Original Article

Standardization and Classification of *In vitro* Biofilm Formation by Clinical Isolates of *Staphylococcus aureus*

Ashish Kumar Singh, Pradyot Prakash, Arvind Achra, Gyan Prakash Singh[1], Arghya Das, Rakesh Kumar Singh[2]

Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, [1]Department of Community Medicine, Division of Biostatistics, Institute of Medical Sciences, Banaras Hindu University, [2]Department of Biochemistry, Institute of Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Abstract

Background: *Staphylococcus aureus* is Gram-positive bacterium commonly associated with nosocomial infections. The development of biofilm exhibiting drug resistance especially in foreign body associated infections has enabled the bacterium to draw considerable attention. However, till date, consensus guidelines for *in vitro* biofilm quantitation and categorization criterion for the bacterial isolates based on biofilm-forming capacity are lacking. Therefore, it was intended to standardize *in vitro* biofilm formation by clinical isolates of *S. aureus* and then to classify them on the basis of their biofilm-forming capacity. **Materials and Methods:** A study was conducted for biofilm quantitation by tissue culture plate (TCP) assay employing 61 strains of *S. aureus* isolated from clinical samples during May 2015–December 2015 wherein several factors influencing the biofilm formation were optimized. Therefore, it was intended to propose a biofilm classification criteria based on the standard deviation multiples of the control differentiating them into non, low, medium, and high biofilm formers. **Results:** Brain-heart infusion broth was found to be more effective in biofilm formation compared to trypticase soy broth. Heat fixation was more effective than chemical fixation. Although, individually, glucose, sucrose, and sodium chloride (NaCl) had no significant effect on biofilm formation, a statistically significant increase in absorbance was observed after using the supplement mix consisting of 222.2 mM glucose, 116.9 mM sucrose, and 1000 mM NaCl ($P = 0.037$). **Conclusions:** The present study puts forth a standardized *in vitro* TCP assay for biofilm biomass quantitation and categorization criteria for clinical isolates of *S. aureus* based on their biofilm-forming capacity. The proposed *in vitro* technique may be further evaluated for its usefulness in the management of persistent infections caused by the bacterium.

Keywords: Biofilm, brain-heart infusion broth, tissue culture plate method, trypticase soy broth

Introduction

Staphylococcus aureus is a leading cause of morbidity and mortality in nosocomial and community-based infections.[1] It is associated with a number of infections ranging from dental caries, periodontitis, stye, carbuncle, impetigo, and pyoderma to persistent tissue infections such as wound infection, otitis media, osteomyelitis, rhinosinusitis, recurrent urinary tract infection, and endocarditis.[2] It is also one of the most important pathogens in implant-related infections.[3,4] Several features of this bacterium render survival fitness in a wide variety of environments of which the biofilm formation is one of the special modes of persistent infections.[5-10]

Biofilm formation is an adaptive protected mode of growth enabling bacteria to survive in hostile environments as in the human host. This mode also enables them to disperse and colonize new niches as per their need which is mediated by their chemical cross-talk called quorum sensing.[11,12] The essential paradox of chronic infections is untreatability, and in most cases, chronic infections are accompanied by the formation of biofilms. The National Institute of Health, USA, claims the involvement of biofilms in 80% of all bacterial infections.[1] Neutrophil entrapment within biofilms leads to tissue injury by release of various inflammatory mediators. It has been observed that dead debris of neutrophils and/or other immune cells also serve as a biological matrix to facilitate biofilm formation. Bacterial genomic DNA liberated from

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Address for correspondence: Dr. Pradyot Prakash, Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi - 221 005, Uttar Pradesh, India. E-mail: pradyotbhu@gmail.com

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biofilms is also an immunostimulant and is recognized by toll-like receptor 9.[12] Therefore, biofilms can be considered as a special mode of persistent bacterial infection.[13]

Further, biofilm formation is dependent on different parameters including the characteristics of the nature of carbon source, its concentration, pH, ionic strength, and temperature, etc.[14] Although investigators have tried to optimize the conditions required for biofilm formation by staphylococcal isolates, some of the parameters such as optimum concentration of sugars, salt, and richness of medium have not been thoroughly investigated.[15] Some investigators have used trypticase soy broth (TSB) with glucose and/or brain-heart infusion (BHI) broth with sucrose supplementations to assess the effect on biofilm phenotype.[16] However, some have comprehensively elucidated sodium chloride (NaCl) dependence of biofilms in *S. aureus*.[17] However, their quantitative interpretation and categorization based on biofilm production criteria were not clear and cannot be replicated in every laboratory settings. Therefore, a simple and consensus guideline for *in vitro* biofilm synthesis by clinical isolates of *S. aureus* is direly needed. To the best of our knowledge, the effect of growth medium, fixation and elution and then supplementation of different sugars and salt levels to a larger range of concentrations on the characteristics of *S. aureus* biofilm has received comparatively little attention as the majority of investigators have not screened the sugar and salt concentration beyond 1%.[14,18] Further, there is no method described till date by which the bacteria can be differentiated on the basis of their biofilm-forming ability.

Therefore, in the present study, we aimed for the standardization of consensus protocol for achieving maximum *in vitro* biofilm formation by clinical isolates of *S. aureus* utilizing the supplementation with the proper concentration of glucose, sucrose, and NaCl. We also tried to put forth categorization criteria for the bacterial isolates on the basis of their biofilm-forming capacity.

Materials and Methods

Bacterial isolates

A study was conducted in which a total of 61 non-repetitive, consecutive strains of *S. aureus* isolated from the clinical samples received in the Microbiology laboratory over a period of 7 months (May 2015–December 2015), from various outpatients (outpatient departments [OPDs]) and inpatients wards of University Hospital, Banaras Hindu University. Of all the clinical isolates, majority were isolated from samples received from the Dermatology and Venereology OPD (n = 17), surgery OPD (n = 17), orthopedics ward (n = 10), high dependency unit (n = 4), pediatrics ward (n = 3), Intensive Care Unit (ICU) (n = 2), Neonatal ICU (n = 2), and one each from obstetrics and gynecology, plastic surgery, otorhinolaryngology, neurology, medicine, and urology wards [Figure 1 and Supplementary Data 1].

The bacterial identification was performed using conventional bacteriological techniques, such as colony morphology,

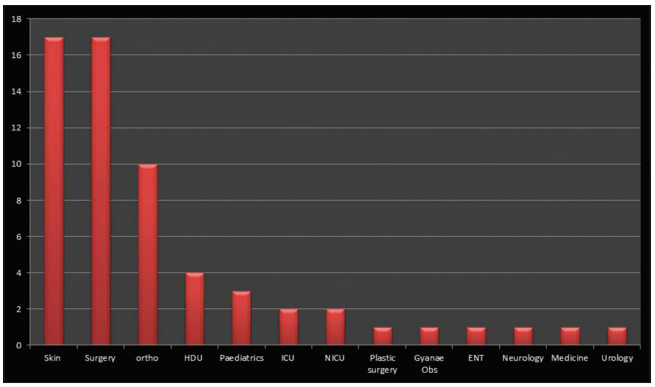

Figure 1: Distribution pattern of isolates of *Staphylococcus aureus* from different outpatient departments and wards

Gram-staining, catalase test, coagulase test, mannitol fermentation, bacitracin susceptibility test, and salt tolerance. *Staphylococcus epidermidis* ATCC 35984 (high slime producer), ATCC 35983 (moderate slime producer), and ATCC 12228 (non-slime producer) were used as reference strains since similar biofilm-producing reference strains of *S. aureus* are not available till date.

Determination of antimicrobial resistance

Antibiotic susceptibility testing of the isolates was performed by modified Kirby–Bauer method in accordance with the Clinical and Laboratory Standards Institute guidelines 2015 using 13 antibiotic discs including penicillin (10 Units), cefoxitin (30 mcg), erythromycin (15 mcg), trimethoprim and sulfamethoxazole (25 mcg), clindamycin (2 mcg), azithromycin (15 mcg), linezolid (30 mcg), ciprofloxacin (5 mcg), netilmicin (30 mcg), moxifloxacin (5 mcg), and amoxicillin/clavulanate (30 mcg). Antimicrobial susceptibility to mupirocin and fusidic acid was interpreted as described by Park *et al*.[19] All the materials needed for the current study were procured from HiMedia Laboratories, Mumbai, otherwise mentioned. Tissue culture plates (TCPs) were procured from Tarsons, Kolkata, India.

Standardization of *in vitro* synthesis of biofilm in tissue culture grade microtiter plates

In the present study, the effect of various parameters on *in vitro* biofilm synthesis was at first observed on *S. epidermidis* American Type Culture Collection (ATCC) strains and *S. aureus* clinical isolates using 96-well flat bottom TCP.

Initial inoculum, media, and incubation

In the first step, we evaluated the effect of growth conditions for the preparation of initial inoculum (solid medium BHI agar vs. liquid medium TSB), effect of nutritional media for generation of biofilm (TSB vs. BHI broth), and incubation time (6, 12, 18, and 24 h) at 37°C.

In the first method, briefly fresh isolates were inoculated in TSB and BHI broth in stationary condition overnight at 37°C and diluted 1 in 100 with fresh medium for subsequent use. Each well of TCP was filled with 200 µl aliquots of the diluted

cultures and then investigated for biofilm formation after 6, 12, 18, and 24 h at 37°C.

While in another method, the isolates were grown on BHI agar overnight at 37°C. Then, colonies from overnight grown BHI agar culture plates were suspended directly into physiological saline (0.89% NaCl), and vortexed to achieve a suspension of 0.5-McFarland turbidity (1.5×10^8 CFU/ml). Each well of TCP was filled with 190 µl aliquots of BHI and then 10 µl of bacterial suspension was added to it. Like above, the plates were read after 6, 12, 18, and 24 h of incubation.

Fixation

After respective incubations, the plates were inverted and gently tapped to remove residual broth. The wells were washed thrice with 200 µl of phosphate buffer saline (PBS) (pH 7.2) to remove planktonic bacteria before fixation.

The two protocols as mentioned above were compared for fixation of cells in the plates by two different methods. In the first method, cells were fixed with 200 µl of sodium acetate (2% w/v) for 30 min, while in another, plates were incubated for heat fixation at 60°C for 20 min. After fixation, the plate with sodium acetate was washed with 200 µl PBS thrice before staining.

Staining and elution

For staining, we used 175 µL of 0.5% crystal violet for 5 min. The excess crystal violet was removed, and the plates were washed with running tap water until runoff was clear. For elution, we used 150 µl ethanol-acetone mixture (80:20) and left at room temperature for 30 min. The elute was then resuspended in wells of new TCP to take optical density (OD) readings at λ_{max} 550 nm in ELISA plate reader (Thermo Scientific, USA).

Supplementation with sugars and salt

Glucose, sucrose, and NaCl in different molar concentrations, namely, 55.6, 111.11, 166.7, and 222.2 mM for glucose; 29.2, 58.5, 116.9, and 175.4 mM for sucrose; and 500, 750, and 1000 mM for NaCl, respectively, were investigated to observe for any possible effect on the biofilm formation individually.

Based on the observations of maximum biofilm yielded by supplementation of the individual ingredient, a solution of optimum concentrations of glucose, sucrose, and NaCl (supplement mix) was selected to supplement the above method and the optimized method was then applied on all the clinical isolates once again.

Categorization of isolates based on biofilm-forming capacity

The following criteria were used for biofilm gradation in clinical isolates.

$OD_{cut} = OD_{avg}$ of negative control + 3 × standard deviation (SD) of ODs of negative control.

1. $OD \leq OD_{cut}$ = Non-biofilm-former (NBF)
2. $OD_{cut} < OD \leq 2 \times OD_{cut}$ = Weak biofilm-former (WBF)
3. $2 \times OD_{cut} < OD \leq 4 \times OD_{cut}$ = Moderate biofilm-former (MBF)
4. $OD > 4 \times OD_{cut}$ = Strong biofilm-former.

In this study, sterile broth and *S. epidermidis* ATCC 12228 served as the negative control. However, *S. epidermidis* ATCC 35984 (high slime producer) and ATCC 35983 (moderate slime producer) were used as positive control. All experiments with clinical isolates were done in quadruplet, i.e., each isolate were inoculated in four wells simultaneously and repeated thrice (on different days), and then, OD values were averaged and SD was calculated.

Statistical analysis

One-way ANOVA and one-tail *t*-test assuming equal variance were used to compute and analyze the differences in OD values obtained with different experimental variables of the *in vitro* synthesis of biofilm by TCP method. MS Excel data analysis tool along with IBM SPSS version 21.0, Armonk, New York was utilized for analysis. $P \leq 0.05$ was considered statistically significant.

Results

The following results were observed for different variables on *in vitro* biofilm synthesis by TCP assay in achieving conditions required for maximum biofilm biomass.

Effect of growth medium for harvesting bacterium for inoculum preparation

Higher biofilm formation was observed as inferred from increased OD when initial bacterial inoculum was prepared from the growth on BHI agar as compared to those grown in broths [Table 1].

Effect of growth medium

The absorbance was significantly higher when BHI broth was used as the nutritional medium as compared to TSB ($P = 0.00019$, $P < 0.05$) [Figure 2 and Supplementary Data 2]. For instance, the average OD for *S. epidermidis* ATCC 35984 was 1.491 ± 0.017 (OD ± SD) in BHI broth, which was 34% higher when compared with average OD in TSB (0.986 ± 0.019). Therefore, BHI broth was selected as the medium for characterization of biofilm formation of clinical isolates of *S. aureus* in the present study.

Effect of incubation period

When ATCC control strains were assessed for the effect of incubation period on biofilm formation, maximum biofilm

Table 1: Absorbance after *in vitro* biofilm assay using tissue culture plates method using different initial inoculums

Strains	OD when grown in broth	OD when grown on BHI agar
ATCC 35984	1.452±0.019	1.961±0.017
ATCC 35983	0.471±0.013	0.577±0.016
ATCC 12228	0.106±0.016	0.197±0.014

BHI: Brain heart infusion, ATCC: American Type Culture Collection, OD: Optical density

yield was found after 24 h with resultant average OD 0.991 ± 0.021 for ATCC 35984, 0.433 ± 0.012 for ATCC 35983, and 0.102 ± 0.017 for ATCC 12228. It was observed that after 6 h of incubation, the majority of the *S. aureus* isolates displayed insignificant absorbances with average OD ranging from 0.147 ± 0.0301 to 0.236 ± 0.0410. After 18 h, all isolates were found to produce biofilms as reflected by relative absorbances. The average OD for one of the isolates of *S. aureus* (Isolate number 27) was 0.358 ± 0.04, 0.511 ± 0.02, and 0.726 ± 0.04 at 12, 18, and 24 h, respectively. The similar pattern was also observed for other isolates. Statistically significant (P = 0.0015) results were observed after 24 h of incubation compared to 18 h of incubation and therefore was considered as the optimum incubation period for the assessment of biofilm-forming capacity of *S. aureus* [Figure 3 and Supplementary Data 3].

Effect of fixation

When ATCC control strains were assessed for fixation by heat, it was found that there is a statistically significant increase in the absorbance as compared to sodium acetate fixation (P = 0.004) with average resultant OD 1.491 ± 0.017 for ATCC 35984, 0.478 ± 0.016 for ATCC 35983, and 0.129 ± 0.014 for ATCC 12228. However, with sodium acetate, average absorbance was found to be 0.973 ± 0.016 for ATCC 35984, 0.311 ± 0.021 for ATCC 35983, and 0.073 ± 0.017 for ATCC 12228.

Upon heat fixation, significantly enhanced absorbance (average OD 0.653 ± 0.075) was observed compared to sodium acetate fixation with average OD ranging from 0.15 ± 0.01 to 0.38 ± 0.09 for most of the *S. aureus* isolates.

Effect of glucose

It was observed that most of the clinical isolates displayed a perceivable biofilm-positive phenotype when BHI broth was supplemented with glucose [Supplementary Data 4]. Glucose in almost all concentrations was positively added to the biofilm formation, but highest absorbance was observed at 222.2 mM glucose. However, individual concentrations of glucose had no significant effect on absorbance (P = 0.135) [Figure 4].

Effect of sucrose

It was noted that less number of clinical isolates displayed a biofilm-positive phenotype when BHI broth was supplemented with sucrose (P = 0.21). Sucrose also had no significant effect on absorbance. However, it has shown maximum absorbance at concentration of 116.92 mM. Beyond 116.92 mM concentration saturation was observed and in some cases, even the loss in the biofilm was observed as reflected by ODs [Figure 5 and Supplementary Data 4].

Effect of sodium chloride

S. epidermidis reference strains have shown enhanced absorbance although observations were not statistically significant (P = 0.67). However, the response of *S. aureus* was varying. It was observed that all the methicillin-sensitive *S. aureus* (MSSA) isolates showed enhanced biofilm phenotype compared to methicillin-resistant *S. aureus* (MRSA) isolates [Supplementary Data 5]. Although, upon supplementation of NaCl, the enhancement was not statistically significant (P = 0.84) [Figure 6], highest absorbance was observed at 1000 mM NaCl.

Biofilm synthesis by clinical isolates of *Staphylococcus aureus* employing proposed modified tissue culture plate method

Based on the observations of different variables of *in vitro* biofilm synthesis including sugars and NaCl concentration as described above, all the stains were subjected to biofilm formation on the selected combination of 222.2 mM glucose, 116.9 mM sucrose, and 1000 mM NaCl (supplement mix). A significant increase in the biofilm formation (P = 0.031) was observed after supplementation as compared to unsupplemented BHI broth [Figures 7, 8 and Supplementary Data 6].

Categorization of *Staphylococcus aureus* isolates based on biofilm-forming capacity

We tried to establish criteria for categorizing *S. aureus* isolates based on their biofilm-forming capacity. Based on the results

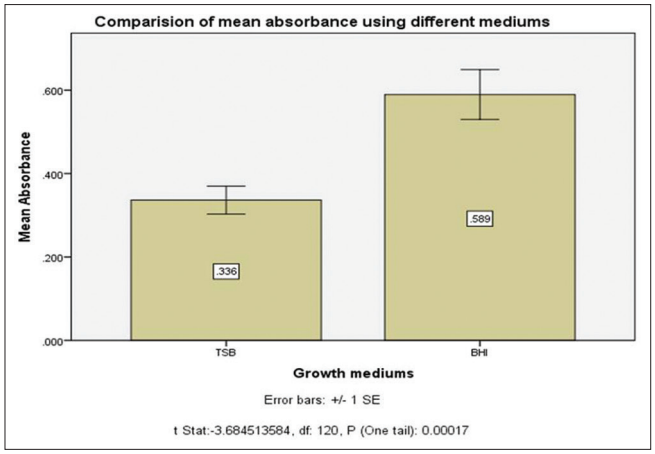

Figure 2: Enhancement in biofilm formation by clinical isolates of *Staphylococcus aureus* using brain heart infusion and trypticase soy broth

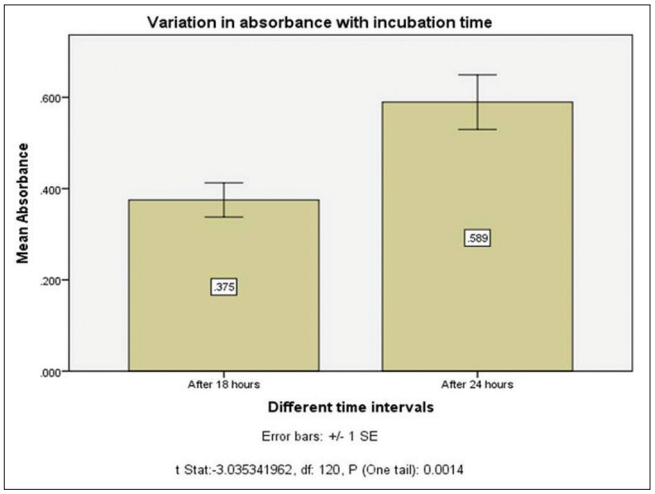

Figure 3: Effect of incubation period on absorbance by clinical isolates of *Staphylococcus aureus*

obtained from TCP assay with supplement mix, a cut-off OD (OD_{cut}) was obtained by taking the average of all the ODs of the negative control ATCC 12228 and thrice the value of SD of the negative control was added to it.

In this study, the average OD of the negative control came to be 0.147 ± 0.0305. Hence, the cutoff OD value in the current study was set as 0.238. The isolates which have OD value lesser than 0.238 were considered as NBFs [Table 2].

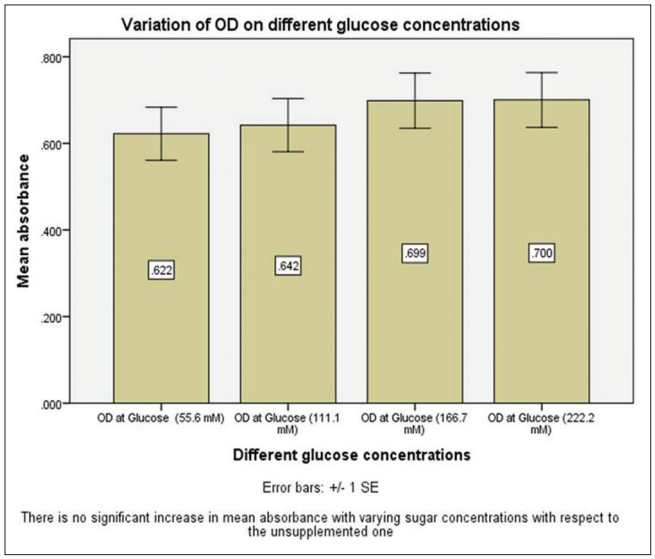

Figure 4: Effect of different concentrations of glucose supplementations on absorbance

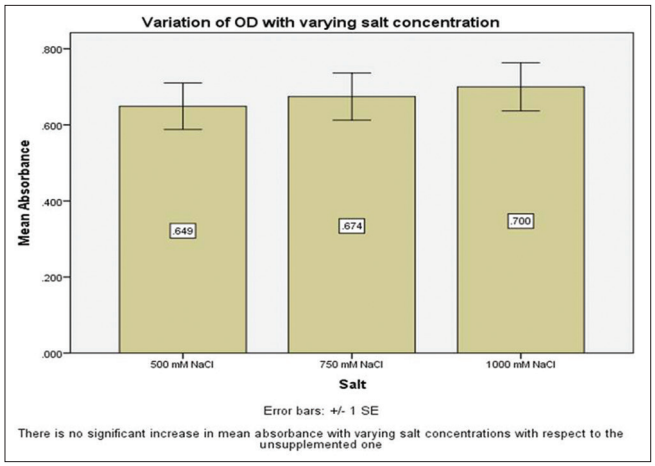

Figure 6: Effect of different concentrations of sodium chloride supplementations on absorbance

Upon employing differentiation criterion adopting OD_{cut}, all the 61 clinical isolates were observed to be biofilm formers by proposed method using supplement mix in this study. However, 15 (24.5%) isolates were observed to be non-former of biofilm by unsupplemented TCP method. Out of these 15 non-former strains, 9 were MSSA and 6 were MRSA. Upon addition of supplement mix, of total 9 NBF MSSA isolates, two (isolate no. 28, 36) showed medium grade biofilm and the rest seven showed low-grade biofilm formation, i.e., no isolate showed the non-biofilm producer phenotype. Similar to MSSA, upon

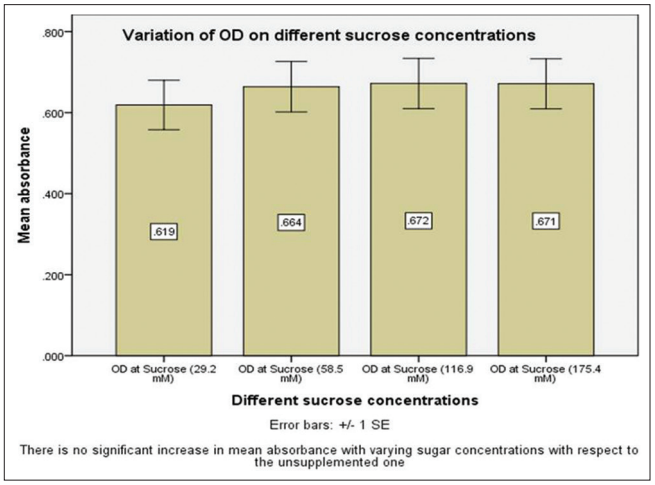

Figure 5: Effect of different concentrations of sucrose supplementations on absorbance

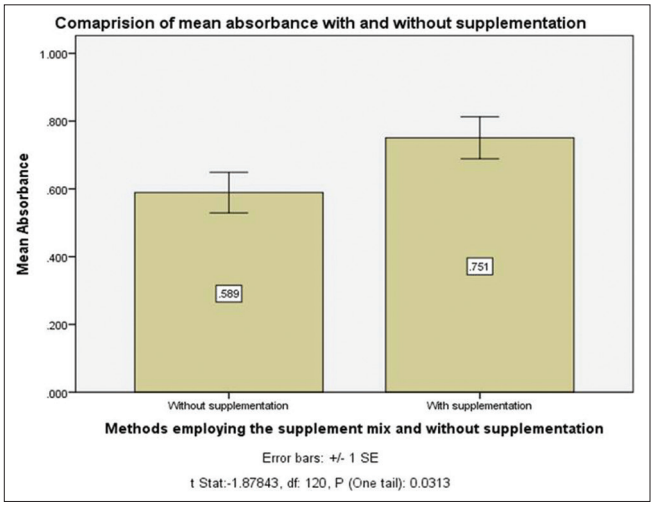

Figure 7: Effect of optimized supplement mix on absorbance

Table 2: Categorization of biofilm made by strains of *Staphylococcus aureus* (*n*=61)

Average OD range	Biofilm grade	Number of strains older method	Number of strains proposed method
<0.238	Non-former	15+1[$]	0+1[$]
≥0.238 but ≤0.477	Low biofilm former	20	13
≥0.477 but ≤0.954	Moderate biofilm former	16+1[@]	32+1[@]
≥0.954	High biofilm former	10+1[#]	16+1[#]

[$]ATCC 12228, [@]ATCC 34983, [#]ATCC 34984. ATCC: American Type Culture Collection, OD: Optical density

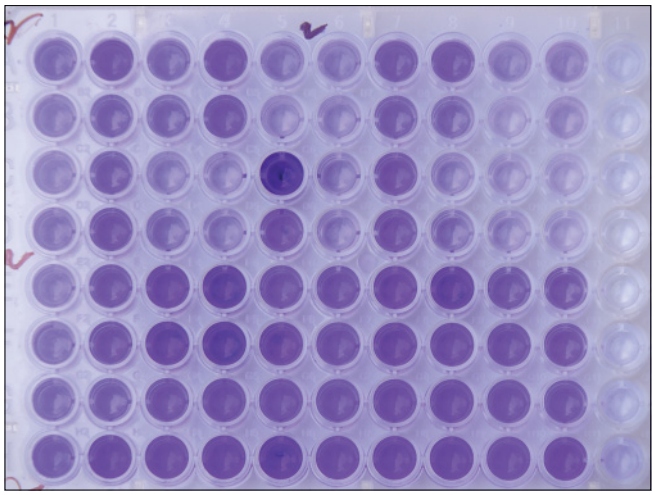

Figure 8: Effect of supplementation - A phenotypic view. Lane 1: Row A, B, C, and D show the unsupplemented brain-heart infusion while Row E, F, G, and H show the supplemented brain-heart infusion for ATCC 1228. Lane 2: Row A, B, C, and D show the unsupplemented brain heart infusion while Row E, F, G, and H show the effect of supplemented brain-heart infusion for ATCC 35983. Lane 7: Row A, B, C, and D show the unsupplemented brain-heart infusion while Row E, F, G and H show the effect of supplemented brain-heart infusion for ATCC 35984. Lane 11: Row A, B, C, and D show the unsupplemented brain-heart infusion while Row E, F, G, and H show the effect of supplemented brain-heart infusion for negative control

supplementation of the supplement mix, all previously NBF MRSA isolates showed enhanced biofilm formation on the addition of supplement mix. Of six NBF MRSA isolates, 5 shifted to low biofilm-former grade while one (isolate no. 52) showed medium-former grade phenotype (more enhance biofilm phenotype). All the low biofilm-former ($n = 4$) showed medium-biofilm forming phenotype except one (isolate no. 41), which retained its low biofilm-forming phenotype.

Without supplementation, only 11 MBF isolates were observed. However, only 5 (45.45%) showed the shift into a high biofilm-former grade (isolate no. 3, 17, 27, 29, and 49) and the remaining 6 isolates (isolate no. 4, 9, 12, 23, 24, and 26) retained their biofilm grade even after adding the supplement mix [Table 3 and Supplementary Data 7].

Effect of opting *Staphylococcus epidermidis* ATCC 12228 as the negative control

It was observed that the ODs of moderate and high biofilm producing ATCC strains of *S. epidermidis* lied repeatedly in the range of $2 \times OD_{cut} < OD \leq 4 \times OD_{cut}$ and $OD > 4 \times OD_{cut}$ respectively with respect to the non-former ATCC 12228 strain. Therefore, opting *S. epidermidis* ATCC 12228 as the negative control was considered to be more useful in deciding the precise cut-off criteria rather than the broth alone [Figure 8 and Table 1].

The optimized protocol for the in vitro synthesis of biofilm by TCP assay for clinical isolates of *S. aureus* has been summarized in Figure 9.

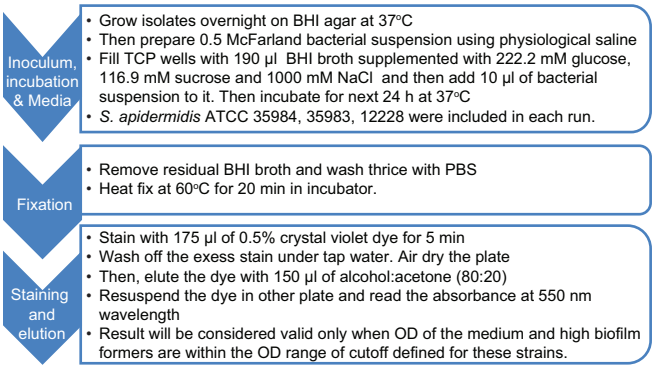

Figure 9: A simplified flowchart of the proposed method

Table 3: Distribution of isolates in different classes in toto and selective distribution of methicillin-susceptible *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* isolates in different classes

Total	Before	After	MRSA	Before	After	MSSA	Before	After
HBF	10	16	HBF	3	4	HBF	7	16
MBF	17	32	MBF	5	8	MBF	11	32
LBF	19	13	LBF	4	6	LBF	16	13
NBF	15	0	NBF	6	0	NBF	9	0

MRSA: Methicillin-resistant *Staphylococcus aureus*, MSSA: Methicillin-susceptible *Staphylococcus aureus*, NBF: Nonbiofilm former, MBF: Moderate biofilm forming, LBF: Low biofilm formers, HBF: High biofilm former

Antimicrobial sensitivity pattern

Out of 61 clinical isolates of *S. aureus*, 18 (29.51%) were MRSA. The majority of *S. aureus* isolates were found to be resistant to more than 9 antibiotics. All the clinical isolates were found to be sensitive to linezolid and netilmicin. Only 3 isolates were penicillin sensitive. Isolates have shown lesser susceptibility toward ciprofloxacin as the majority was either resistant or intermediate susceptible. The majority of isolates ($n = 37$) showed intermediate resistance to the erythromycin. However, compared to azithromycin, the incidence of resistance was lesser with erythromycin. Most of the isolates ($n = 44$) were resistant to co-trimoxazole. Four isolates were resistant to fusidic acid while mupirocin resistance was detected in only one strain [Supplementary Data 1]. Strong and moderate biofilm-producing isolates were found to be more resistant to commonly used antibiotics compared to weak producing ones [Table 4].

Discussion

Biofilm is a sessile microbial community wherein cells are attached to a surface (biotic or abiotic) and are enmeshed within a self-produced protective extracellular polymeric matrix. This extracellular polymeric matrix in *S. aureus*/*S. epidermidis* is poly-N-acetyl glucosamine (PNAG).[20] There are cases where PNAG-independent proteinaceous biofilms are also reported in *S. aureus*.[21,22]

Schleifer and Kroppenstedt reported the surface association of the infecting bacteria and speculated similarity of solid agar

Table 4: Biofilm-forming ability of strains of different resistance pattern

Name of antibiotic	Isolate resistance including intermediate percentage	Biofilm grade
Penicillin (10 units)	95.08 (58/61)	2 sensitive strains are weak biofilm formers while one is strong former
Cefoxitin (30 mcg)	29.51 (18/61)	5 resistant strains were high former, 7 were medium formers, and 6 were weak formers
Erythromycin (15 mcg)	83.6 (51/61)	5 resistant strains were high formers, 2 were medium formers while 7 were weak formers
Trimethoprim and sulfamethoxazole (25 mcg)	83.6 (51/61)	22 were weak formers, 11 were strong formers, and 11 were medium formers
Clindamycin (2 mcg)	8.19 (5/61)	3 were weak formers and 2 were medium formers
Azithromycin (15 mcg)	40.98 (25/61)	8 were strong formers, 4 were medium formers, and 7 were weak formers
Ciprofloxacin (5 mcg)	93.44 (57/61)	9 were strong formers, 11 were medium formers, and 12 were weak formers
Moxifloxacin (5 mcg)	72.13 (44/61)	11 were strong formers, 10 were medium formers, and 13 were weak formers
Amoxicillin and clavulanate (30 mcg)	45.90 (28/61)	7 were strong formers, 11 were medium formers, and 9 were weak formers
Mupirocin (200 mcg)	1.63 (1/61)	1 is medium former
Fusidic acid (10 mcg)	6.55 (4/61)	1 is strong former, 2 are medium formers, and 1 is weak former

grown bacteria to natural infection settings and then to the pathogens grown in liquid media.[23] When initial inoculum was prepared from the bacteria grown on BHI agar, we noticed their comparatively higher efficiency in biofilm production as compared to those grown in broths. This could be probably a result of the higher expression of surface proteins required for adherence when bacteria are grown on solid media. The expression of these proteins is also reported as a prerequisite for infectivity in various studies.[24]

The richness of nutrients is another important factor which influences the ability of bacteria to produce biofilm.[15] Some investigators have utilized TSB for biofilm quantitation.[25,26] In the current study, BHI broth was found to be significantly more effective in biofilm formation [Supplementary Data 2]. Proteins especially rich in leucine, proline, serine, and aspartate are abundant in BHI broth since these amino acids may be essential for the production of adhesins such as fibronectin-binding protein and clumping factors which are necessary for adherence. The presence of lipids such as choline and sphingosine in BHI may have added advantage in biofilm formation and provide resistance from desiccation. Further, it is a source of sugars such as inositol/myoinositol which cannot be fermented by *S. aureus* leading to resistance in pH fall, which, in turn, may be needed for robust biofilm architecture. These results indicate a strong dependence of biofilm formation in *S. aureus* and the environmental conditions required for growth, which seems to be even more pronounced in *S. aureus* than in *S. epidermidis*.[22,27-29] Similarly, while observing the effect of incubation period on *in vitro* biofilm formation, it was noticed that after 6 h of incubation, the majority of the *S. aureus* isolates remained NBF and for some of the isolates biofilms were even non-detectable. Adhesion of bacterial cells to microtiter plate appeared to be a function of time and increased linearity was observed with time progression. Although biofilm formation was observed in all isolates after 18 h of incubation, the maximum biofilm yield as reflected in ODs was observed after 24 h of incubation as also noticed by other investigators.[15,16]

The fixation of attached cells by heating at 60°C for 20 min was found to be statistically more significant than fixation by sodium acetate in our study. Therefore, we opted for heat fixation. Heat disrupts hydrogen bonds and non-polar hydrophobic interactions of bacterial cell surface proteins leading to coagulation and in some cases its denaturation. Further, it dehydrates the sugar content leading to the crude biomass estimation. While sodium acetate has a protective effect against denaturation.[30] These results are in consonance with the observations of Baldassarri *et al*.[31]

During elution step, only 150 μL of eluent (ethanol:acetone [80:20]) was added per well, to evade interference with the stained matter at the liquid–air interface, which is not considered to be indicative of biofilm formation.

We examined the biofilm formation in both MRSA and MSSA isolates in media supplemented with different concentration of glucose, sucrose, and NaCl. Although the addition of sugars and salts individually has increased the biofilm phenotype as manifested by an increase in OD, it was not statistically significant ($P > 0.05$). On the other hand, when the supplement mix was added to the broth in a defined ratio, the significant increase in OD was observed ($P = 0.037$, $P < 0.05$). Therefore, it is strongly recommended to use the proposed method for *in vitro* biofilm quantitation.

Among MSSA isolates, isolate-to-isolate variation was observed with respect to biofilm-forming ability with nature of supplementation used. Glucose in almost all concentrations was positively added to the biofilm formation while sucrose at concentration beyond 116.92 mM showed almost saturation and in some cases even the loss in the biofilm. NaCl at 1000 mM concentration showed the maximum increase in absorbance. This observation was found consistent with Lim *et al*. who found enhanced expression of *rbf* gene involved in the signal transduction pathway for biofilm production when the NaCl concentration is above 1.6% but not when it is below 1.6%.[17]

While observing biofilm synthesis by MRSA isolates, the strong correlation existed between the biofilm phenotype

and the concentration of the sugar supplemented. Even some isolates showed exceptional behavior to this generalized rule [Supplementary Data 3-5]. Although this sort of heterogeneity in biofilm-forming capacity of MRSA has been addressed earlier, isolate-wise exceptional behavior has never been highlighted. Each isolate responded differently from one another regarding response to the sugar and, in turn, in biofilm phenotype. Pozzi *et al*. (2012) proposed that acquisition of methicillin resistance appears to repress polysaccharide-type biofilm production and promote the formation of proteinaceous biofilms as evidenced by biofilm phenotype observations made in the present study.[32,33] However, there are certain MRSA isolates which showed the exception to this generalized rule. The universality to this generalized rule is just an enigma. Biofilm development in MRSA isolates is primarily glucose induced but not solely, and apparently, involves a protein adhesin.[21,34]

Till date, there is no consensus view regarding categorization of *S. aureus* isolates based on their biofilm-forming capacity. Therefore, the definition of a strong, medium, weak, and non-biofilm producer varies greatly among the studies.[15,16,35,36] Mathur *et al*. have recently proposed the criteria for grading the isolates based on their ability to form a biofilm which considered non-former isolates when the OD was <0.120, while OD range for medium-former was >0.120–≤0.240 and for those of high former was >0.240.[16] Similarly, Stepanovic *et al*. have also proposed the criteria for biofilm classification and used the same old gold standard of Christensen *et al*. using the same ATCC 35984, 35983, and 12228 reference isolates.[15] Christensen *et al*. have used only an approximation of distance plotted in a graph, by dividing the graph into three portions: nonadherent (OD in both media, <0.120), weakly adherent (OD in either medium, >0.120 but 0.240), and strongly adherent (OD in either medium, >0.240).[37]

In the present study, a need of new cut-off criteria was felt because of the aforesaid reason and significant increase of the OD expanding the limit of OD in previously described non, moderate, and high biofilm-former category. A plethora of literature is available where only broth was taken as the negative control. In this study, *S. epidermidis* ATCC 12228 as the negative control was found to be more accurate in deciding the precise cutoff criteria rather than the broth alone. Broth can be used to ensure the sterility during the execution of the experiment. As negative and positive controls are a must in any experimental setup, we propose the OD cutoff criteria based on the OD of the negative control and the addition of some factor to its SD value. And then, various multiples (even) of OD_{cut} can be used to distinguish clinical isolates based on their biofilm-forming capacities. By adopting the proposed method and criteria, it was observed that reference strains ATCC 35984, 35983, and 12228 remained in their respective classes as high, medium, and non-formers. However, it was interesting to observe that when the new criterion was applied on all the clinical isolates of *S. aureus*, all the previously declared nonformer isolates were either shifted to low former or to the medium-former category. Therefore, instead of using uninoculated broth, ATCC 12228 may be used as negative control for error free and concordant results. This method can, therefore, be unequivocally used for all clinical staphylococcal isolates to adapt the low/WBFs as reported by other investigators also.[15,16,28,38]

It was observed that ODs of a number of clinical isolates of *S. aureus* lied between the non and the moderate biofilm range. Therefore, a new category of WBFs is needed to be introduced in the study of biofilm quantitation and also for the sake of uniformity. To further strengthen the validity of results on biofilm quantitation, one may need a higher number of reference strains of both *S. epidermidis* as well as *S. aureus* of all the four grades of biofilm producers.

In the present study, strong and MBF isolates were found to be more resistant to commonly used antibiotics compared to WBFs. Strong biofilm producers are more adapted pathogenic strains and have acquired resistance over the period due to continuous exposure to the antibiotics or by acquiring genes through horizontal gene transfer or by both. This may be the consequence of biofilm providing an appropriate environment for the transfer of drug resistance determinants.[39]

Further, investigators claimed that as much as thousand times increased MIC of biofilm-dwelling cells than the planktonic cells.[1] This may be due to interruptions posed by the biofilm slimy matrices in the form of electrostatic repulsion and/or sequestration of antibacterial substances apart from being diffusion barrier.[40,41] There are attempts which were made to design a number of anti-biofilm compounds mainly short peptides, which seems to be promising strategy against staphylococcal biofilm.[42] However, in the future, for these and several other candidate drugs, there will be a need for a standardized method for *in vitro* biofilm synthesis by *S. aureus* along with classification criterion for conclusive authentication of drugs as potential antibiofilm agents.

However, the limitation of the current study is that the method of biofilm formation proposed here may not be useful for Gram-negative isolates. This is because, among Gram-negative bacteria, altogether, different operon arrays are responsible for controlling biofilm biogenesis. In Gram-negative bacteria, some of the polysaccharides are neutral or polyanionic due to the presence of uronic acids or ketal-linked pyruvates.[40] However, classification criteria can be used with properly established negative control.

Conclusions

The results indicate that the different variables including supplement mix containing glucose, sucrose, and NaCl in a defined ratio enhances the biofilm-forming ability of *S. aureus* significantly in the proposed method of *in vitro* biofilm formation assay employing TCP. The present study puts forth a standardized *in vitro* TCP assay for biofilm synthesis by *S. aureus* and its categorization indicating their differential ability to produce biofilm. The proposed *in vitro* technique may be further evaluated for its usefulness in the management of persistent infections caused by the bacteria.

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Conflict of interest

There are no conflicts of interest.

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Supplementary Data 1: Epidemiological profile and resistance pattern of *Staphylococcal* strains

Strain number	Clinical strain number	Resistant to antimicrobials	Ward/OPD
1	1114/2015	1, 4, 8, 11	Skin/pus
2	1115/2015	1, 4, 10, 11	Skin/pus
3	774/2015	10	ICU/ET aspirate
4	1269/2015	1, 8, 12, 10, 11,12	ICU/ET aspirate
5	792/2015	1, 2, 13, 8,10, 11	Orthopedics/pus
6	1229/2015	1, 4, 8, 10, 11	Skin/pus
7	1975/2015	1, 4, 8, 11	Skin/pus
8	1968/2015	1, 4, 8, 10	Skin/pus
9	1360/2015	1, 3, 4, 6, 8, 10, 11	Pediatrics emergency/pleural fluid
10	1458/2015	1, 4, 10	Orthopedics/pus
11	1659/2015	1, 4, 8, 10	Skin/pus
12	1573/2015	1, 8, 10, 11	Skin/pus
13	2037/2015	1, 4, 11	Orthopedics/pus
14	2018/2015	1, 3, 4, 6, 8,10	Skin/pus
15	2034/2015	1, 3, 4, 6, 8, 10	Skin/pus
16	771/2015	1, 2, 4, 8, 10	SOPD/pus
17	775/2015	1, 6, 8	NSW/pus
18	876/2015	1, 3, 4, 6, 8, 10	SOPD/pus
19	2028/2015	1, 2, 3, 6, 8, 10, 11	NSW/tracheal aspirate
20	1104/2015	1, 4, 10, 11	Skin/pus
21	699/2015	1, 2, 3, 4, 6, 10, 11	HDU/IJV tip
22	1115/2015	1, 4, 10, 11	Skin/pus
23	1114/2015	1, 4, 6, 8	Skin/pus
24	749/2015	1, 4	SOPD/pus
25	1371/2015	1, 4, 11	ENT/pus
26	1378/2015	1, 4, 8	Pediatrics emergency/pus
27	753/2015	1, 4, 11	SOPD/pus
28	704/2015	4, 8, 11	SOPD/pus
29	3862/2015	1, 4, 6	SOPD/pus
30	4042/2015	1, 2, 3, 4, 6, 8, 10, 11	Skin/pus
31	492/2015	1, 11	Orthopedics/pus
32	962/2015	1, 2, 3, 4, 6, 8, 10	Urology/pus
33	2179/2015	1, 2, 4, 11	Skin/wound
34	619/2015	1, 4	Gynecology/HVS
35	961/2015	1, 4, 8	Orthopedics/pus
36	742/2015	1, 3, 5, 6	Orthopedics/pus
37	619/2015	1, 2, 4, 8, 10, 11	HDU/pus
38	625/2015	1, 5, 6	Neurology/pus
39	758/2015	1, 2, 10	HDU/IJV tip
40	756/2015	1, 2, 4, 8, 10, 11	SOPD/pus
41	699/2015	1, 2, 3, 4, 5, 8, 10, 11	HDU/IJV tip
42	994/2015	1, 4, 11, 12	SOPD/pus
43	935/2015	4, 8, 10	ENT/pus (ear swab)
44	869/2015	1, 4, 11	SOPD/pus
45	394/2015	1, 2, 3, 4, 5, 6	SOPD/pus
46	775/2015	1, 8, 10	NSW/pus
47	676/2015	1, 4, 8, 10, 11	Orthopedics/pus
48	617/2015	1, 2, 3, 4, 6	Skin/pus
49	517/2015	1, 3, 6, 12	SOPD/pus
50	999/2015	1, 4, 8, 10	SOPD/pus
51	740/2015	1, 11	Medicine/pus
52	408/2015	1, 2, 3, 6, 13	NICU/pus
53	719/2015	1, 2, 8, 10	Orthopedic/pus
54	328/2015	1, 4	SOPD/pus

Contd...

Supplementary Data 1: Contd...

Strain number	Clinical strain number	Resistant to antimicrobials	Ward/OPD
55	907B/2015	1, 4, 10	Orthopedic/pus
56	643/2015	1, 4, 10	Orthopedic/pus
57	545/2015	1, 6, 8, 10, 12	Skin/pus
58	1823/2015	1, 4, 8, 10	SOPD/pus
59	771B/2015	1, 2, 4, 8, 10, 11	SOPD/pus
60	2972/2015	1, 2, 4, 11	Skin/pus
61	394/2015	1, 2, 3, 6, 10	SOPD/pus

Penicillin (1), Cefoxitin (2), Erythromycin (3), Trimethoprim and Sulfamethoxazole (4) , Clindamycin (5), Azithromycin (6), Linezolid (7), Ciprofloxacin (8), Netilmicin (9), Moxifloxacin (10), Amoxicillin and clavulanate (11), Fusidic Acid (12), Mupirocin (13). OPD: Outpatient department, ICU: Intensive Care Unit, SOPD: Surgical outpatient department, HDU: High dependency unit, ENT: Ear, nose, and throat, NICU: Neonatal Intensive Care Unit, ET: Endotracheal aspirate, HVS: High vaginal swab, IJV: Internal jugular venous catheter tip

Supplementary Data 2: Comparison of OD's when media used was trypticase soy broth and brain-heart infusion over biofilm formation

Strain number	With TSB	With BHI
1	0.197	0.253
2	0.211	0.298
3	0.438	0.827
4	0.329	0.594
5	0.327	0.595
6	0.981	2.006
7	0.183	0.269
8	0.199	0.376
9	0.349	0.606
10	0.207	0.36
11	0.276	0.445
12	0.342	0.579
13	0.873	1.715
14	0.826	1.667
15	0.823	1.404
16	0.867	1.631
17	0.329	0.697
18	0.798	1.13
19	0.812	1.351
20	0.849	1.606
21	0.753	1.024
22	0.831	1.462
23	0.506	0.827
24	0.379	0.614
25	0.237	0.428
26	0.411	0.666
27	0.527	0.726
28	0.103	0.247
29	0.627	0.901
30	0.103	0.22
31	0.173	0.325
32	0.091	0.194
33	0.397	0.663
34	0.206	0.318
35	0.197	0.306
36	0.124	0.236
37	0.631	0.927

Contd...

Supplementary Data 2: Contd...

Strain number	With TSB	With BHI
38	0.122	0.233
39	0.193	0.349
40	0.147	0.28
41	0.185	0.301
42	0.186	0.329
43	0.185	0.328
44	0.069	0.153
45	0.073	0.174
46	0.213	0.428
47	0.207	0.362
48	0.239	0.472
49	0.403	0.768
50	0.117	0.303
51	0.079	0.174
52	0.109	0.211
53	0.053	0.157
54	0.069	0.172
55	0.097	0.144
56	0.062	0.141
57	0.091	0.187
58	0.257	0.409
59	0.328	0.563
60	0.103	0.199
61	0.421	0.627

TSB: Trypticase soy broth, BHI: Brain-heart infusion, OD: Optical density

Supplementary Data 3: Comparative OD's when subjected to four different incubation periods

Strain number	After 6 h	After 12 h	After 18 h	After 24 h
1	-	0.061	0.120	0.253
2	-	0.068	0.137	0.298
3	0.151	0.342	0.528	0.827
4	0.097	0.143	0.337	0.594
5	0.089	0.152	0.362	0.595
6	0.316	0.581	1.211	2.006
7	-	0.062	0.171	0.269
8	-	0.087	0.229	0.376
9	0.105	0.294	0.459	0.606
10	-	0.083	0.213	0.36
11	0.061	0.192	0.306	0.445
12	0.084	0.216	0.389	0.579
13	0.187	0.396	0.838	1.715
14	0.187	0.394	0.832	1.667
15	0.181	0.387	0.828	1.404
16	0.192	0.401	0.913	1.631
17	0.163	0.307	0.511	0.697
18	0.422	0.681	0.904	1.13
19	0.487	0.624	0.926	1.351
20	0.513	0.731	0.964	1.606
21	0.399	0.573	0.869	1.024
22	0.483	0.631	0.915	1.462
23	0.161	0.391	0.623	0.827
24	0.114	0.309	0.481	0.614
25	0.093	0.119	0.207	0.428
26	0.142	0.286	0.434	0.666
27	0.185	0.358	0.511	0.726
28	-	0.087	0.111	0.247
29	0.245	0.417	0.689	0.901
30	-	0.083	0.108	0.22
31	-	0.093	0.194	0.325
32	-	-	0.096	0.194
33	0.153	0.321	0.469	0.663
34	-	0.089	0.194	0.318
35	-	0.097	0.204	0.306
36	-	0.084	0.121	0.236
37	0.369	0.583	0.716	0.927
38	-	0.076	0.109	0.233
39	0.094	0.123	0.211	0.349
40	0.076	0.114	0.186	0.28
41	0.086	0.128	0.195	0.301
42	0.098	0.154	0.216	0.329
43	0.098	0.155	0.213	0.328
44	-	-	0.083	0.153
45	-	-	0.094	0.174
46	0.091	0.157	0.239	0.428
47	-	0.134	0.209	0.362
48	0.103	0.211	0.342	0.472
49	0.218	0.467	0.532	0.768
50	-	0.131	0.214	0.303
51	-	-	0.086	0.174
52	-	-	0.153	0.211
53	-	-	0.096	0.157
54	-	-	0.102	0.172
55	-	-	0.088	0.144
56	-	-	0.087	0.141
57	-	-	0.107	0.187
58	0.104	0.211	0.297	0.409
59	0.159	0.267	0.385	0.563
60	-	-	0.088	0.199
61	0.166	0.310	0.423	0.627

Contd...

OD: Optical density

Supplementary Data 4: Effect of glucose and sucrose supplementation in different concentration over biofilm formation by proposed method

Serial number	OD at glucose (55.55 mM)	OD at glucose (111.11 mM)	OD at glucose (222.22 mM)	OD at glucose (333.33 mM)	OD at sucrose (29.23 mM)	OD at sucrose (58.47 mM)	OD at sucrose (116.92 mM)	OD at sucrose (175.38 mM)	OD without any sugar
1	0.359	0.362	0.397	0.411	0.352	0.359	0.361	0.363	0.253
2	0.371	0.379	0.403	0.415	0.37	0.378	0.382	0.382	0.298
3	0.83	0.851	0.897	0.898	0.829	0.828	0.837	0.836	0.827
4	0.594	0.613	0.679	0.679	0.591	0.626	0.631	0.629	0.594
5	0.598	0.613	0.627	0.629	0.597	0.609	0.613	0.613	0.595
6	2.013	2.097	2.169	2.182	2.009	2.017	2.157	2.161	2.006
7	0.431	0.472	0.498	0.498	0.441	0.463	0.469	0.469	0.269
8	0.383	0.397	0.418	0.423	0.381	0.398	0.419	0.417	0.376
9	0.612	0.619	0.647	0.649	0.619	0.638	0.641	0.639	0.606
10	0.367	0.371	0.383	0.385	0.368	0.378	0.378	0.373	0.36
11	0.447	0.451	0.468	0.467	0.445	0.458	0.456	0.46	0.445
12	0.581	0.589	0.596	0.598	0.581	0.587	0.591	0.589	0.579
13	1.781	1.792	1.851	1.857	1.737	1.793	1.797	1.787	1.715
14	1.673	1.681	1.837	1.838	1.669	1.783	1.769	1.719	1.667
15	1.405	1.521	1.581	1.589	1.459	1.539	1.517	1.514	1.404
16	1.636	1.641	1.724	1.729	1.633	1.685	1.639	1.646	1.631
17	0.713	0.727	0.893	0.854	0.722	0.849	0.798	0.824	0.697
18	1.139	1.142	1.267	1.249	1.131	1.187	1.191	1.159	1.13
19	1.362	1.367	1.457	1.413	1.356	1.394	1.369	1.373	1.351
20	1.616	1.621	1.763	1.753	1.61	1.735	1.714	1.719	1.606
21	1.049	1.057	1.126	1.129	1.024	1.098	1.107	1.108	1.024
22	1.476	1.491	1.587	1.551	1.462	1.498	1.463	1.469	1.462
23	0.831	0.837	0.869	0.865	0.829	0.854	0.856	0.856	0.827
24	0.618	0.621	0.659	0.657	0.616	0.643	0.643	0.643	0.614
25	0.613	0.628	0.643	0.648	0.599	0.629	0.634	0.631	0.428
26	0.669	0.673	0.741	0.743	0.668	0.724	0.729	0.726	0.666
27	0.729	0.732	0.791	0.793	0.726	0.751	0.757	0.759	0.726
28	0.249	0.257	0.353	0.361	0.247	0.339	0.327	0.318	0.247
29	0.912	0.933	0.967	0.971	0.917	0.958	0.961	0.964	0.901
30	0.224	0.239	0.297	0.291	0.225	0.231	0.263	0.261	0.22
31	0.331	0.348	0.409	0.417	0.329	0.389	0.396	0.394	0.325
32	0.206	0.218	0.237	0.263	0.201	0.214	0.269	0.268	0.194
33	0.670	0.677	0.729	0.732	0.668	0.687	0.693	0.691	0.663
34	0.321	0.329	0.441	0.452	0.318	0.396	0.379	0.379	0.318
35	0.311	0.319	0.458	0.461	0.313	0.321	0.436	0.438	0.306
36	0.241	0.249	0.354	0.355	0.236	0.327	0.33	0.331	0.236
37	0.934	0.941	1.027	1.033	0.934	0.987	0.997	0.989	0.927
38	0.239	0.243	0.328	0.331	0.235	0.306	0.314	0.31	0.233
39	0.352	0.357	0.373	0.362	0.352	0.351	0.362	0.362	0.349
40	0.289	0.297	0.309	0.316	0.282	0.287	0.304	0.306	0.28
41	0.311	0.324	0.331	0.334	0.307	0.319	0.327	0.329	0.301
42	0.337	0.342	0.425	0.429	0.336	0.391	0.397	0.395	0.329
43	0.334	0.357	0.478	0.481	0.331	0.437	0.442	0.442	0.328
44	0.159	0.172	0.269	0.273	0.159	0.247	0.253	0.251	0.153
45	0.181	0.187	0.194	0.196	0.176	0.182	0.189	0.193	0.174
46	0.434	0.497	0.583	0.59	0.434	0.517	0.523	0.524	0.428
47	0.369	0.376	0.458	0.462	0.367	0.427	0.439	0.443	0.362
48	0.478	0.487	0.512	0.523	0.475	0.489	0.516	0.516	0.472
49	0.775	0.813	0.871	0.883	0.771	0.828	0.841	0.84	0.768
50	0.379	0.385	0.436	0.447	0.374	0.414	0.415	0.415	0.303
51	0.196	0.265	0.305	0.313	0.183	0.287	0.298	0.301	0.174
52	0.219	0.227	0.231	0.239	0.211	0.221	0.229	0.226	0.211

Contd...

Supplementary Data 4: Contd...

Serial number	OD at glucose (55.55 mM)	OD at glucose (111.11 mM)	OD at glucose (222.22 mM)	OD at glucose (333.33 mM)	OD at sucrose (29.23 mM)	OD at sucrose (58.47 mM)	OD at sucrose (116.92 mM)	OD at sucrose (175.38 mM)	OD without any sugar
53	0.168	0.172	0.187	0.187	0.163	0.168	0.183	0.184	0.157
54	0.181	0.251	0.293	0.309	0.179	0.264	0.281	0.281	0.172
55	0.157	0.237	0.27	0.276	0.153	0.249	0.261	0.268	0.144
56	0.151	0.231	0.272	0.272	0.149	0.243	0.251	0.251	0.141
57	0.188	0.241	0.279	0.287	0.19	0.252	0.26	0.264	0.187
58	0.419	0.496	0.571	0.58	0.412	0.511	0.52	0.523	0.409
59	0.569	0.581	0.617	0.619	0.566	0.593	0.606	0.609	0.563
60	0.217	0.226	0.239	0.241	0.222	0.222	0.236	0.241	0.199
61	0.638	0.649	0.686	0.689	0.621	0.642	0.661	0.659	0.627
62	1.993	2.021	2.124	2.117	1.972	2.094	2.121	2.112	1.961
63	0.589	0.597	0.609	0.615	0.582	0.592	0.604	0.601	0.577
64	0.202	0.211	0.218	0.216	0.2	0.209	0.21	0.214	0.197

OD: Optical density

Supplementary Data 5: Effect of sodium chloride supplementation in different concentration over biofilm formation by proposed method

Serial number	OD at 500 mM NaCl concentration	OD at 750 mM NaCl concentration	OD at 1000 mM NaCl concentration	OD without supplementation
1	0.363	0.387	0.419	0.253
2	0.384	0.397	0.421	0.298
3	0.852	0.871	0.892	0.827
4	0.621	0.653	0.676	0.594
5	0.597	0.602	0.614	0.595
6	2.107	2.162	2.193	2.006
7	0.443	0.467	0.498	0.269
8	0.391	0.422	0.443	0.376
9	0.615	0.639	0.653	0.606
10	0.373	0.382	0.406	0.36
11	0.457	0.492	0.509	0.445
12	0.588	0.595	0.593	0.579
13	1.767	1.793	1.816	1.715
14	1.69	1.715	1.742	1.667
15	1.419	1.543	1.578	1.404
16	1.635	1.657	1.691	1.631
17	0.734	0.829	0.883	0.697
18	1.171	1.235	1.327	1.13
19	1.361	1.37	1.377	1.351
20	1.621	1.644	1.72	1.606
21	1.068	1.099	1.114	1.024
22	1.647	1.704	1.769	1.462
23	0.846	0.872	0.891	0.827
24	0.627	0.649	0.67	0.614
25	0.644	0.671	0.676	0.428
26	0.729	0.757	0.783	0.666
27	0.742	0.787	0.799	0.726
28	0.358	0.358	0.381	0.247
29	0.951	0.963	0.977	0.901
30	0.229	0.237	0.242	0.223
31	0.373	0.399	0.413	0.325
32	0.208	0.237	0.244	0.194
33	0.674	0.687	0.706	0.663
34	0.352	0.397	0.449	0.318

Contd...

Supplementary Data 5: Contd...

Serial number	OD at 500 mM NaCl concentration	OD at 750 mM NaCl concentration	OD at 1000 mM NaCl concentration	OD without supplementation
35	0.319	0.333	0.459	0.306
36	0.299	0.347	0.361	0.236
37	0.932	0.939	0.94	0.927
38	0.267	0.308	0.334	0.233
39	0.356	0.36	0.367	0.349
40	0.287	0.293	0.309	0.281
41	0.309	0.311	0.316	0.301
42	0.364	0.383	0.427	0.329
43	0.369	0.389	0.467	0.328
44	0.251	0.263	0.274	0.153
45	0.179	0.186	0.191	0.174
46	0.497	0.517	0.579	0.428
47	0.429	0.455	0.491	0.362
48	0.479	0.483	0.487	0.472
49	0.791	0.838	0.874	0.768
50	0.389	0.424	0.447	0.303
51	0.289	0.312	0.327	0.174
52	0.222	0.231	0.243	0.211
53	0.163	0.169	0.172	0.157
54	0.296	0.323	0.338	0.172
55	0.197	0.267	0.294	0.144
56	0.251	0.268	0.275	0.141
57	0.246	0.271	0.283	0.187
58	0.526	0.558	0.581	0.409
59	0.568	0.571	0.577	0.563
60	0.219	0.227	0.239	0.199
61	0.632	0.638	0.641	0.627
62	1.993	2.013	2.117	1.961
63	0.582	0.596	0.611	0.577
64	0.198	0.208	0.215	0.197

OD: Optical density NaCl: Sodium chloride

Supplementary Data 6: Comparative OD's obtained with and without supplementation in proposed method

Serial No	OD without supplementation	OD at final supplementation
1	0.253	0.479
2	0.298	0.477
3	0.827	0.962
4	0.594	0.688
5	0.595	0.693
6	2.006	2.224
7	0.269	0.482
8	0.376	0.521
9	0.606	0.654
10	0.36	0.487
11	0.445	0.536
12	0.579	0.603
13	1.715	1.857
14	1.667	1.893
15	1.404	1.588
16	1.631	1.771

Contd...

Supplementary Data 6: Contd...

Serial No	OD without supplementation	OD at final supplementation
17	0.697	0.959
18	1.13	1.329
19	1.351	1.537
20	1.606	1.796
21	1.024	1.324
22	1.462	1.793
23	0.827	0.886
24	0.614	0.667
25	0.428	0.678
26	0.666	0.781
27	0.726	0.993
28	0.247	0.491
29	0.901	0.98
30	0.22	0.397
31	0.325	0.478
32	0.194	0.298
33	0.663	0.733
34	0.318	0.483
35	0.306	0.494
36	0.236	0.487
37	0.927	1.103
38	0.233	0.391
39	0.349	0.492
40	0.28	0.511
41	0.301	0.427
42	0.329	0.483
43	0.328	0.481
44	0.153	0.297
45	0.174	0.286
46	0.428	0.597
47	0.362	0.509
48	0.472	0.619
49	0.768	0.981
50	0.303	0.492
51	0.174	0.318
52	0.211	0.481
53	0.157	0.283
54	0.172	0.341
55	0.144	0.297
56	0.141	0.28
57	0.187	0.302
58	0.409	0.587
59	0.563	0.648
60	0.199	0.353
61	0.627	0.739
62	1.961	2.125
63	0.577	0.669
64	0.197	0.216

OD: Optical density

Supplementary Data 7: Comparative class obtained with and without supplementation in proposed method

Grading of biofilm without supplementation	Grading after	Isolate number
LBF	MBF	1
LBF	MBF	2
MBF	HBF	3
MBF	MBF	4
MBF	MBF	5
HBF	HBF	6
LBF	MBF	7
LBF	MBF	8
MBF	MBF	9
LBF	MBF	10
LBF	MBF	11
MBF	MBF	12
HBF	HBF	13
HBF	HBF	14
HBF	HBF	15
HBF	HBF	16
MBF	HBF	17
HBF	HBF	18
HBF	HBF	19
HBF	HBF	20
HBF	HBF	21
HBF	HBF	22
MBF	MBF	23
MBF	MBF	24
LBF	MBF	25
MBF	MBF	26
MBF	HBF	27
NBF	MBF	28
MBF	HBF	29
NBF	LBF	30
LBF	MBF	31
NBF	LBF	32
MBF	MBF	33
LBF	MBF	34
LBF	MBF	35
NBF	MBF	36
MBF	HBF	37
NBF	LBF	38
LBF	MBF	39
LBF	MBF	40
LBF	LBF	41
LBF	MBF	42
LBF	MBF	43
NBF	LBF	44
NBF	LBF	45
LBF	MBF	46

Contd...

Supplementary Data 7: Contd...

Grading of biofilm without supplementation	Grading after	Isolate number
LBF	MBF	47
LBF	MBF	48
MBF	HBF	49
LBF	MBF	50
NBF	LBF	51
NBF	MBF	52
NBF	LBF	53
NBF	LBF	54
NBF	LBF	55
NBF	LBF	56
NBF	LBF	57
LBF	MBF	58
MBF	MBF	59
NBF	LBF	60
MBF	MBF	61
HBF	HBF	62
MBF	MBF	63
NBF	NBF	64

NBF: Non biofilm former, MBF: Moderate biofilm former, LBF: Low biofilm formers