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Diversity of culturable detrimental biofilm-forming bacteria in wastewater treatment system

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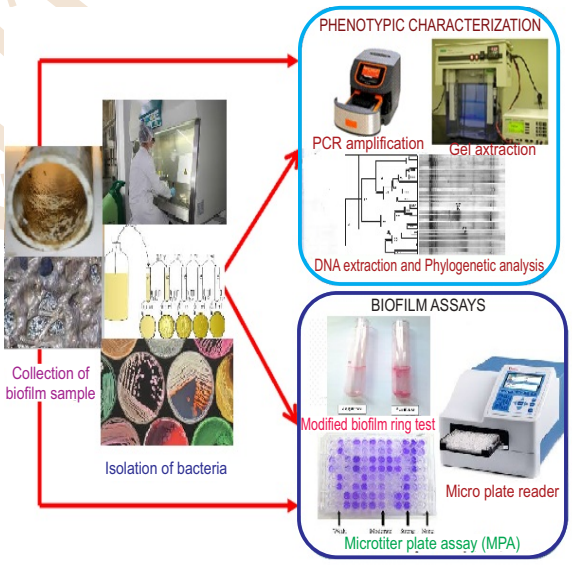
Abstract

Aim: Biofilms are sessile communities of cells attached to a surface or to each other, usually embedded in polymeric substances produced by the bacteria. The community structure of bacteria in the biofilm formed in industrial systems is the basic knowledge to understand the complexity and mechanisms of biocorrosion. The major objective of the present study was to find out the bacterial biodiversity of detrimental biofilm masses from an industrial water treatment system in Köyceğiz-Muğla, Turkey.

Methodology: The biofilm sample was collected from the waste water treatment plant of Köyceğiz-Muğla, Turkey. For phenotypic identification of bacterial isolates from sample, basic morphological and biochemical tests were performed, as reported in Bergey's Manual of Systematic Bacteriology. These bacteria were identified based on 16S rRNA gene sequence analysis for phylogenetic characterization. Additionally, the biofilm-forming ability of all the strains were evaluated in the study by modified biofilm ring test and by semi-quantitative method (microtiter plate assay). Microtiter plate assay is based on the colorimetric measure of a dye such as crystal violet incorporated by sessile cells.

Results: All identification tests indicated that nineteen strains belonged to four different groups. Among four firmicutes (74.0%) were found to be dominant phylum. The majority of isolated strains were affiliated to family Bacillaceae (69.5%). Other strains from the biofilm were affiliated to Enterobacteriaceae (10.5%), Comamonadaceae (5.0%), Microbacteriaceae (5.0%) and Nocardiaceae (5.0%), family respectively in Group Gammaproteobacteria (10.5%), Betaproteobacteria (5.0%) and phylum Actinobacteria (10.5%). Generally, all the nineteen strains were able to form biofilm strongly.

Interpretation: Biofilms may be a source of recalcitrant and xenobiotic contaminations, causing environmental detriment and possible source of public health problems such as outbreaks of waterborne pathogens. The study revealed a high bacterial diversity in the biofilm.



Introduction

Microbial biofilms are found in the environment inhabited by bacteria: natural, industrial or clinical. Only the presence of a hydrated environment and a minimum amount of nutrients is required, and they can develop on hydrophobic or hydrophilic, biotic or abiotic surfaces (Terry *et al.*, 2003). Biofilms are complex communities that consist of bacteria, protozoa, microalgae and micrometazoa living in an extracellular polymeric matrix. Their development is affected by biotic and abiotic characteristics of the ambit, and is therefore connective of environmental conditions (Snyder *et al.,* 2004; Nocker *et al.,* 2007). The adhesion of microorganisms to the surfaces and the subsequent biofilm development are very complex processes, affected by several variables (Chae *et al.*, 2006; Oulahal *et al.*, 2008; Simões *et al.*, 2010). Bacteria and other colonizing microorganisms secrete extracellular polymeric substances (EPS) which anchors to the substratum, thereby modifying the surface chemistry which can stimulate further growth and the support and emplacement of macroorganisms resulting in biofouling (Chambers *et al.*, 2006).

Biofilms are commonly related to economic, processing, yield, health and contamination problems in industrial and medical settings (Donlan, 2001; Hall-Stoodley and Stoodley 2002; Saravanan *et al.*, 2007). Biofouling and microbial communities associated with the biofilms promote corrosion of water-related industrial systems (Beech *et al.*, 2005). Biofilms constitute a protected growth that allows microorganisms to keep alive in hostile environments, being their physiology and behavior expressive different from their planktonic counterparts. Biofilms are difficult to eradicate due to their resistant phenotype. Nevertheless, usual cleaning and disinfection applications may also contribute to inefficient biofilm control and for the propagation of resistance. In many industries, biofilms may be a source of recalcitrant and xenobiotic contaminations, causing environmental detriment and are possible source of public health problems such as outbreaks of waterborne pathogens (Neria-González *et al.*, 2006; Simões *et al.*, 2010). In many industrial processes, microorganisms attach to the surfaces, forming biofilms with detrimental results, often causing significant financial loss. The negatory effects of undesirable biofilms include obstacle to heat transfer across heat exchanger surfaces, mechanical congestions in cooling conduits and condenser tubes and other installations and enhanced perforation/biocorrosion of structural materials (Hamilton, 2003; Ludensky, 2003; Beech *et al.*, 2005; Saravanan *et al.*, 2006).

In few surveys, the isolation of bacterial species presented in pipeline transportation systems and cooling water system exploitation do not reflect the complexity of the bacterial community. However, only few environmental microorganisms can be studied by using culture-based techniques (Amann *et al.*, 1995). Therefore, last works have focused on the use of molecular methods to better define and detect drinking water pipe microbiota (Szewzyk *et al.*, 2000). Some researches on bacterial communities in their several habitats were applied (Zhang and Fang, 2001; Jan-Roblero *et al.*, 2004; Neria-González *et al.*, 2006; Cuzman *et al.*, 2010; Inbakandan *et al.*, 2010; Elhariry *et al.*, 2012; Revdiwala *et al.*, 2012). There is no such report about characterization of detrimental biofilms in water treatment system of industrial plant. But for any kind of study to prevent biofilm formation, the knowledge of the industrial environments and bacterial composition of the target detrimental biofilm layer would be of considerable importance. The aim of the present study was to analyse bacterial diversity of detrimental biofilm mass developed in water treatment system of an industrial plant with evident biofouling and biocorrosion signals.

Materials and Methods

Collection of biofilm samples: The area of sample collection in waste water treatment plant of Köyceğiz, Muğla, Turkey (July, 2011) included; the water distribution channel from treatment tank to treated water storage. Microbial biofilm sample was wet, viscous and very slimy brown. This work focussed on the biofilm which was produced by non-phototrophic bacteria, growing without sunlight. The sample was taken by swapping and/or scraping the surface of distribution channel and then collected in sterilized bottles. Collected sample was transferred under aseptic conditions in a refrigerated container to the laboratory.

Isolation and characterization of bacteria: Culturable bacterial strains were isolated on ESP medium and incubated for 24 hrs at 30°C. A total of 20 bacterial isolates were collected. All media used in this study were sterilized at 121 °C for 15 min and plates were incubated at 30 °C for 24 hrs, unless otherwise indicated. All isolates investigated in this study were routinely grown on ESP agar plates. After incubation, slimy colonies in these plates were purified for production of EPS. All bacteria were purified by repeated procedures and subcultured on plates at 30 °C. Pure cultures of these isolates were stored at -80 °C in the corresponding isolation medium, supplemented with 25% (v/v) of glycerol. These frozen stocks were used for further identification at genus and species level (Horan *et al.*, 1988).

Phenotypic characterization: For phenotypic identification of isolates, the following morphological and biochemical tests were performed following in Bergey's Manual of Systematic Bacteriology: Gram stain, growth temperature, cell morphology, endospore forming, motility, presence of pigment, oxidase and catalase tests, oxidative or fermentative acid production from carbohydrates, reduction of nitrate; hydrolysis of starch, Tween 80 and gelatin; citrate, urease, lysine decarboxylase, esterase, H2S production; indole, metile red, Voges-Proskauer tests. All tests were prepared in duplicate. All these characteristics were then compared with the standard description of Bergey's Manual of Determinative Bacteriology (Krieg and Holt, 1984). Moreover, nineteen strains were tested for the ability to grow on various solid selective-media (Collins *et al.*, 1989).

Evaluation of biofilm formation by modified biofilm ring test: Ability of selected twenty isolates to form biofilm was tested using

the Biofilm Ring Test® as described by Chavant *et al.* (2007) and Leroy *et al.* (2009), with modifications. Overnight cultures of the isolates cultured three times in Trypticase Soy Broth (TSB; Oxoid) were diluted in fresh medium. Controls were sterile ESP or TSB medium. Biofilms developed on glass tubes were retrieved at different time intervals and stained with 0.1% safranine (Sigma) for 30 min at room temperature. At the end of staining, unbound stain was removed by rinsing the test tubes three times with sterile phosphate-buffered saline (PBS). Stain was placed into biofilm inner surface of the test tubes. The biofilm containing stain was dried at room temperature and then, the degree of biofilm formation was defined visually. The results were evaluated as weak, moderate, strong and no biofilm production respectively, (+), (++), (+++) and (-).

Semi-quantitative evaluation of biofilm formation by microtiter plate assay (modified crystal violet staining method): As described by Musk *et al.* (2005) the microtiter plate assay (MPA), with modifications, was performed. Briefly, the wells of sterile 96-well polystyrene microtitre plates (MP) (Nunc, USA) were filled with 150 µl of TSB. A 50 µl of each vegetative cell culture was put into each well. The negative control wells contained TSB only. MP were incubated under static conditions at 30 °C for 48 hrs. The content of the plates was poured off and the wells were washed three times with 200 µl of phosphate-buffered saline (PBS). After incubation, the medium was removed and wells were washed three times with 200 µl distilled water to remove non-adherent bacterial cells. The wells were air dried for 45 min and 200 µl per well of 1% (v/v) crystal violet solution in distilled water was added for 15 min. After staining step of adhered cells, the wells were washed three times with 300 µl of distilled water to remove surplus stain. The dye absorbed by the adherent cells was solubilised with 200 µl of 33% (v/v) acetic acid (Sigma-Aldricht, France). The optical density of each plate-well was measured at 570 nm by using a MP reader (Thermo Scientific-Multiskan FC). Based on the absorbance (OD570nm) produced by bacterial films, the strains were classified into four categories according to Christensen *et al.* (1985), with modification by Stepanovic *et al.* (2007). For classification of isolates according to their ability to form biofilms, cut-off optical density values were calculated by the following formula :

ODc = Average OD of negative control + 3 x S.D. of negative control. According to ODc values, the biofilm production capabilities of isolates were valuated as: nonbiofilm production (-) (OD ≤ ODc), weak biofilm production (+) (ODc < OD ≤ 2 × ODc), moderate biofilm production (++) (2 × ODc <OD ≤ 4 × ODc), and strong biofilm production (+++) (4 × ODc > OD). The assay was performed in triplicate with three repeats for each experiment (3 wells). The wells with broth and without inoculating the isolate were used as negative control. For statistical analysis, the mean values and standard deviations of tests were determined for each isolate (Stepanovic *et al.*, 2007)

DNA extraction and PCR amplification: The genomic DNA of strains were extracted from 1 ml of isolate cultured in Lactose Broth with agar using genomic DNA Extraction kit (Zymo Research, USA). Bacterial small subunit rRNA genes were amplified from the purified genomic DNA using the universal forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and the universal reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Kwon *et al.,* 2002; Sambrook, 2006).

PCR amplification was performed in 50 reaction mixtures containing 5 µl of each primer, 200 to 500 ng of genomic DNA, PCR reaction-polymerase buffer (with 20 mM MgCl2), 1 µl of PCR Grade Nucleotid Mix (dNTP Mix), 5 uw^{-1} of Taq DNA polymerase and brought to 50 µl of deionized water. Identical reaction mixtures without target DNA were used as negative controls and did not yield products. A 1000 bp DNA ladder was used as a molecular size standard (Fermentas). The PCR conditions were as follows :

PCR stage	Cycle	Time (minute)	Temperature (°C)
Initial denaturation	1	1	95
Denaturation		1	95
Annealing	30	1	54
Elongation		1	72
Final extension	1	10	72
Cooling	-	Unlimited	4

Gel extraction: PCR products were analyzed using agarose gel electrophoresis; the gel was stained with ethidium bromide and visualized by UVP Basic Jel (Biosan). All PCR products were separated on 1% agarose gels, excised, and purified using the GeneJET Gel Extraction Kit (Fermantas) by following the manufacturer's instructions (Shivaji *et al.*, 2005).

Phylogenetic analysis: Basic Local Alignment Searching Tool (BLAST) search program of the National Center for Biotechnology Information (NCBI) to determine whether they aligned with closely related microorganisms (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Adiguzel *et al.*, 2009). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The tree with the sum of branch length = 0.63587192 is shown. The percentage of replicate trees in which the related taxa clustered together in the bootstrap assay (10000 replicates) are shown next to the branches (Felsenstein, 1985). The optimal tree is created to scale, with branch lengths in the same units as those of the evolutionary distances used to make out the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base shifts per site. All positions containing gaps and missing data were eliminated. There were a total of 1283 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 software (Molecular Evolutionary Genetics Analysis version 6.0) (Tamura *et al.*, 2013). Identification of some isolates (ZZ13, ZZ28, ZZ40 and ZZ82) were done based on the sequence obtained from Sanger sequencing reaction by the primer 1492R. The others were

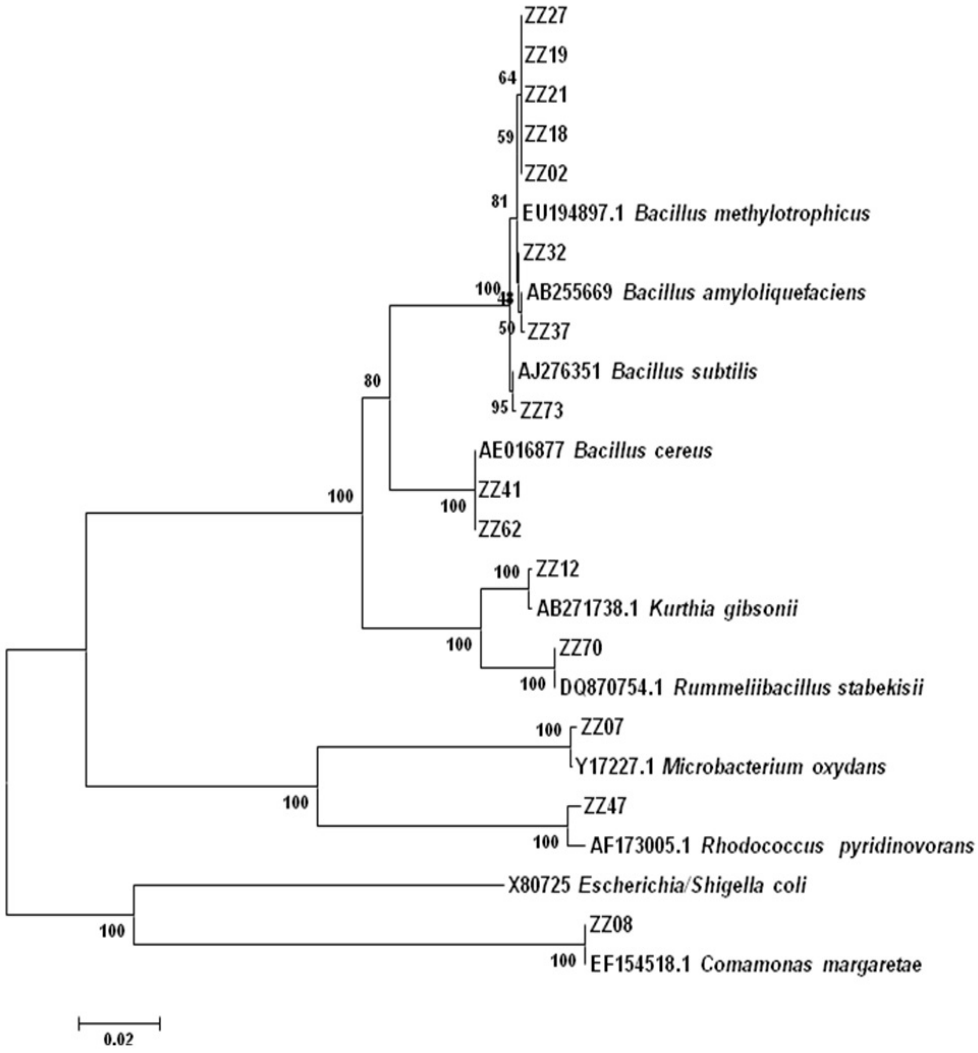


Fig. 1: Phylogenetic positions of the selected detrimental biofilm-forming bacteria in waste water treatment system. The tree was constructed with similarity and neighbor by MEGA6. Bar scale indicates 0.02 (2%) sequence divergence

identified by the full gene sequence analysis using the primers 27F and 1492R.

Accession numbers: For each isolates, the fragments of 16S rRNA gene sequences were deposited in NCBI/GenBank. GenBank accession numbers are given in Table 2.

Results and Discussion

Gram-staining was applied followed by estimation of oxidase and catalase activity, oxidative/fermentative properties in Oxidation/Fermentation (OF) medium and Triple Sugar Iron (TSI) agar medium. Most of the isolated strains were Gram-positive, except ZZ08, ZZ13 and ZZ40 (Table 1). Gram-positive reaction was obtained from two phyla: Firmicutes (except for the classes Mollicutes and Negativicutes) and *Actinobacteria.* Considering the origin of Gram-positive forms, they made up the phylum Firmicutes, a name now used for the largest group. It contains many well-known genera such as *Bacillus*, *Staphylococcus, Streptococcus, Enterococcus, Listeria* and *Clostridium.* Most bacterial phyla are Gram-negative, including cyanobacteria, spirochaetesde and green sulfur bacteria and most Proteobacteria and *Escherichia coli* (Madigan *et al.*, 2004; Brenner *et al.*, 2005).

Also, the strains creating typical colonies were given in some selective media in the table. Only ZZ07, ZZ08, and ZZ82 were yellowish-pigmented, while ZZ47 was salmon-pink-pigmented.

Table 1: Phenotypic properties of isolates.

Phenotypic property	Isolate number																		
	ZZ02	ZZ07	ZZ08	ZZ12	ZZ13	ZZ18	ZZ19	ZZ21	ZZ27	ZZ28	ZZ32	ZZ37	ZZ40	ZZ41	ZZ47	ZZ62	ZZ70	ZZ73	ZZ82
Gram reaction	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Cell morphology	r	rc	r	r	c	c	r	r	r	r	c	r	r	r	rc	r	r	r	r
Motility	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Endospore forming		-	-	-	-	+	+	+		+	+	+	-	+	-	+	+	+	+
Colony pigmentation	-	+ (yellowish)	+ (yellowish)	-	-	-	-	-	-	-	-	-	-	-	+ (salmon-pink)	-	-	-	+ (yellowish
Utilization as a carbon source : Citrate	-	-	+	-	+	-	-	-	-	+	+	+	+	+	+	+	-	+	+
Biochemical tests																			
Urease	+	-	-	-	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-
Lysine decarboxylase	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	+
Indole formation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Metil red reaction	+	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-
Voges- proskauer reaction	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	+
Oxidative/Fermentative	OF	O	O	O	F	OF	OF	OF	OF	OF	OF	OF	F	O	O	O	OF	OF	OF
Nitrate reduction	+	-	+	-	+	+	+	+	+	+	+	+	+	-		-	+	+	+
H_2S formation	-	+	-	-	-	-	-	-	-	+	+	+	-	-	+	-	+	+	-
Esterase	+	-	-	-	-	+	+	+	+	+	+	+	-	-		-	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on various solid media creating typical colonies :																			
EA	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
EMB	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
SSA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MSA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PA	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TSI	K/A	K/A	K/A	K/A	A/A	K/A	K/A	K/A	K/A	A/A	A/A	A/A	A/A	K/A	K/A	K/A	A/A	A/A	K/A
Acid production from starch:	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of gelatin	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-	-	+	+	+
Hydrolysis of Tween 80	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+

r:rod-shaped; c:coccoid shaped; rc:rod-coccoid; EA:Enterococcus agar; EMB:Eosin metilen blue agar; SSA:Salmonella shigella agar; MSA:Mannitol salt agar; PA:Pseudomonas agar; TSA:Triple sugar iron agar; A/A:Acidic slant and acidic butt; K/A:Alkaline slant and acidic butt; +:positive reaction; -:negative reaction.

Table 2: 16S rRNA gene based identification results for ZZ isolates

Isolate name	Sequence length (bp)	Percent similarity (%)	Identification result based on NCBI-BlastN	GenBank accession numbers
ZZ02	1435	99	*Bacillus methylotrophicus* EU194897	KU234670
ZZ07	1446	99	*Microbacterium oxydans* Y17227	KU234671
ZZ08	1387	100	*Comamonas margaretae* EF154518	KU234672
ZZ12	1447	99	*Kurthia gibsonii* AB271738	KU234673
ZZ18	1430	99	*Bacillus methylotrophicus* EU194897	KU234674
ZZ19	1430	99	*Bacillus methylotrophicus* EU194897	KU234675
ZZ21	1435	99	*Bacillus methylotrophicus* EU194897	KU234676
ZZ27	1435	99	*Bacillus methylotrophicus* EU194897	KU234677
ZZ32	1424	99	*Bacillus amyloliquefaciens* AB255669	KU234678
ZZ37	1450	99	*Bacillus amyloliquefaciens* AB255669	KU234679
ZZ41	1440	100	*Bacillus cereus* AE016877	KU234680
ZZ47	1432	99	*Rhodococcus pyridinovorans* AF173005	KU234681
ZZ62	1446	100	*Bacillus cereus* AE016877	KU234682
ZZ70	1432	99	*Rummeliibacillus stabekisii* DQ870754	KU234683
ZZ73	1406	99	*Bacillus subtilis* AJ276351	KU234684
ZZ13a	961	100	*Enterobacter* sp. LN907776	KU234685
ZZ28a	1041	100	*Bacillus* sp. KR029176	KU234688
ZZ40a	951	99	*Enterobacter* sp. KF052587	KU234686
ZZ82a	981	99	*Bacillus* sp. HM242249	KU234687

[a] Identification was done based on the sequence obtained from Sanger Sequencing reaction by the primer 1492R. The others were identified by the full gene sequence analysis using the primers 27F and 1492R.

When biochemical analysis provide to ambiguous identification, the strains were signalized by molecular identification.

The biofilms represent microbial communities containing both culturable and non-culturable bacterial population. In the culture-based method, only a part of the microbial groups were cultured, not the microbial community as a whole. Meanwhile, the culture-dependent method can be used for better exploration of the physiological peculiarities of the biofilm isolates.

Nineteen colonies of bacteria were isolated from the biofilm in the water-treatment system and morphologically distinct strains were isolated and pure cultured. The isolates were renamed from ZZ02 to ZZ82 (Table1). The pure isolates were subjected to 16S rRNA gene sequencing. The different lengths of nucleotides generated from the present study were subjected to BLAST analysis. Sequence resemblance was analyzed through the medium of NCBI nucleotide BLAST with a non-redundant database (Table 2) and accession numbers of the strains are given and species identification and prokaryotic phylogeny was viewed through MEGA6 (Fig. 1).

The 16S rRNA sequencing of nineteen biofilm forming strains were submitted to GenBank. Based on 16S rRNA sequencing, these isolates were identified as *Bacillus, Microbacterium, Comamonas, Kurthia, Enterobacter, Rhodococcus* and *Rummeliibacillus genus.* Among the nineteen strains, Firmicutes (low GC Gram-positive endospore-forming bacteria) were found to be dominant (74.0%) as compared to high GC gram-positive bacteria as Actinobacteria (10.5%), Gammaproteobacteria (10.5%) and Betaproteobacteria (5.0%), respectively. Phylogenetic analysis using 16S rRNA nucleotide sequences indicated that the nineteen strains belonged to four different groups. Firmicutes (ZZ02, ZZ18, ZZ19, ZZ21 and ZZ27 *Bacillus methylotrophicus*, ZZ12 *Kurthia gibsonii,* ZZ32 and ZZ37 *Bacillus amyloliquefaciens,* ZZ73 *Bacillus subtilis,* ZZ41 and ZZ62 *Bacillus cereus,* ZZ70 *Rummeliibacillus stabekisii,* ZZ28 and ZZ82 *Bacillus sp.*), *Actinobacteria*-High GC-Gram positive bacteria (ZZ07 *Microbacterium oxydans*, ZZ47 *Rhodococcus pyridinovorans*), *Gammaproteobacteria*(ZZ13 *Enterobacter sp.* and ZZ40 *Enterobacter sp.*) and *Betaproteobacteria* (ZZ08 *Comamonas margaretae*). In another biofilm investigation, partial sequences of 16S rRNA gene in the fifty seven isolates ensured the presence of only twenty two different strains in biofilm samples from drinking water distribution networks. Gram-negative *Aeromonas hydrophila* was the most formed bacterium in the microbial biofilm obtained from the purified-water storage tanks followed by Gram negative *Pseudomonas sp.* (Elhariry *et al.*, 2012).

Bacillus, Rummelii bacillus and *Kurthia* genera of Phylum Firmicutes are all low GC Gram-positive endospore-forming bacteria. Firmicutes were found to be dominant phylum

compared to others in the present study. These results are similar to another investigation, *B. cereus* and *B. subtilis* was the most common Gram-positive bacteria in the microbial biofilm collected from the water-related storage tanks and pipes (Elhariry *et al.*, 2012). In recent years, *B. subtilis* has become a model organism for the study of biofilm production (Branda *et al.*, 2001; Hamon and Lazazzera, 2001). The ability to produce endospores should be advantageous for a microorganism because microbial habitats can be highly variable environments in terms of nutrient levels, temperature and water activity. Thus, a heat- and desication-resistant structure capable of remaining dormant for long periods should offer considerable survival value in industrial systems. Moreover, *Firmicutes* species that are pathogenic to humans or other animals are primarily saprophytic organisms, and infect hosts only incidentally (Madigan *et al.*, 2005). In addition, *Bacillus species* biofilms exhibit heterogeneity in matrix of extracellular polymeric substance production; large groups of cells dramatically increase EPS secretion when they convert into the biofilm phenotype (Vlamakis *et al.*, 2008). EPS plays a major role in retaining cells together within the biofilm while simultaneously protecting them from external agents.

The biofilm contained spore forming bacteria (Bacillaceae), nonsporulating non-motile Gram-positive bacteria (Nocardiaceae- *R. pyridinovorans*), Gram-negative bacteria (Comamonadaceae and Enterobacteriaceae), rod-shaped bacteria *(Kurthia sp., Bacillus sp., Rumellibacillus sp., Enterobacter sp., Comamonas sp., Microbacterium sp.)*, obligate aerobes like *M. oxydans* and *R. pyridinovorans* and facultative anaerobic forms like *Enterobacter* species and Microbacteriaceae. Therefore, the undesirable bacterial biofilm community in industrial system contains bacteria with diversified physiology and tolerance capabilities.

In the present study, most of the isolated strains were Gram-positive except *Comamonas* and *Enterobacter* strains. Neighbour joining phylogenetic trees inferred from 16S rRNA gene sequences of nineteen biofilm strains indicated that they were distributed over a wide range of bacterial taxonomic groups (Fig. 1). Two Gram-negative genera; *Enterobacter* and *Comamonas* belonged to the *Gammaproteobacteria* and *Betaproteobacteria* subclass, respectively. From the partial sequencing data of 16S rRNA gene, it is clearly understood that the most occurrence frequency in the microbial biofilm was recorded by Gram-negative *Proteobacteria*, especially *Enterobacter species* in the industrial system followed by the Gram-positive *Bacillus sp. Enterobacter* strains (ZZ13 and ZZ40) reside in the gamma subdivision, and ZZ08 *C. margaretae* resides in the beta subdivision of Proteobacteria. *Enterobacter species* have been reported to form biofilms in abiotic or biotic environments by some researchers (Kim *et al.*, 2006; Jacob and Kim, 2010; Naher *et al.*, 2014). *Comamonas sp.* is also phylogenetically related to Pseudomonads and is characterized as no spores, aerobic Gram-negative rods (Buchanon and Gibbons, 1974). Based on the other investigations of water-related systems using molecular tools (Schwartz *et al.*, 1998; Schmeisser *et al.* 2003), the consensus was that bacteria from the *alpha, beta* and *Gammaproteobacteria* consist of the majority of the cells in water distribution networks (Kalmbach *et al.*, 1997a, 1997b). In previous studies, most of the isolated strains from drinking water distribution networks were facultative anaerobes except *Pseudomonas, Micrococcus* and *Bacillus strains.* Neria-González *et al.* (2006) reported that bacterial diversity in corrosive biofilms associated to steel pipelines, basically included *Enterobacter sp., Citrobacter sp., Halanaerobium sp*. and *Desulfovibrio alaskensis.*

Rhodococcus is a genus of nonsporulating, nonmotile Gram-positive bacteria closely related to *Mycobacterium* and *Corynebacterium* (Burkovski, 2008). While a few species are pathogenic, most are benign, and have been found to thrive in a broad range of environments, including water, soil and eukaryotic cells. *Rhodococcus* is also an experimentally favored system owing to a relatively fast growth rate and simple developmental cycle, thus its asset is important (McLeod *et al.*, 2006). In the present study, among the coryneform bacteria, the phenotypically and phylogenetically closely related isolates ZZ47 *(R. pyridinovorans)* and ZZ07 (*M. oxydans*) (Fig. 1) were observed. Gneiding *et al.* (2008) reported eleven strains belonging to *M. oxydans* from human clinical biofilm specimens were further characterized by phenotypic and molecular genetic methods.

The present study explains the taxonomic position of round-spore-forming strain ZZ70. 16S rRNA gene sequence similarities demonstrated that strain ZZ70 was most closely affiliated with the species of Bacillus strains and with *Kurthia gibsonii* as the next nearest relatives, which was identified as *R. stabekisii* (Fig.1). Vaishampayan *et al*. (2009) reported that differences in the molecular structure of the cell-wall peptidoglycan could not differentiate *R. stabekisii* strains sufficiently from other closely related genera (*Bacillus* and *Kurthia*) (Vaishampayan *et al.*, 2009).

Enterobacter species reported in the present study (ZZ13 and ZZ40) may emerge as pathogens responsible for hospital-acquired infections, as several *Enterobacter* strains are opportunistic organisms that can cause an array of diseases and *Enterobacter* sp. belongs to the *E. cloacae* complex, which has been often isolated from nosocomial infections (Paauw *et al*., 2008).

The majority of biofilms occured in fouling of pipes and industrial equipment is undoubtedly included by multiple microbial species (Stoodley *et al.*, 2002). Biofilms are a complex mixture of microbial species, phenotyping and molecular identification studies focus on the microbial diversity of detrimental biofilms (Mielich-Süss and Lopez, 2015).

The mean (±SD) biofilm production was calculated for each bacteria. Biofilm forming abilities of bacterial strains (optical density of cut off=ODc) varied from 0.38 to 14.10 at 37°C. Though the strains were moderate to strong biofilm producers, analysis

did not reveal significant differences in the biofilm formation abilities among the two methods ($p>0.05$). Accordingly results, the isolates were divided into four groups based upon the OD_{570} of bacterial biofilm : nonformer, weakly = 1; 5.26%), moderately = 2, 10.53%) or strongly = 16; 84.21%) biofilm formers. All the isolates were biofilm producers, although of them majority were strong biofilm producers. The percentage of isolates that were moderate biofilm producers was clearly higher than the percentage obtained for weak biofilm producers. The results exhibited that the key bacterial strains in biofilm production were Gram-positives *Bacillus species* (eleven strains), *R. pyridinovorans, R. stabekisii* and Gram-negative *Enterobacter species* (two strains) and *C. margaretae,* where they were defined as strong biofilm producer (+++).

Similarly, among the twenty two isolated aquatic bacteria from microbial biofilms formed in drinking water distribution networks, eleven strains were strong biofilm producers at 30 and 37°C (Elhariry *et al.*, 2012). In the same survey, at these temperatures, all strains were strong biofilm producers (+++) including Micrococcus flavus (TUB1 and TUB2), *Escherichia coli* TUB17, Pseudomonas alcaligenes (TUB12 and TUB13), *Pseudomonas aeruginosa* TUB14, *Aeromonas sp.* (TUB19, 20 and 21) and *Lactobacillus delbrueckii subsp. Lactis* (TUB5 and TUB7).

It is quite evident from the present study that bacterial biofilm in an industrial system harbors a diverse group of bacteria. From the present study it is understood that effective control of unwanted biofilms can be achieved by identification of biofilm microbial contents and the microorganisms to be removed from industrial environments.

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References

Adiguzel, A., H. Ozkan, O. Baris, K. Inan, M. Gulluce and F. Sahin: Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey. *J. Microbiol. Meth*., **79,** 321-328 (2009).

Amann, R.I., W. Ludwig and K.H. Schleifer : Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.,* **59**, 143-169 (1995).

Beech, I.B., J.A. Sunner and K. Hiraoka : Microbe surface interactions in biofouling and biocorrosion process. *J. Ind. Microbiol. Biotechnol.,* **8,** 157-168 (2005).

Branda, S.S., J.E. Gonzalez-Pastor, S. Ben-Yehuda, R. Losick and R. Kolter: Fruiting body formation by *Bacillus subtilis. Proc. Natl. Acad. Sci.,* **98,** 11621-11626 (2001).

Brenner, D.J., N.R. Krieg and J.T. Staley: Introductory Essays. Bergey's Manual of Systematic Bacteriology (Ed.: G.M. Garrity.). 2nd Edn., Springer, New York, p. 304 (2005).

Buchanon, U.R.E. and N.E. Gibbons: Bergey's Manual of Determinative Bacteriology. 8th Edn., The Williams and Wilkins Company Ltd., Baltomore, pp. 22-35 (1974).

Burkovski, A. (Ed.): Corynebacteria: Genomics and Molecular Biology. Caister Academic Press, New York., pp. 30-41 (2008).

Chae, M.S., H. Schraft, L. Truelstrup and R. Mackereth: Effects of physicochemical surface characteristics of *Listeria monocytogenes* strains on attachment to glass. *Food Microbiol.,* **23,** 250–259 (2006).

Chambers, L.D., K.R. Stokes, F.C. Walsh and R.K.J. Wood: Modern approaches to marine antifouling coatings. *Surf. Coat. Technol.*, **201,** 3642-3652 (2006).

Chavant, P., B. Gaillard-Martinie, R. Talon, M. Hébraud and T. Bernardi: A new device for rapid evaluation of biofilm formation potential by bacteria. *J. Microbiol. Meth*., **68**, 605–612 (2007).

Christensen, G.D., W.A. Simpson, J.J. Younger, L.M. Baddour, F.F. Barrett and D.M. Melton: Adherence of coagulase-negative staphylococci to plastic tissue culture plates: A quantitative model for the adherence of staphylococci to medical devices. *J. Clin. Microbiol.*, **22,** 996-1006 (1985).

Collins, C.H., P.M. Lyne and J.M. Grange: Microbiological Methods.Oxford: Butterworth- Heinemann, pp. 380 (1989).

Cuzman, O.A., S. Ventura, C. Sili, C. Mascalchi, T. Turchetti, L. D'Acqui and P. Tiano: Biodiversity of phototrophic biofilms dwelling on monumental fountains. *Microbial. Ecol.,* **60,** 81–95 (2010).

Donlan, R.M.: Biofilm formation: A clinically relevant microbiological process. *Clin. Infec. Dis.,* **33,** 1387–1392 (2001).

Elhariry, H., Y. Gherbawy, B. El-Deeb and A. Altalhi: Molecular identification and biofilm-forming ability of culturable aquatic bacteria in microbial biofilms formed in drinking water distribution networks. *Geomicrobiol., J*., **29,** 561-569 (2012).

Felsenstein, J. : Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,* **39,** 783-791 (1985).

Gneiding, K., R. Frodl and G. Funke: Identities of *Microbacterium sp.* encountered in human clinical specimens. *J. Clin. Microbiol.,* **46,** 3646- 3652 (2008).

Hall-Stoodley, L. and P. Stoodley: Developmental regulation of microbial biofilms. *Curr. Opin. Biotechnol.,* **13,** 228–233 (2002).

Hamon, M.A. and B.A. Lazazzera: The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis. Mol. Microbiol.,* **42,** 1199–1209 (2001).

Hamilton, W.A.: Microbially influenced corrosion as a model system for the study of metal microbe interactions: unifying electron transfer *hypothesis. Biofouling,* **19,** 65–76 (2003).

Horan, N.J., A.M. Bu'Ali and C.R. Eccles. Isolation, identification and characterization of filamentous and floc-forming bacteria from activated sludge flocs. *Environ. Technol.,* **9,** 449–457 (1988).

Inbakandan, D., P.S. Murthy, R. Venkatesan and S.A. Khan: 16S rDNA sequence analysis of culturable marine biofilm forming bacteria from a ship's hull. *Biofouling,* **26,** 893–899 (2010).

Jan-Roblero, J., J.M. Romero, M. Amaya and S. Le Borgne: Phylogenetic characterization of a corrosive consortium isolated from a sour gas pipeline. *Appl. Microbiol. Biotechnol.,* **64,** 862–867 (2004).

Jacob, B.M. and E. Kim: Inhibiting biofilm formation of *Enterobacter sp*. prevented premature withering in cut flowers. *Korean J. Chem. Eng.,* **27,** 1252-1257 (2010).

Kalmbach, S., W. Manz and U. Szewzyk: Dynamics of biofilm formation in drinking water: Phylogenetic affiliation and metabolic potential of single cells assessed by formazan reduction and *in situ* hybridization. *FEMS Microbiol. Ecol.,* **22,** 265–279 (1997a).

Kalmbach, S., W. Manz and U. Szewzyk: Isolation of new bacterial species from drinking water biofilms and proof of their in situ dominance with highly specific 16S rRNA probes. *Appl. Environ. Microbiol.,* **63,** 4164–4170 (1997b).

Kim, H., J.H. Ryu and L.R. Beuchat: Attachment of and biofilm formation by *Enterobacter sakazakii* on stainless steel and enteral feeding tubes. *Appl. Envir. Microbiol.,* **24,** 5846–5856 (2006).

Kimura, M.: A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.,* **16,** 111-120 (1980).

Krieg, N.R. and C.G. Holt: Bergey's Manual of Systematic Bacteriology, Vol. 1, Williams & Wilkins Press, Baltimore, p. 787 (1984).

Kwon, K.K., H.S. Lee, S.Y. Jung, J.H. Yim, J.H. Lee and H.K. Lee: Isolation and identification of biofilm-forming marine bacteria on glass surfaces in Dae-Ho Dike, Korea. *J. Microbiol.,* **50,** 260-262 (2002).

Leroy, S., I. Lebert, J.P. Chacornac, P. Chavant, T. Bernardi and R.Talon: Genetic diversity and biofilm formation of *Staphylococcus equorum* isolated from naturally fermented sausages and their manufacturing environment. *Int. J. Food Microbiol.,* **134,** 46–51 (2009).

Ludensky, M.: Control and monitoring of biofilms in industrial applications. *Int. Bacteriol. Bioeng.,* **51,** 255-253 (2003).

Madigan, M.T., J. Martinko and J. Parker: Brock Biology of Microorganisms 10th Edn., Lippincott Williams & Wilkins, Philadelphia, pp. 369 (2004).

Madigan, M., J. Martingo and J. Parker: Brock Biology of Microorganisms. 11th Edn., UpperSaddle River (NJ), Prentice-Hall, p. 550 (2005).

McLeod, M.P., R.L. Warren, W.W. Hsiao, N. Araki, M. Myhre, C. Fernandes, D. Miyazawa, W. Wong, A.L. Lillquist, D. Wang, M. Dosanjh, H. Hara and A. Petrescu: The complete genome of *Rhodococcus sp.* RHA1 provides insights into a catabolic powerhouse. *PNAS,* **103,** 15582–15587 (2006).

Mielich-Süss, B. and D. Lopez: Molecular mechanisms involved in *Bacillus subtilis* biofilm formation. *Envir. Microbiol.,* **17,** 555–565 (2015).

Musk, D.J., D.A. Banko and P.J. Hergenrother: Iron salts perturb biofilm formation and disrupt existing biofilms of *Pseudomonas aeruginosa*. *Chem. Biol.,* **12,** 789–796 (2005).

Naher, J., S.A. Chowdhury, A.A. Mamun, N. Mahmud, W. Shumi and R.A.Khan : A comparative study on the biofilm formation of *Enterobacter agglomerans* and *Serratia rubideae* in different environmental parameter under single culture condition. *Open J. Med. Microbiol.,* **4,** 70- 76 (2014).

Neria-González, I., E.T. Wang, F. Ramírez, J.M. Romero and C.H. Rodríguez: Characterization of bacterial community associated to biofilms of corroded oil pipelines from the southeast of Mexico. *Anaerobe,* **12,** 122–133 (2006).

Nocker, A., J.E. Lepo, L.L. Martin and R.A. Snyder: Response of estuarine biofilm microbial community development to changes in dissolved oxygen and nutrient concentrations. *Microbial. Ecol.,* **54,** 532-542 (2007).

Oulahal, N., W. Brice, A. Martial and P. Degraeve: Quantitative analysis of survival *Staphylococcus aureus* or *Listeria innocua* on two types of surfaces: Propylene and stainless steel in contact with three different dairy products. *Food Control,* **19,** 178–185 (2008).

Paauw, A., M. Caspers, F. Schuren, H.M. Leverstein-van , A. Delétoile, R. Montijn, J. Verhoef and A. Fluit: Genomic diversity within the *Enterobacter cloacae* 3 complex. *PLoS One,* **3,** 3018-3028 (2008).

Revdiwala, S., B.M. Rajdev and S. Mulla: Characterization of bacterial etiologic agents of biofilm formation in medical devices in critical care setup. *Critical Care Research* and *Practice.* **Vol. 2012**, Article id. 945805 (2012).

Sambrook, J.: Molecular Cloning a Laboratory Manual. 3rd Edn., Cold Spring Harbor Laboratory Press, Boston, pp, **143** (2006)

Saravanan, P., Y.V. Nanharaiah, V.P. Venugopalan, T.S. Rao and S. Jayachandran: Biofilm formation by *Pseudoalteromonas ruthenica* and its removal by chlorine. *Biofouling,* **22,** 371-381 (2006).

Saravanan, P., S.R. Prabagaran, Y.V. Nancharaiah, M. Krishnaveni, V.P. Venugopalan and S. Jayachandran: Isolation and characterization of *Pseudoalteromonas ruthenica* (SBT033), an EPS-producing biofilm bacterium from the seawater intake point of a tropical power station EPS producing *Pseudoalteromonas ruthenica. World J. Microbiol. Biotechnol.,* **24,** 509-515 (2007).

Saitou, N. and M. Nei : The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.,* **4,** 406-425 (1987).

Schmeisser, C., C. Stockigt and C. Raasch: Metagenome survey of biofilms in drinking-water networks. *Appl. Environ. Microbiol.,* **69,** 7298–7309 (2003).

Schwartz, T., S. Kalmbach, S. Hoffmann, U. Szewzyk and U. Obst: PCR-based detection of mycobacteria in biofilms from a drinking water distribution system. *J. Microbiol. Meth.,* **34,** 113–123 (1998).

Shivaji, S., G.S. Reddy, K. Suresh, P. Gupta, S. Chintalapati, P. Schumann, E. Stackebrandt and G.I. Matsumoto: *Psychrobacter vallis sp.* nov. and *Psychrobacter aquaticus sp.* nov., from Antarctica. *Int. J. Syst. Evol. Micr.,* **55,** 757–762 (2005).

Simões, M., L.C. Simões and M.J. Vieira: A review of current and emergent biofilm control strategies. *LWT - Food Sci. Tech.,* **43,** 573–583 (2010).

Stepanovic, S., D. Vukovic, V. Hola, G. Di-Bonaventura, S. Djukic and I. Cirkovic: Quantification of biofilm in MPs: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Apmis,* **115,** 891-899 (2007).

Stoodley, P., K. Sauer, D.G. Davies and J.W. Costerton: Biofilms as complex differentiated communities. *Annu. Rev. Microbiol.,* **56,** 187–209 (2002).

Snyder, R.A., M.A. Lewis, A. Nocker and J.E. Lepo: Microbial biofilms as integrative sensors of environmental quality. In: Estuarine Indicators, (ed.: S.A. Bort-one). CRC Press, Boca Raton, FL., pp 111–126 (2004).

Szewzyk, U., R. Szewzyk, W. Manz and K.H. Schleifer: Microbiological safety of drinking water. *Annu. Rev. Microbiol.,* **54,** 81–127 (2000).

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar: MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.,* **30,** 2725-2729 (2013).

Terry, A., E. Curtis, R. Pantle and S.A. Penny : Boundaries for biofilm formation: Humidity and temperature. *Appl. Environ. Microbiol.,* **69,** 5006- 5010 (2003).

Vaishampayan, P., M. Miyashita, A. Ohnishi, M. Satomi, A. Rooney, M.T. La-Duc and K. Venkateswaran: Description of *Rummeliibacillus stabekisii* gen.nov., sp.nov. and reclassification of *Bacillus pycnus* Nakamura *et al.* 2002 as *Rummeliibacillus pycnus* comb. nov. *Int J Sys Evol Microbiol.,* **59,** 1094–1099 (2009).

Vlamakis, H., C. Aguilar, R. Losick and R. Kolter: Control of cell fate by the formation of an architecturally complex bacterial community. *Genes Dev.,* **22,** 945 –953 (2008).

Zhang, T. and P.H. Fang: Phylogenetic diversity of SRB- rich marine biofilm. *Appl. Microbiol. Biotechnol.,* **57,** 437–440 (2001).