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Research Article
Quantification of the Metabolic Activities of Natural Biofilm of Different Microenvironments

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Abstract

Background and Objective: The study of the microbial biofilm of drainage pipes has traditionally been based on culturing organisms. The development and application of culture independent methods have supplied new tools for examining the microbial diversity and activity of biofilm samples. Therefore, the purpose of this research was to quantify some microbial metabolic activities of natural biofilm to establish a relationship between the populations of biofilm cell and their metabolic activities and to compare the biofilm cell densities in biofilm sample collected from different microenvironments. **Materials and Methods:** Natural biofilm samples were collected from different microenvironments. Total Bacterial Counts (TBC) of biofilm were enumerated at two different temperatures (37 and 22°C). Adenosine triphosphate (ATP), polysaccharides and protein level were quantified. Statistical analyses were performed using GraphPad Prism version 5.0 (USA) software. **Results:** There is a well relationship between TBC and ATP level, thus ATP can be used as a quick and direct technique for enumeration of bacterial biofilm populations. Also, results cleared that, the biofilm cells densities were greater in the kitchen and hospital drainage pipes than others. The quantities of protein in each whole and EPS biofilm were more than polysaccharide. **Conclusion:** It is concluded that determination of biofilm cells was depending on their metabolic products and the viability of the bacteria. The amounts of ATP, protein and polysaccharides are dissimilar according to the bacterial cell numbers and the types of microenvironment.

Key words: Natural biofilm, microenvironments, total bacterial counts, metabolic activities, ATP

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Biofilm is a mixture of attached microbial cells which present in sessile state embedded in self-produced Extracellular Polymeric Substance (EPS) by these microorganisms. Frequently, EPS comprise of different amounts of polysaccharides, proteins, fatty acids and DNA[1].

Biofilm can be accumulated on the inner surface of pipes, it considers as a main reservoir of several strict and opportunistic pathogenic microbes in drainage and water pipes[2]. Approximately, 95% of total microbial cells in water and drainages systems are attached in biofilm on pipe surfaces[3]. Therefore, the pipe decaying and changes in the water organoleptic properties can be caused by the accumulation biofilms in water systems and drainage pipes while the main problem is associated with the public health concern. Additionally, biofilm considers as the main source and reservoirs for pathogens in water systems[4].

The development of biofilm present in any solid surface colonized by different types of microorganisms. Furthermore, it can either be helpful or harmful to public health of people that exposed to the microenvironments (bathroom, kitchen, laboratories sink drainage pipes and others) anywhere biofilm is presented[5]. Thus, for all hygiene and sanitation enhancement program, many works must be carried out to as possible control pathogens points. For improved household sanitation, the assessment of fomite contamination as an indicator must be done[6].

Adenosine triphosphate (ATP) bioluminescence method is one of the most important alternative methods for assessment of bacterial populations which can be used as a quantifiable assay to enumerate the total bacterial counts in different types samples[7]. Moreover, this method is a rapid, cost-effective and easy when comparing with conventional methods for estimation of microbial densities in water and biofilm samples and it has been a good relation with Heterotrophic Plate Counts (HPC) data[8,9]. Therefore, the main aim of this study was to establish a relationship between natural biofilm cell populations and their metabolic activities. Moreover, to evaluate the ATP and extracellular components as fast and easy approaches for determining of the biofilm cells densities. So, this study will help to find the rapid, easy to use and economic methods for detection of bacterial cells in biofilm.

MATERIALS AND METHODS

Biofilm sampling: The present study was done during the period from 1/3/2016 to 1/3/2017 and all analyses were carried out at Environmental Microbiology Lab., Water Pollution Research, National Research Centre.

Sixty one natural biofilm samples were taken from different source microenvironments such as bathroom, kitchen, hospitals and laboratory sink drainage pipes. The samples were harvested by scraping of 10 cm^2 from inner surface of pipes using sterile cotton swabs. Swabs were submerged into tubes each one containing 10 mL sterile water and homogenized by using vortex agitator for 5 min[10]. Then, it was preserved in ice box and immediately transferred to lab for analyses according to APHA[11].

Estimation of cultivable bacterial counts: According to APHA[11], the pour plate technique was used to enumerate the total bacterial counts at different temperature (37 and 22°C) which involved in natural biofilm.

Measurements of polysaccharides and protein contents in whole and EPS biofilm: The EPS was extracted from biofilm samples using Cation Exchange Resin method according to Denkhaus *et al.*[12] and Hemdan *et al.*[13].

Measurement of polysaccharide contents: The polysaccharide content of crude EPS and whole biofilm was determined using phenol-sulfuric acid method according to the protocol described by DuBois *et al.*[14].

Measurement of protein contents: Folin method for estimation of crude EPS and whole protein biofilm was used according to Lowry *et al.*[15].

ATP quantification: Series of standard ATP solution were prepared with sterilized tris acetate buffer solution. In luciferase assay protocol, aliquot of 30 µL of the ATP solution was added to an optical sensing cell and aliquot of 270 µL of luciferin-luciferase was added subsequently. The luminescence patterns and luminescence intensity were recorded by using the ATP luminometer. Luminescence intensity was recorded as Relative Light Unit (RLU) [16].

Direct microscopic examinations

Scanning Electron Microscopy (SEM) examination: Four natural biofilm types were examined by Scanning Electron Microscopy (SEM) model JEOL JXA-840A, electron probe micro-analyzer, Japan. Pipe coupons (1×1 cm^2) were prepared for examination using scanning electron microscopy. The samples were prepared by fixation with 2.5% glutaraldehyde for 1 h and with 1% osmium tetroxide for 1 h. And then they were dehydrated in 50, 70, 90 and 95% ethanol for 10 min per each step and 2 times in 100% ethanol for 10 min. They were dehydrated 2 times in HMDS (hexamethyldisilazane) for 10 min, air dried and coated with gold. Their images were analyzed using SEM[17].

Transmission Electron Microscopy (TEM) examination: Four natural biofilm types (Kitchen, bathroom, hospital and lab.) were examined by Transmission Electron Microscopy (TEM) model JEM 2100-HRTEM. Biofilm samples were investigated by the negative contrast method and the method of ultrathin sections. For the investigation of total samples by the negative contrast method, the biofilm cells suspensions were applied to a Formvar coated grid stabilized with carbon. The samples were stained by a 1% water solution of the ammonium molybdate (Sigma, UK) for 30 sec then examined under TEM[18,19].

Statistical analysis: Statistical analyses were performed using GraphPad Prism version 5.0 (USA) software. A Linear Regression (R^2) analysis was performed ($p<0.05$). The Linear Regression was applied to evaluate positive or negative correlations between ATP amounts and TBC at different temperature (37 and 22°C). The critical p-value for the test was set at <0.05 [20].

RESULTS AND DISCUSSION

Biofilm contains different types of microorganisms including bacteria, virus, parasites, fungi and exogenous substances that are surrounded in gelatinous polymeric substances and attached to solid surfaces. Furthermore, the biofilm formation consists of 50-90% EPS and 5-25% different types of microorganisms[21].

Regarding to the results of kitchen sink drainage pipes, the average log counts of TBC at 22°C and ATP were at the same log (Fig. 1). In case of bathroom sink drainage pipes, results of this research showed that, the average log counts of TBC at 37 and 22°C were less than biofilm of kitchen pipe. In addition to that, results of average ATP level were equal of TBC at 22°C (Fig. 1). Concerning of results illustrated graphically (Fig. 1) noticed that, the average counts of TBC at 37and 22°C in lab biofilm were visualized.

The obtained results cleared that the bacterial densities in the kitchen and hospital were largest compared other drainage pipes. These results in well-matched with Berger *et al.*[22] who showed that, the kitchen considered as a best refuge of pathogenic bacteria.

Additionally, the results indicated that, there is relationship between TBC and ATP level, so ATP may be used as a rapid and direct method for estimation of bacterial biofilm communities. Whereas, the viable microbial cell can produce ATP through their breathing, ATP can be used to transfer essential cellular activities for their living, development and

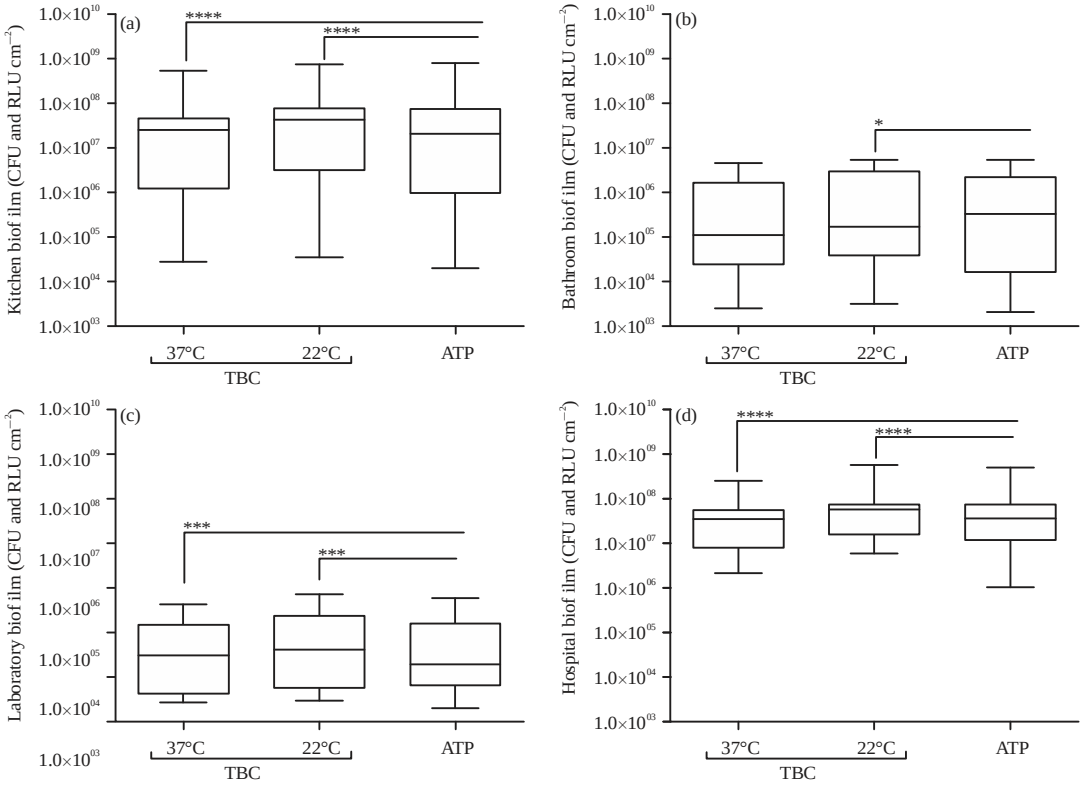

Fig. 1(a-d): Log_{10} of the average level of TBC at (37 and 22°C) and ATP of natural biofilm from different microenvironments, (a) Kitchen biofilm, (b) Bathroom Biofilm, (c) Laboratory biofilm and (d) Hospital biofilm
*Indicated to low correlation, ***Indicated to highly correlation, ****Indicated to extremely high correlation

multiplication. Moreover, inside bacteria cell, the roles of ATP is used to protect and provide energy and enzymatic reactions in metabolism[23].

As well, these results found that, there is a well correlation with significance between the TBC and ATP level. So, results in agreement with Deininger and Lee[16] they stated that, there are a great correlation between ATP and TBC. The correlation coefficient between ATP and TBC was 0.84 with highly significant ($p<0.05$). Also, the obtained results compatible with Mempin *et al.*[24] who revealed that, ATP which presented the supernatant of bacterial culture of several Gram-positive and Gram-negative bacterial species. The extracellular ATP level in all bacterial strains tested was growth-phase dependent with the highest level at the late log phase of growth. The presence of extracellular ATP and the dynamic changes in its levels suggest that ATP may have important extracellular functions in addition to its long-established intracellular roles.

The EPS consist of polysaccharides, proteins, DNA and lipids with different percentages. These substances participate in the steadiness mechanical of microbial biofilm[25]. Moreover, Flemming and Wingender[26]demonstrated that, the EPS play an important role in the biofilm accumulation. Furthermore, Tsuneda *et al.*[27] stated that, the amounts of polysaccharides and proteins are ranged between 75-89% of the biofilm EPS composition, which are considered as a foremost substances.

The quantity of each measured extracellular component (polysaccharides and protein) in whole and EPS biofilm is shown in Fig. 2. The amounts of polysaccharides and protein in whole and EPS biofilm varied from pipe to pipe. Results shown in (Fig. 2a) indicated that, the highest amount of extracellular component (polysaccharides and protein) was higher in kitchen drainage pipe than lab drainage pipe.

Results illustrated graphically in (Fig. 2b) established that, the maximum amounts of polysaccharides in EPS biofilm were hospital biofilm. While, the maximum level of protein was kitchen biofilm.

The obtained results cleared that, the highest amount of extracellular component was shown in the kitchen than hospital. This is probably the higher microbial populations in biofilm. So, the densities of microbial community may be reflected the amount of polymeric matrices in biofilm. Numerous former investigations proposed that, there is a strong correlation between the ability of biofilm formation and the amount of released polysaccharide[28,29].

Additionally, results showed that, the levels of extracellular component in whole biofilm were more highly than in EPS biofilm. This may be due to, extracellular component consists of the bacteria cell and EPS compositions. These are in agreements with Sutherland[30] who confirmed

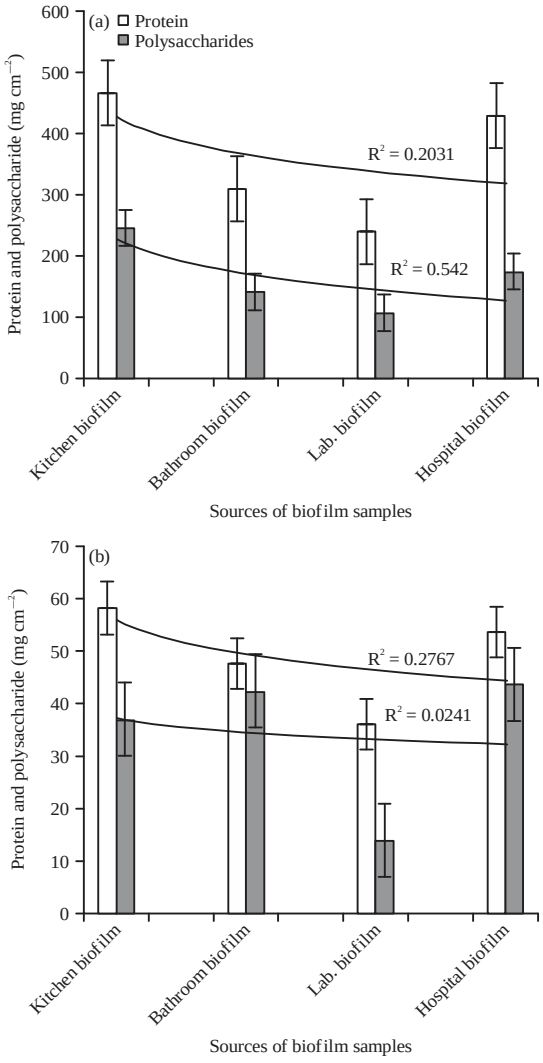

Fig. 2(a-b): Standard Deviation (SD) of the amount of protein and polysaccharides in (a) Whole biofilm and (b) EPS of natural biofilm from different microenvironments

that, biofilm are mainly possessed of the polymeric matrix, which is a complex assimilation of polysaccharide, nucleic acids and proteins. These substances can play a vital role in protection of structure constancy and protective role for the biofilm.

Additionally, the microbial metabolic activities and products of biofilm, especially, the EPS substances and microbial cells (whole biofilm) may be measured and evaluated by using different bacteriological chemical techniques or direct microscopic examination methods. Even though, the isolation of EPS, the cellular fraction from whole biofilm required to use chemical approach of the biofilm[31].

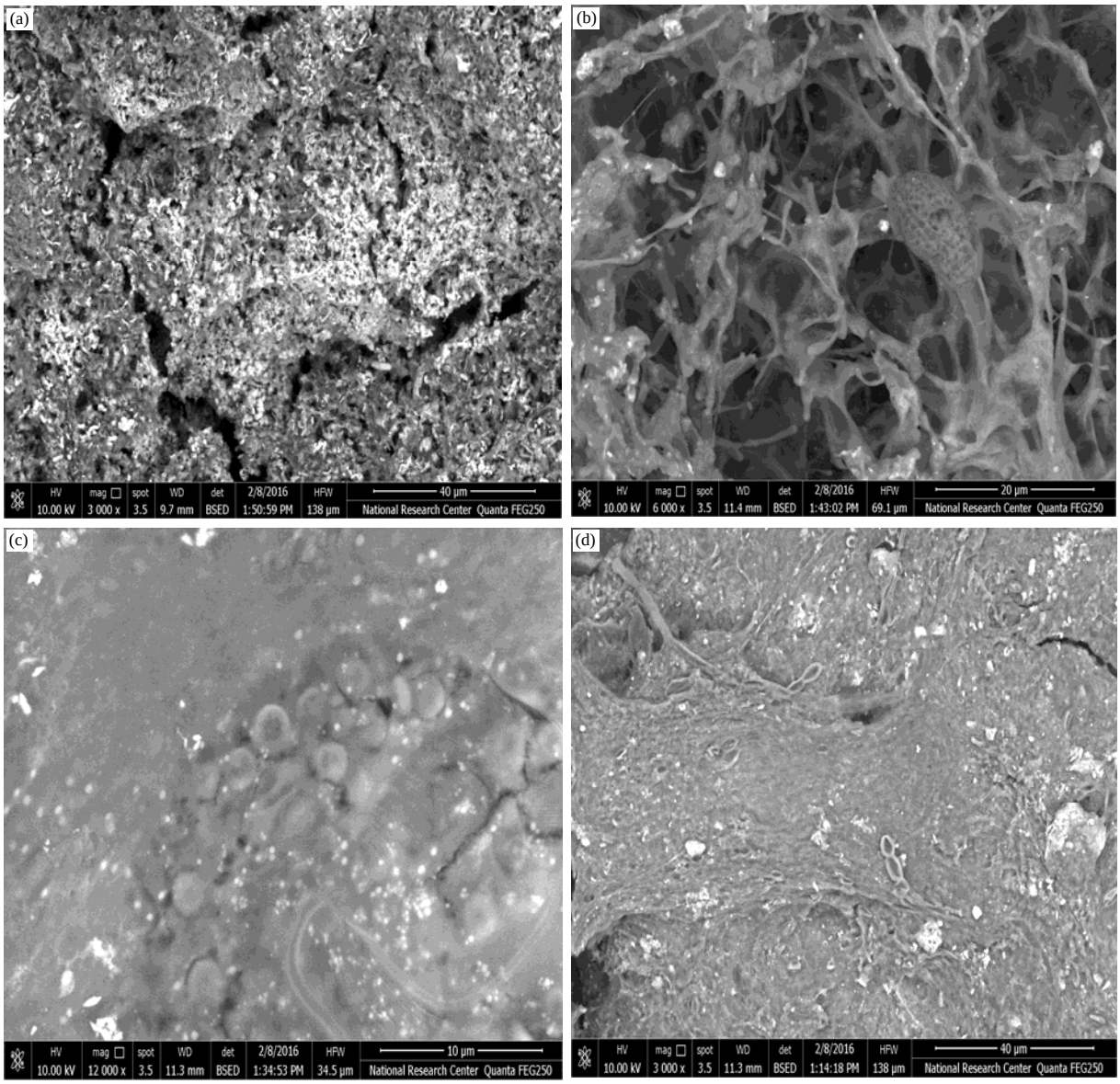

Fig. 3(a-d): SEM images for biofilm collected from (a) Kitchen, (b) Bathroom, (c) Lab. and (d) Hospital

Furthermore, results showed the concentrations of EPS protein were highest when comparing with polysaccharide amounts. Because the extracellular protein was the most abundant component, followed by DNA, while polysaccharides were the least represented component in most conditions[32,33]. In addition to Jahn *et al.*[34] who established that, proteins are a main portion of EPS, in spite of, polysaccharides are a considered as a most rich constituent of EPS.

Direct microscopic characterization of natural biofilm

Scanning electron microscopy: Another useful technique to visualize biofilms is Scanning Electron Microscopy (SEM), this has been used to obtain 3D images of biofilms on surfaces of drinking water networks[35]. In this study, the structures of 4 biofilm collected from kitchen, bathroom, lab. and hospital sink drainage pipes were examined by using SEM. SEM images showed that, the thickness of biofilm scraped from kitchen drains was greater than others. While, the lowest was in biofilm collected from lab. drains (Fig. 3). These results were compatible with the results of Feazel *et al.*[36] and Hong *et al.*[37], they demonstrated that, the PVC pipe contained a layer of EPS in which microbes embedded in polymeric material.

Transmission electron microscopy: Transmission Electron Microscopy (TEM) is one of the well methods which used

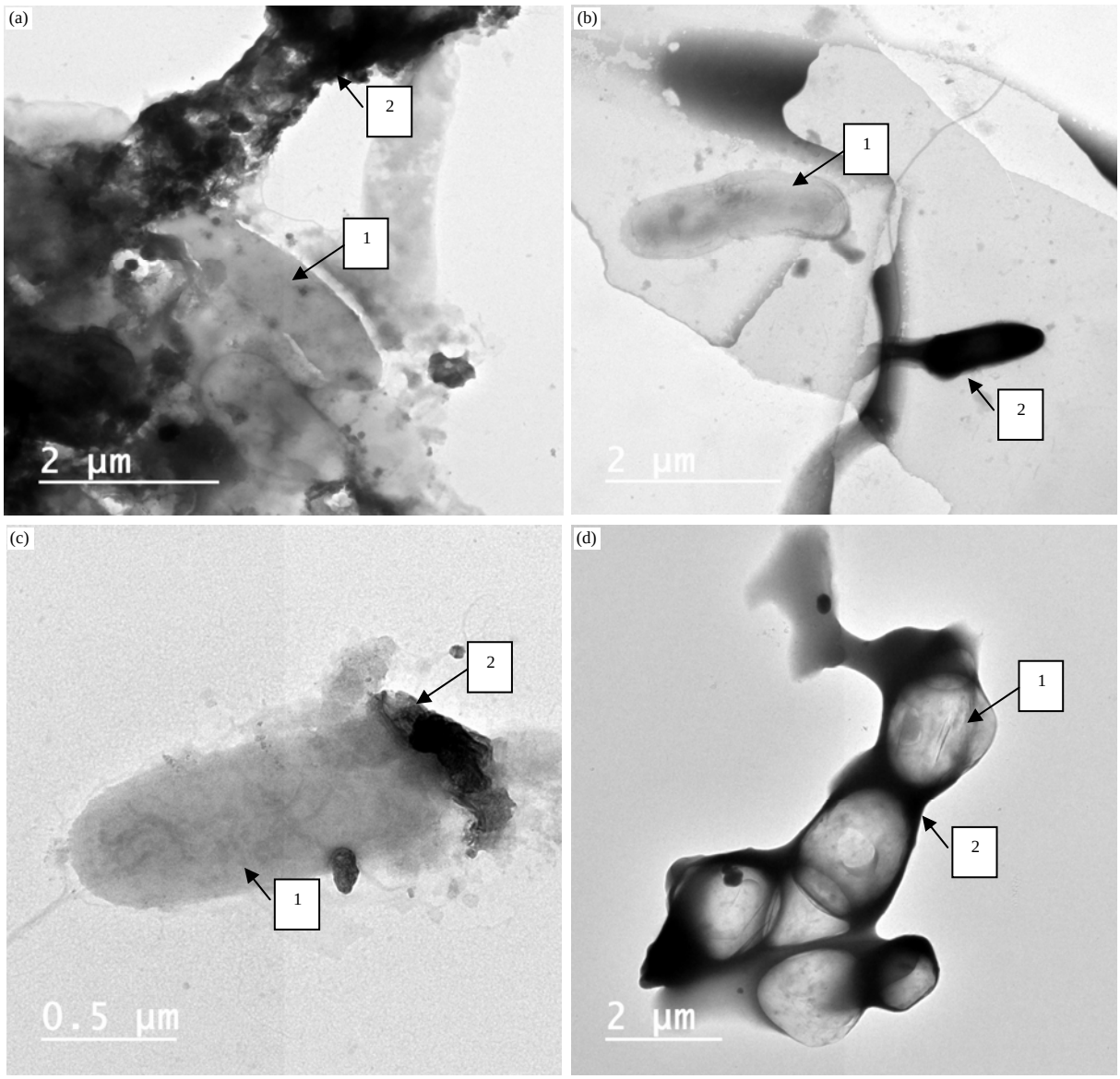

Fig. 4(a-d): TEM photomicrograph for natural biofilm collected from (a) Kitchen, (b) Bathroom, (c) Lab. and (d) Hospital
1: Bacterial cell, 2: EPS structure

to evaluate the microbial composition of biofilm[38]. The photomicrograph of TEM indicated that, the cells were embedded in a polymer matrix and exopolysaccharides. Also, it was produced from the biofilm cells in different shapes.

It was found that type of environment and cell densities were affecting on the quantity and shape of the exopolysaccharides which produced from biofilm cells as shown in Fig. 4. Additionally, the variation in the amount and sizes of the EPS of cell aggregates were well-known[39].

In the present study, it can be implicated that, the determination of microbial metabolic activities of biofilm cells will help to enumerate the bacteria densities of bacterial biofilm cells. In addition to, these rapid methods can be applied in outbreak. In this study, it can be recommended that, the determination of ATP and EPS amounts of biofilm is better than culture methods. The main limitation of this research may need to professional persons.

CONCLUSION

It was concluded that:

- A large numbers of biofilm populations were shown in kitchen and hospital then bathroom and lab drainage pipes

- Furthermore, the extracellular components were highest in whole biofilm compared with in biofilm EPS
- The amounts of protein were more than polysaccharide in both whole and EPS biofilm
- The microbial metabolic activities (extracellular components and ATP assay) may help for solving the problems encountered in the detection of viable bacteria. Whereas, the main function of the assay is to quantify ATP, the important compound in metabolism that is found within all living cells

SIGNIFICANCE STATEMENT

This study screened the densities of bacterial biofilm cells and their metabolic activity. The characterization of natural biofilm compositions may help to develop new approaches to rapidly detect and estimate the bacterial numbers.

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