Quantification of Biofilm Production and Molecular Identification For Highly Yielding Production Isolates

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Abstract

Background: Biofilms are aggregation extracellular matrix produce by some bacteria considered important for the existence of it, in inadequate environmental conditions, which are organized into structural communities and subpopulations if produce provide exo – polysaccharide (EPS) matrix as a major component structure for their stability.

Objectives: In the present study, we investigated biofilm forming ability in two different media beside the molecular identification for high yield biofilm production isolates.

Methods: In this study a 32 isolates obtained from Central Public Health Laboratory in Karbala City. The ability for biofilm production were evaluated and compared in two distinct media (Luria Bertani- LB broth and Tryptic soy broth - TSB). Microtitre plate (MTP) assay was used to quantify the biofilm production ability from bacterial isolates. DNA was extracted, molecular identification was accomplished by fragmentary Sequences of 16s rRNA gene in nine more effective biofilm production isolates.

Results: From the total number of isolates 12.5% could be categorized as a weakly biofilm former ; 62.5% moderate and 25% strongly produced to biofilm in LB broth media, whereas (37.5%, 53.1% and 9.4% ) were weakly; moderately and strongly produced to biofilm respectively in TSB media . Molecular identification of the selected isolates showed S.1 had 99% similarity to Bhargavaea cecembebsis ;S.2 had 100% similarity to Staphylococcus aureus; S.3 had 99% similarity to Bacillus licheniformis; S.4 had 99% similarity to Bacillus sonorensis; S.5 had 100% similarity to Stenotrophomona maltophilia and S.6 had 100% similarity to Enterococcus faecalis; while no PCR amplification results were given in S.7, S.8 and S.9 isolates.

Conclusion: All of isolates in present study had biofilm forming ability, 9 isolates can be categorized as strong biofilm former in LB broth media, while 3 isolates can be categorized as strong biofilm former in TSB broth media. Also, the ability of biofilm production in LB media was higher than when TSB media was used (p= >0.0001). The appropriate medium for studying and detection the ability of bacteria to the production of biofilm varies according to the type of bacteria as it has been shown Tryptic soy was more suitable of Bacillus sonorensis for detection biofilm formation as compared to the Luria Bertani broth in the present investigation.
Introduction

One of Multidrug-resistant of pathogenic microorganism mechanism have ability to forming plastic surface architecturally complex communities termed biofilms [1,2]. In biofilms, cells of bacteria or fungi grow in multicellular aggregates that are encased in surface membrane [2]. The types of biofilms production depending on specific strain of cell, medium, growth conditions and substrates [3,4]. Biofilms are biopolymer contain from an extracellular polysaccharide result of different steps attached with other especially composed such as teichoic acid, phospholipids, extracellular proteins, nucleic acids [5,6]. In general, found some small molecules play an important role in the maintenance and development of biofilm communities such as antibiotics, homoserine lactones and other secondary metabolites (pigments and siderophores) by inhibition of the quorum - sensing system and enhance the genes expression responsible for exopolysaccharide synthesis [7-9]. In Pseudomonas aeruginosa consider Imipenem antibiotics induced expression of alginate (exopolysaccharide) and the phenazine pyocyanin pigment Is the first sign of an increase in the ability to make biofilms but marine furanones and quinupristin-dalfopristin in Staphylococcus aureus which may result in the use of the wrong bacterial antibiotic to induce mucoid phenotypes, which make more thicker biofilms with additional components [10]. These structures help opportunistic bacteria to invade the host body and therapies resistant through its contribution for the initial attachment, prevent detection on bacterial cell walls by phagocytes, reduce the lymphoproliferative response, limits the ability of leukocytes to penetrate and movement through biofilm, attenuates their ability to exposing bacterial to inhibitory antibiotic treatment concentrations [11-13]. Through that you become biofilm-related bacteria are notoriously hard to eradicate and associated with chronic bacterial infections in human organs according to a publication by the National Institutes of Health including P. aeruginosa, the main causal cystic fibrosis (CF), Escherichia coli and Klebsiella, the causative of the urinary tract infections (UTIs), dental caries caused by Streptococcus mutans, upper respiratory tract infections such Mycobacterium tuberculosis, which causes tuberculosis and Chronic wounds (CWs) infection by S. aureus, P. aeruginosa, Enterococcus faecalis [14-16].

The aim of present study was investigate biofilm forming ability in two different media beside the molecular identification for high yield biofilm production isolates.

Materials and Methods

Bacterial strains:

A total of 32 identified isolates coded as (S.1 to S.32) were obtained from Central Public Health Laboratory in Karbala City, maintained in glycerol stock at 4º C. Bacterial isolates identified were examined on the basis of the colony exterior shape, gram reaction, cell morphology and standard biochemical tests [17,18].
Quantification of Biofilm Formation

The LB and TSB were used as growth medium. The commonly used microtitre dish as important tool for determining bacterial the early stages in adhesion to plastic surface (biofilm formation) used batch-growth conditions was applied in the present study. Add 180 μL of culture medium (LB or TSB) in a 96 well of microtitre-dish (BioTek, USA), Briefly, A 20 μl of each vegetative cell culture suspension (0.5 O.D600) was added in to each well, the negative control well contain media only. Plates were incubated at 37°C for 18-24 h, the content of the microtitre plates were poured off and the wells were washed with autoclaved distilled water, followed by vigorous tapping on absorbent paper, the remaining cohesion cells were fixed for 30 min 80ºC and staining by addition 125 μl of 0.3% crystal violet solution in water for 10 min at room temperature, the excess of stain was shake out by washing with sterile distilled water. After the microtitre plates were air dried for a few hours at room temperature, 125 μl of decolouring solution (ethanol/acetone 80:20) was added to each well for 15 min to solubilize the crystal violet stain from surface of flat bottomed microtitre. The absorption of the eluted stain was measured in a plate reader (Synergy, Biotek Winooski, VT, USA) at 630 nm using ethanol/acetone 80:20 as the blank. Based on optical density (O.D.) reading of biofilm, Bacterial isolates were classify in to three categories; weakly, moderately and strongly biofilm producers as previously described [19-21].

Molecular Identification for Highly Yielding Biofilm producing Bacteria:

Pure cultures bacteria were grown overnight in nutrient broth for isolation genomic DNA purification kit (Promega wizard® / USA). Deoxyribonucleic acid (DNA) concentration and purity were determined by using a spectrophotometer (BioTek’s). The isolates were identified by 16S ribosomal RNA gene sequencing.16S rDNA was amplified by using the universal bacterial 16S rDNA primers. Forward: GTTTGATCCTGGCTCAG Reverse: AAGGAGGTGATCCAGCC (22). The PCR master mix was (Syntol/ Russia) and the PCR was performed in a thermo cycler under conditions that mentioned in [23]. The PCR products were loaded to electrophoresis with gel electrophoreses in 1% agarose gels stained with ethidium bromide (10 mg/ml), photographed and analyzed using gel documentation system (Harvard/UK) to check the amplification of the desire amplicons of rDNA which is approximately 1538 bp A 100 bp DNA ladder (Bioneer - South Korea) were used as a size marker. The resolved PCR amplicons were commercially sequenced from both (forward and reverse) termini according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of local bacterial samples with the retrieved neighboring DNA sequences of the NCBI Blastn engine, the virtual positions and other details of the retrieved PCR fragments were identified. The sequencing results of the PCR products of different samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome.

Comprehensive phylogenetic tree construction:

A specific comprehensive bacterial tree was constructed in this study according to the protocol described by Al-Shuaib et al. [24]. The observed bacterial variants were compared with their neighbour homologous reference sequences using NCBI-BLASTn server [25]. Then, the blast results of the observed variants were combined and aligned together using a Clustal Omega based tools. A full inclusive tree, including the observed variant, was visualized as polar
cladogram using Figtree tool [26]. The bacterial sequences of each classified phylogenetic species-group in the comprehensive tree were coloured appropriately.

**Statistical Analysis**

Statistical analysis was carried out using SPSS version 23 (SPSS, IBM Company, Chicago, USA). Chi square test was used to compare the variation of biofilm formation by each isolate growth in LB and TSB media. P value for all tests was taken as and considered significant if < 0.05.

**Results and Discussion**

**Screening for biofilm producing ability of bacterial isolates:**

The ability of biofilm production were tested in two types of culture media (LB and TSB) media as shown in (Table 1), depended on the result applied primarily for the study and obtained, by Microtiter Dish assay method, The more concentrated stain, greater biofilm former (Figure 1). A total of 32 isolates 12.5% and 37.5% categorized as weak biofilm producer, additionally, 62.5% and 53.1% were classified as moderate biofilm producer, whereas 25% and 9.4% have a strong capacity to form biofilm in LB and TSB media respectively (Table 2). The difference between the strongly biofilm producing isolates in both media were found to be statistically significant (p = >0.0001).

**Table 1:** Screening of isolates for biofilm formation by Microtitre dish method in LB and TSB media

<table>
<thead>
<tr>
<th>Samples/L.B</th>
<th>O.D.</th>
<th>Biofilm respond**</th>
<th>Samples/T.S.B</th>
<th>O.D.</th>
<th>Biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.09</td>
<td>weakly adherent</td>
<td>control</td>
<td>0.067</td>
<td>weakly</td>
</tr>
<tr>
<td>S.1</td>
<td>0.386</td>
<td>strongly adherent</td>
<td>S.1</td>
<td>0.156</td>
<td>moderately</td>
</tr>
<tr>
<td>S.2</td>
<td>0.392</td>
<td>strongly adherent</td>
<td>S.2</td>
<td>0.172</td>
<td>moderately</td>
</tr>
<tr>
<td>S.3</td>
<td>0.678</td>
<td>strongly adherent</td>
<td>S.3</td>
<td>0.488</td>
<td>strongly</td>
</tr>
<tr>
<td>S.4</td>
<td>0.434</td>
<td>strongly adherent</td>
<td>S.4</td>
<td>0.716</td>
<td>strongly</td>
</tr>
<tr>
<td>S.5</td>
<td>0.433</td>
<td>strongly adherent</td>
<td>S.5</td>
<td>0.132</td>
<td>weakly</td>
</tr>
<tr>
<td>S.6</td>
<td>0.319</td>
<td>strongly adherent</td>
<td>S.6</td>
<td>0.176</td>
<td>moderately</td>
</tr>
<tr>
<td>S.7</td>
<td>0.346</td>
<td>strongly adherent</td>
<td>S.7</td>
<td>0.151</td>
<td>moderately</td>
</tr>
<tr>
<td>S.8</td>
<td>0.342</td>
<td>strongly adherent</td>
<td>S.8</td>
<td>0.150</td>
<td>moderately</td>
</tr>
<tr>
<td>S.9</td>
<td>0.184</td>
<td>moderately</td>
<td>S.9</td>
<td>0.345</td>
<td>strongly</td>
</tr>
<tr>
<td>S.10</td>
<td>0.227</td>
<td>moderately</td>
<td>S.10</td>
<td>0.089</td>
<td>weakly</td>
</tr>
<tr>
<td>S.11</td>
<td>0.188</td>
<td>moderately</td>
<td>S.11</td>
<td>0.119</td>
<td>weakly</td>
</tr>
<tr>
<td>S.12</td>
<td>0.162</td>
<td>moderately</td>
<td>S.12</td>
<td>0.286</td>
<td>moderately</td>
</tr>
<tr>
<td>S.13</td>
<td>0.266</td>
<td>moderately</td>
<td>S.13</td>
<td>0.100</td>
<td>weakly</td>
</tr>
<tr>
<td>S.14</td>
<td>0.209</td>
<td>moderately</td>
<td>S.14</td>
<td>0.108</td>
<td>weakly</td>
</tr>
<tr>
<td>S.15</td>
<td>0.155</td>
<td>moderately</td>
<td>S.15</td>
<td>0.131</td>
<td>weakly</td>
</tr>
<tr>
<td>S.16</td>
<td>0.302</td>
<td>moderately</td>
<td>S.16</td>
<td>0.136</td>
<td>moderately</td>
</tr>
<tr>
<td>S.17</td>
<td>0.276</td>
<td>moderately</td>
<td>S.17</td>
<td>0.166</td>
<td>moderately</td>
</tr>
<tr>
<td>S.18</td>
<td>0.229</td>
<td>moderately</td>
<td>S.18</td>
<td>0.277</td>
<td>moderately</td>
</tr>
<tr>
<td>S.19</td>
<td>0.176</td>
<td>moderately</td>
<td>S.19</td>
<td>0.132</td>
<td>weakly</td>
</tr>
<tr>
<td>S.20</td>
<td>0.163</td>
<td>moderately</td>
<td>S.20</td>
<td>0.079</td>
<td>weakly</td>
</tr>
<tr>
<td>S.21</td>
<td>0.258</td>
<td>moderately</td>
<td>S.21</td>
<td>0.095</td>
<td>weakly</td>
</tr>
<tr>
<td>S.22</td>
<td>0.213</td>
<td>moderately</td>
<td>S.22</td>
<td>0.181</td>
<td>moderately</td>
</tr>
<tr>
<td>S.23</td>
<td>0.150</td>
<td>moderately</td>
<td>S.23</td>
<td>0.144</td>
<td>moderately</td>
</tr>
</tbody>
</table>
Table 2: Distribution of the isolates for biofilm producing in LB and TSB media

<table>
<thead>
<tr>
<th>Biofilm responds</th>
<th>Samples NO. (%) in LB</th>
<th>Samples NO. (%) in TSB</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weakly adherent</td>
<td>4 (12.5%)</td>
<td>12 (37.5%)</td>
<td>0.05&gt;</td>
</tr>
<tr>
<td>Moderately adherent</td>
<td>20 (62.5%)</td>
<td>17 (53.1%)</td>
<td>0.05&gt;</td>
</tr>
<tr>
<td>Strongly adherent</td>
<td>8 (25%)</td>
<td>3 (9.4%)</td>
<td>0.0001&gt;</td>
</tr>
</tbody>
</table>

*p value ≤ 0.05
The Microtittere Plate Method (MTP) was used in many studies as quantitative method for biofilm production. Sheriff and Sheena [27] value judgment of biofilm formation in two groups of Clinically critical Isolates of *Staphylococcus epidermidis* and matching of quantitative and qualitative assay methods of biofilm formation in a tertiary care hospital. They found the more quantitative and sensitive method was MTP method. In contrast the tissue culture plate (TCP) method was found to be most sensitive, accurate and reproducible screening method among others Tube method (TM) and Congo red agar (CRA) method for disclosure statement of biofilm production by *Staphylococci* and has the improve and advantage of being a quantitative model to study the cohesion and coherence of *Staphylococci* on biomedical devices when evaluate three methods for disclosure statement of biofilm formation in *Staphylococci* [28-30].

**Molecular Identification for Highly Yielding Biofilm producing Bacteria**

The highly biofilm producing bacterial isolates were identified by 16S rRNA gene sequence analysis which is the most useful molecular chronometer to infer phylogenetic relationships, because they are present in all organisms [31]. The gene of 16S rRNA was amplified using universal primer set with PCR product of 1538 bp in size (Figure 2).

![Electrophoresis pattern of PCR product for 16S rRNA gene](image)

**Figure 2:** Electrophoresis pattern of PCR product for 16S rRNA gene in 1% agarose, 75 V, for 3 h (10 µl of PCR product loaded in each well).

Since 16S rDNA sequences are highly conserved within this locus, six samples were included in the present study that had shown to amplify 16S rRNA genetic sequences in several bacterial isolates. Concerning the supposed 1492 bp amplicons, NCBI BLASTn engine shown about 99% to 100% sequences similarities between the sequenced samples and several bacterial reference target sequences. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences, the approximate positions and other details of the retrieved PCR fragments were identified.

The phylogenetic tree showed evolutionary distance among bacteria based on 16S rRNA gene sequence (Figure 3). The phylogenetic tree showed S.1 has 99% homology with *Bhargavaea cecembensis* (GenBank acc. no. NR_042537.1), S.2 has similarity with *Staphylococcus aureus* strain about 100% (GenBank acc. no. LR134193.1), S.3 has 99% similarity with *Bacillus licheniformis* (GenBank acc. no. MK577398.1), S.4 has 99% homology with *Bacillus sonorensis* (GenBank acc. no. NR_042537.1).
acc. no. CP021920.1), S.5 has 100% homology with *Stenotrophomonas maltophilia* (GenBank acc. no. GQ267817.1) and S.6 has 100% homology with *Enterococcus faecalis* (GenBank acc. no. CP028720.1).

**Figure 3:** Comprehensive phylogenetic tree of genetic variants of 16S rRNA – based phylogenetic tree of six isolated bacterial species. The black color refers to the sequenced local bacterial S1-S6 variants, while the other colors refer to other referring NCBI deposited bacterial species. All the mentioned numbers referred to Genbank acc. no. of each referring species. The number “4.0” at the bottom of the tree refers to the degree of scale range among the comprehensive tree categorized organisms.

Between the studies have mainly concentrated on molecular characterization of biofilm formation bacteria. Sujana and his colleagues test the ability of soil strains bacteria to produce biofilm by microtitre dish. The molecular identification for two highly yielding isolates matches with *Acinetobacter sp.* and *Acinetobacter bouvetii* according to 16S rRNA sequencing [31]. Related study was accomplished on copper resistant bacteria isolates and the identity of selected isolates
was based on 16S rRNA gene sequence analysis the results express C38 strain had a 99% similarity to B. cereus strain HM85, C43 strain had a 99% similarity to B. subtilis strain E16, shown 99% similarity to Lycinibacillus fusiformis of C40 strain had a, but in MB52 and C53 isolates had a 98% similarity to P. aeruginosa strain GGRJ21 [32,33].

**Conflict of interest**

None of the authors have any conflicts of interest relevant to this research subject.

**Ethical Approval**

Ethical Committee at the Karbala Health Directorate approved the study.

**References**


