditions for	pH	Growth	6.5	8.5	6.5	8.0
		Biofilm	8.0	8.5	6.0	6.0
	NaCl	Growth	1.5%	3.0%	3.0-3.5%	0.0%
		Biofilm	4.0%	0.0%	0.0%	0.0%
	Temperature	Growth	35°C	50°C	50°C	50°C
		Biofilm	30°C	45°C	50°C	55°C
EPSs content	Protein (µg/mL)		831	1103	2343	3235
	Carbohydrate (µg/mL)		38	42	82	32
	eDNA (ng/µL) - MW (kb)		2255 – 20.1	826 – 17.7	1150 – 18.4	2565 -21.4
Biofilms on polystyrene plates at optimal conditions ($OD_{595\ nm}$)			≤4,000	3,550	3,040	0,414
Viable cell numbers on stainless steel at optimal conditions ($logCFU/cm^2$)			5,91	5,00	5,31	2,49
Colour formation by interactions between amyloid fibres of EPSs matrix and CR			+ (red)	+ (red)	+ (orange)	+ (orange)
Change in the colony diameter in CR during 96 h			1.15 cm	0.4 cm	0.9 cm	0.45 cm
Presence of fluorescence by binding of β(1-3) /β(1-4) linkages of EPSs polysaccharides to calcofluor			+	+	+	⊥
Pellicle type			Rigid	Rigid	Rigid	Fragile

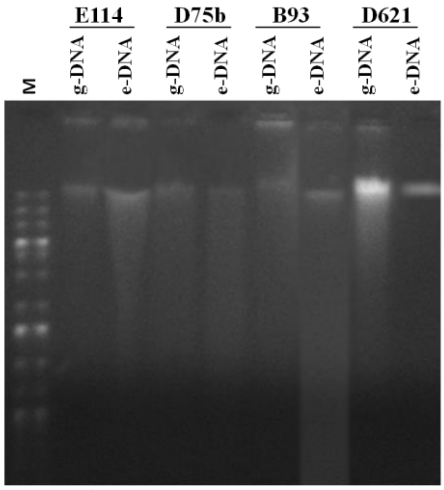

Figure 5 Comparison of genomic (g-DNA) and extracellular DNA (e-DNA) contents of E114, D75b, B93, and D621 isolates (Marker: Fermentas Gene Ruler 1 kb Plus DNA Ladder, 75-20000 bp).

DISCUSSION

The biofilm characteristics and EPSs components of many endospore-forming bacilli are still unclear and their EPSs properties seem to offer numerous applications in different fields of industry **(Guezennec, 2002)**. The importance of some *Bacillaceae* family members is already known as they are able to produce high levels of EPSs. Of those bacilli, it is revealed that facultative thermophilic *Brevibacillus* species can be used as a factory for the expression of many biotechnologically important enzymes and heterologous proteins including cytokines, antigens, and adjuvants **(Mizukami *et al.*, 2010; Panda *et al.*, 2014)** and can be exploited as an excellent tool for structural and functional biology. Although *Brevibacillus* EPSs has high exopolysaccharide or protein content, and there is also a strong relationship between biofilm and EPSs production, there is no information about the biofilm generation for this biomaterial factory **(Radchenkova *et al.*, 2011; Yasar Yildiz *et al.*, 2013)**.

In recent years, *B. licheniformis* has also become a model microorganism as in the case of *Bacillus subtilis* for the study of its biofilm formation and EPSs matrix. There are *B. licheniformis* isolates that are known to produce EPSs containing trisaccharide units **(Spanò *et al.*, 2013)**, exopolymer levan which was used in oil recovery **(Ghaly *et al.*, 2007)** or extracellular proteins having anti-biofilm activity **(Sayem *et al.*, 2011; Dusane *et al.*, 2013)**. In addition, as *B. licheniformis* is a major contaminant of dairy products besides *B. subtilis*, *Geobacillus stearothermophilius* and *Anoxybacillus flavithermus* species, their biofilms were also studied as they could easily attach and form biofilms on stainless steel surfaces that were used in industrial applications **(Parkar *et al.*, 2001; Yuan *et al.*, 2012; Lücking *et al.*, 2013)**.

In the case of facultative thermophilic *Bacillus pumilus*, it is found in milk as a contaminant and may produce toxins that lead to food poisoning (**Lücking *et al.*, 2013)**. There are solely a few investigations on the general biofilm formation capabilities of some *B. pumilus* isolates on glass or stainless steel surfaces **(Parkar *et al.*, 2001; Dusane *et al.*, 2010)**. Except the other three bacilli used in this study, the biofilm formation and EPSs characteristics of thermophilic *A. caldiproteolyticus* was firstly reported in this area.

In all the bacterial isolates, a ring formation characterized by attaching to the glass surface after 6 h of growth was observed, with a subsequent gradually increasing volume of pellicle at the air-liquid interface. It is known that strains having rigid pellicle mostly fluoresced strongly under UV light on calcoflour agar plates, whereas the ones forming fragile pellicle fluoresced with lower intensity as in the case of thermophilic D621 isolate having fragile pellicle **(Solano *et al.*, 2002)**. From other researches dealing with the endospore-forming bacilli, regarding *Bacillus subtilis* 1A700 biofilms, a ring like structure, which

mostly harbours the sporulated cells, was observed when it was grown on MOPS-based agar **(Veening *et al.*, 2006)**.

The previous findings on *Bacillus subtilis* CR morphotypes revealed that CR was found to be binding to TasA protein, and turning into red in wild type strains **(Romero *et al.*, 2010)**. Moreover, when a strain is unable to produce extracellular matrix, the colony morphotype was found to be completely flat **(Aguilar *et al.*, 2007)**. Therefore, TasA is defined as a kind of amyloid curli fibers as found in the pathogenic species from *Enterobactericeace* like *E. coli* and *S. enterica* serotype Typhimurium, and also used to understand the presence of amyloid fibers in the EPSs matrix component of a few *Bacillaceae* members **(Romero *et al.*, 2010; Milanov *et al.*, 2015b)**. In the case of *Enterobactericeace* family, different matrix components were expressed on CR plates according to the EPSs and biofilm production capabilities of the bacteria, and this was used to compare general colony phenotypes. Only thin, aggregative fibers called curli fimbriae are produced, in "bdar" phenotype generating brown, dry and rough colonies. In addition, only cellulose is produced in EPSs, in "pdar" morphotype give rise to the colonies appearing pink, dry and rough. But when bacteria is able to produce both the curli fimbriae and cellulose in its EPSs matrix, then colonies having dark red colour, dry and rough (rdar) surface are formed. If there is no expression of matrix components, then smooth and white colony phenotype called saw morhotype grows on plates supplemented with Congo red dye **(Solano *et al.*, 2002; Milanov *et al.*, 2015b)**.

According to the morphotype explanations above, the most invasive isolate on solid agar plates was *B. licheniformis* E114. Besides, although the colony colours of both mesophilic E114 and facultative thermophilic D75b were red, orange colour was observed in facultative thermophilic B93 and thermophilic D621 isolates. Both E114 and B93 isolates had darker coloured rings containing bundles in the centre of the colony, whereas bundles were located in the colony edges of D75b and D621. Thus, in all isolates the change of general colony colours into red and orange indicates the presence of cellulose-like carbohydrates in the EPSs matrixes. In addition, the area with darker colours in the rings could contain amyloid curli fibres that can bind to CR.

It was also revealed that especially E114, D75b and B93 isolates were able to form strong biofilms not only on polystyrene plates, but also on stainless steel surfaces as well as at the air-liquid interfaces. The mesophilic *B. licheniformis* E114 was found to be superior when compared with the other isolates in terms of the viable cell enumerations on stainless steel and also the amount of biomass on polystyrene wells. On the other hand, the least biofilm formation on polystyrene and cell attachment on steel surface was observed in the thermophilic isolate D621.

It was revealed that both *B. pumilus* D75b and *B. licheniformis* E114 isolates require alkaline pH and decreased temperature values for triggering their biofilm formation whereas, they differ in their salinity requirements. In the case of *B. thermoruber* B93 and *A. caldiproteolyticus* D621 isolates, they require lower pH and salinity values, but higher temperatures for biofilm formation. In addition, the optimal temperature for biofilm generation was higher than for its optimal growth condition for thermophilic D621 and increased temperatures positively influenced its biofilm generation, when compared with the others. Therefore, it could be concluded that both the pH and salinity requirements for biofilm formation are not directly proportional with the optimal growth conditions of these bacilli, but there is a direct correlation between biofilm formation and temperature requirement in both mesophilic and thermophilic isolates. While increased temperature values sharply decreased the biofilm formation of mesophilic ones, in the case of thermopiles, it induced the biofilm generation, thus the biomass.

When the optimal conditions were applied in order to produce biofilms on polystyrene surfaces and stainless steel for each isolate, especially increased production of biomass on surfaces of the polystyrene wells were achieved for all the isolates. At optimal conditions, *B. licheniformis* E114 was found to be the superior in terms of its adherence capability both on polystyrene and steel surfaces, whereas the least biofilm former on these substratum was the thermophilic D621 isolate (Fig. 4). The surface preference changed according to the isolate. On stainless steel, viable cell number enumerations were found in E114, B93, D75b and D621 isolates from highest to the lowest respectively. In the case of polystyrene surfaces, biomass production was achieved in the decreasing order from E114, D75b, B93 to D621 isolates.

According to the EPSs characterization studies carried out at the optimal conditions, the protein concentration of the purified EPSs of the isolates were all found to be very high, when compared with the other carbohydrate and nucleic acid components. The highest protein content was found in the thermophilic *A. caldiproteolyticus* D621 in contrast to its mesophilic *B. licheniformis* E114 counterpart (Table 1). Therefore, a direct proportion was revealed between the thermostability and protein content of EPSs. Also the amount of eDNA content was the highest in thermophilic D621 isolate. It was obvious that especially as the EPSs contents of the isolates were mostly found to be composed of eDNA and proteins, they were probably very important in the rigidity and especially thermostability of their biofilms.

As *Brevibacillus* species are known to be good EPSs producers, they can be used in many applications in biotechnology. In the studies carried out with *B. thermoruber* T1E **(Bihari *et al.*, 2010)** the EPSs product was obtained when bacteria was grown at 50°C, pH 7.0 under aerobic conditions. It is known that *B. thermoruber* 438 was able to grow well in a wide pH range (6.0-8.0), while the maximum EPSs production was achieved at 50°C, pH 8.0 **(Radchenkova *et al.*, 2011)**. In addition, the maximum EPSs production of *B. thermoruber* 423 was accomplished when bacterium was grown at 55°C, pH 6.5 **(Yasar Yildiz *et al.*, 2013)**. Moreover, both *B. thermoruber* 438 and 423 are able to produce high amount of EPSs mostly composed of exopolysaccharides **(Radchenkova *et al.*, 2011; Yasar Yildiz *et al.*, 2013**). The EPSs content of *B. thermoruber* T1E mostly consists of proteins including products of small oligopeptides, keratinase and proteases **(Bihari *et al.*, 2010)**. *B. thermoruber* B93 generally resembles the strain T1E regarding with its high protein content. Different from those studies, B93 isolate had broader pH stability (6.0 to 9.5) in biofilm production and might be more useful in harsh industrial processes considering its EPSs production in addition to its thermostability, non-pathogenicity and fast productivity.

Despite its low biomass production, *A. caldiproteolyticus* D621 isolate was found to be the one harbouring the highest protein content in its EPSs matrix. There is also no any other literature, dealing with the biofilm or EPSs of this thermophilic bacilli. The EPSs production of D621 isolate with high protein content at relatively high temperatures (up to 65°C), and its non-pathogenicity would also be an advantage in many industrial processes.

In the researches dealing with *B. licheniformis* EPSs, the ones harbouring trisaccharide units, exopolymer levan or some extracellular proteins with anti-biofilm activity were detected **(Sayem *et al.*, 2011; Dusane *et al.*, 2013; Spanò *et al.*, 2013)**. This study revealed that mesophilic *B. licheniformis* E114 isolate can be considered as a strong biofilm manufacturer. It is obvious that this mesophilic isolate was superior on adhering to the surfaces when compared with its facultative thermophilic and thermophilic counterparts. Not only the *B. licheniformis* but also the *B. pumilus* species are known as milk contaminants, and the data obtained in this study revealed their strong attachment capabilities in the biofilm formation processes in comparison with their thermophilic counterparts and might explain their higher abundance especially in diary related products.

CONCLUSIONS

As a conclusion, this is the first comparative report on the basic biofilm characteristics and biofilm formation capabilities of four endospore-formers that exhibit mesophilic or thermophilic growth. As biofilm production is directly associated with the EPSs production of an organism, these four bacilli had some specific features with the advantages of fast productivity and were proved to be very promising model organisms for microbial EPSs production. Therefore, the data of biofilm formation and EPSs production capabilities of these isolates would be a base for the identification and characterization of their EPSs in further studies.

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