Original Article
ISSN (Online): 2350-0530
ISSN (Print): 2394-3629

International Journal of Research - GRANTHAALAYAH
March 2022 10(3), 42-49

INSIGHT INTO EXTRACELLULAR POLYMERIC SUBSTANCES EPS EXTRACTION AND QUANTIFICATION TECHNIQUES: CAUSES, MECHANISMS, AND APPLICATIONS MINI REVIEW

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ABSTRACT

Microorganisms have a secret world, which is full of baffling phenomena. Without studying this world, we cannot understand the concept of biofilm formation, which is based on the aggregation of different kinds of microorganisms to create micro-colonies. The mysterious world of biofilm is fairly understood. Due to the sophisticated complexity of its structure and architecture, biofilm has many essential properties which had made it an important study field for many researchers in different aspects, public health, water treatment, and especially microbiology. By going into the complexity of biofilm structure, studying Extracellular Polymeric Substances (EPS) for the last decade has provided researchers with great evidence regarding biofilm formation. EPS extraction is the first step toward being able to study the composition and properties of various microbial communities. This review covers EPS extraction techniques and their significant in life.

Received 05 February 2022
Accepted 03 March 2022
Published 31 March 2022

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DOI
10.29121/granthaalayah.v10.i3.2022.4518

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Keywords: Biofilm, Extracellular Polymeric Substances (EPS), Microorganisms, Lysis

1. INTRODUCTION

Extracellular polymeric substances (EPS) have been a continually growing point of interest in research over the past decades Donlan (2002). New methods in the extraction and quantification of EPS have been developed as a result and there is still much to be learned from these techniques Comte et al. (2006), Spaeth and Wuertz (2000). The effect of EPS composition and structure on biofilms and activated sludge is the main driver for this research activity Spaeth and Wuertz (2000).

It is important to define the different types of associations between EPS particles when discussing any aspect of this complex matrix. Common terms include pure and mixed cultures, as well as bound and soluble EPS. Past studies focused on the idea that EPS is made solely of polysaccharides (pure cultures), however now it is known that they contain many more components Donlan (2002), Flemming et al. (2007). These mixed cultures include polysaccharides, proteins, DNA, lipids, and uronic acids. Bound EPS refers to capsular, loosely bound EPS and other attached particles. On the other hand, soluble EPS relates to "slime," colloidal material, and soluble macromolecules Spaeth and Wuertz (2000). The nature of the biofilm, in addition to the research questions being asked, plays a major role in determining the best type of extraction and quantification methods to use. These two laboratory processes are tightly linked, and the following literature review will show that the type of information obtained through quantification depends on the extraction methods used Liu and Fang (2002).

2. EXTRACTING EXTRACELLULAR POLYMERIC SUBSTANCES (EPS)

EPS extraction is the first step toward being able to study the composition and properties of various microbial communities Sheng et al. (2010), Sheng et al. (2005). These communities can include microbial mats, biofilms, activated sludge, etc. The methods available are diverse and continually evolving, as this is a relatively new field. Choosing the appropriate extraction method is a complex decision that must take into account what specifically is being studied and the various EPS properties. EPS have many characteristics, such as cohesiveness, mechanical stability, van der Waals forces, electrostatic forces, hydrogen bonds, cation bridging, and hydrophobic and hydrophilic interactions, that result in the need for multiple methods to break these binding forces Donlan (2002), Flemming et al. (2007), Spaeth and Wuertz (2000). Many times, the ultimate goal is to get the highest extraction efficiency without cell lysis or disruption of macromolecules. There are many physical and chemical techniques, as well as combinations of the two that may be suitable Nielsen and Jahn (1999).

3. EXTRACTION METHODS

3.1. PRETREATMENT

Samples need to be homogenized (without changing the cells) in order to get the best extraction results. A variety of homogenizers are available for this process. Many times, the aggregate also needs to be washed in order to remove soluble EPS. The ionic strength of the washing buffer should be similar to the sample so as not to wash bound EPS components away from the matrix Nielsen and Jahn (1999).

3.2. PHYSICAL METHODS

These methods, such as centrifugation, mixing, shaking, sonication ion exchange (resins), and heat treatment, extract EPS by applying a shear force. Ion exchange is one way to apply this force. Divalent cations, like Mg2+ and Ca2+, create bridges between charged compounds that need to be broken in order to extract the EPS. Resins or complexing agents can be used to remove the cations. Individual physical methods often have a lower yield compared to using them in combination with other methods. For example, centrifugation is commonly used as the primary extraction method for soluble EPS but is almost always used as a secondary treatment after the chemical extraction of bound EPS Nielsen and Jahn (1999).

3.3. CHEMICAL METHODS

These methods work by adding a chemical to the sample, which breaks links in the EPS matrix and releases EPS into the water. Examples include alkaline treatment by NaOH, acidic treatment by H2SO4, ion exchange (Ethylenediaminetetraacetic acid EDTA, (ethylene glycol-bis (β- aminoethyl ether)-N, N, N′, N′-tetra acetic acid) EGTA, NaCl), the addition of formaldehyde/glutaraldehyde, enzymatic digestion, and hydrophobic extracellular fraction. Alkaline treatments cause charged groups, like carboxylic groups in proteins and polysaccharides, to be ionized due to their isoelectric points of less than pH 4 – 6. This causes repulsion among particles in the EPS gel and results in higher water-soluble compounds. Extracting the hydrophobic components of EPS is difficult to do because of the lack of knowledge in determining

how much cell lysis occurs Flemming et al. (2007). However, detergents can be used to extract specific hydrophobic compounds Nielsen and Jahn (1999).

4. CASE STUDIES - COMPARING EXTRACTION YIELDS

Two different studies compared the extraction efficiencies of physical, chemical, and combinations of extraction methods Comte et al. (2006), Sheng et al. (2005). Sheng et al. (2005) tested the extraction of EPS from the photosynthetic bacterium Rhodopseudomonas acidophila using a control (centrifugation), chemical (EDTA, NaOH, H2SO4), and physical methods (heat treatment). They compared their results to those of other studies on activated sludge and Rhodovulum sp. using similar methods in addition to the physical method, cation exchange resin (CER). Comte et al. (2006) compared extraction methods from activated sludge with a control (centrifugation), chemical (EDTA, formaldehyde + NaOH, glutaraldehyde), and physical methods (sonication, CER, sonication + CER, heat treatment).

Sheng et al. (2010) found that EDTA yielded the highest EPS with the lowest occurrence of cell lysis, determined by nucleic acid content, for R. acidophila. When comparing their results to other cultures, EDTA had the highest extraction efficiency based on yield and low amount of cell lysis. CER has a much higher yield; however, cell lysis may occur in CER due to the long extraction time and high stirring intensity needed. NaOH and heating are also known to cause a significant amount of cell lysis compared to EDTA Sheng et al. (2005). Comte et al. (2006) determined the highest EPS yield by using formaldehyde + NaOH and that in general, the chemical methods resulted in higher yields. However, they propose that the choice of extraction methods should be determined by more than just the EPS yield and nucleic acid content because chemical methods can result in contamination by extracting reagents and physical methods in varying qualitative compositions. Furthermore, using nucleic acid content as an indicator for cell lysis might not be the most accurate because it is known that the EPS matrix normally contains nucleic acids Comte et al. (2006). This emphasizes the fact that choosing an EPS extraction method is not a black and white decision and that there are multiple factors to take into account.

5. QUANTIFYING BIOFILM STRUCTURE

Most investigations of biofilm growth utilize microbiological sampling and microscopic imaging. Microbiological sampling methods only provide the number of bacterial colonies for a single point in time. Microscopic methods provide information on the morphology of the biofilm but have a limited working distance Reipa et al. (2006). However, there are methods that are able to obtain data in real-time and provide important information about the dynamic properties of biofilms Reipa et al. (2006).

6. WHY QUANTIFY BIOFILM STRUCTURE?

Biofilm structure affects the rate of nutrient transport to the deeper layers of biofilm and influences microbial activity and the rate of biofilm accumulation. It has been shown in different studies that there is a significant effect of biofilm structure on the mass transport mechanism and rates in the biofilm porous area and within the biofilm, respectively Zbigniew and Beyenal (2019). Environmental factors can

affect the rate of biofilm accumulation and activity, causing varied structural characteristics among different biofilms. This variation can include:

- Hydrodynamic-chemical composition of the solution
- Chemical-physical properties of the surface on which the biofilm grows

Thus, it can be hypothesized that there is an important relationship between the structure and processes that occur within the biofilm. If this hypothesis is true, then quantifying biofilm structure will aid in understanding the processes happening within the biofilm Zbigniew and Beyenal (2019).

In addition, there are factors affecting the development of the EPS of the biofilm matrix which are not yet understood:

- The concentration of nutrients and substrate in the bulk phase
- Hydrodynamic conditions according to the flow and reactor design

In order to understand these factors, there is a need for sensitive techniques that provide additional information on biofilm matrix, EPS composition, and distribution Wagner et al. (2009).

Biofilm researchers are mainly interested in quantifying two characteristics of the biofilm:

1) **Biofilm structure –** distribution of biomass in the space occupied by the biofilm
2) **Biofilm heterogeneity –** non-uniform distribution of any feature within the space occupied by the biofilm (i.e., different chemical or physiological groups of the microorganisms)

These two terms are interrelated. There are many parameters that need to be defined so they can be used to characterize the EPS composition and distribution Yang et al. (2000). Parameters are defined as features that quantify a measured structural element of the biofilm Yang et al. (2000).

7. HOW IS BIOFILM STRUCTURE QUANTIFIED?

Depending on the question asked, there are different kinds of techniques that are used in order to study the structure and composition of biofilms. These techniques can be categorized as follows Spaeth and Wuertz (2000)

Summary parameters Not possible to analyze the composition or structure of EPS	Single substance analysis	Invasive microscopy methods	Direct and noninvasive methods
1. Gravimetric measurements. 2. TOC	Classical biochemical methods	TEM* SEM* CLSM*	RM* QCM*

Discussed below

8. TEM (TRANSMISSION ELECTRON MICROSCOPY)

Used when analysing at a scale of resolution smaller than the dimensions of cells, and when the analysis of individual cells can improve our knowledge of the biofilm process Zbigniew and Beyenal (2019). This technique uses an energy

dispersive detector that determines the distribution of elements like Ca+2 or Fe+2 Wagner et al. (2009).

9. SEM (SCANNING ELECTRON MICROSCOPY)

This has been traditionally used in biofilm research, however, its main disadvantage is the need to use a high vacuum, to prepare the samples by fixation in some resin and to dehydrate Zbigniew and Beyenal (2019).

The two techniques above illustrate the spatial distribution and the structure of the cells; however, they need some sample preparation that causes a loss of the 3-D image of the biofilm Spaeth and Wuertz (2000).

10. QUARTZ CRYSTAL MICROBALANCE (QCM)

A sensitive technique to study the solid-solution interface based on a shift of the quartz crystal resonance frequency due to interaction with solution components. Optical methods complement the data obtained from QCM techniques Reipa et al. (2006). The combination of QCM and reflectance has the advantage of being non-destructive and allows complementary measurements in situ and in real-time Reipa et al. (2006).

11. CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

A tool that provides clear images of the space occupied by fully hydrated biofilms Zbigniew and Beyenal (2019). The use of CLSM in combination with different staining protocols allows the visualization and quantification of biofilm structure and is often combined with other methods that provide additional information about biofilm components. The application of combined techniques can result in obtaining a broader picture of the biofilm matrix Wagner et al. (2009). The CSLM images are acquired in layers parallel to the biofilm surface. Each layer corresponds to an image in the X and Y directions and the layers are treated as the Z direction Beyenal et al. (2004). Quantitative parameters describing biofilm physical structure have been extracted from three-dimensional CLSM images and used to compare biofilm structures, monitor biofilm development, and quantify environmental factors affecting biofilm structure Beyenal et al. (2004).

12. RAMAN MICROSCOPY (RM)

RM is an application that aids in the study of the biofilm matrix and provides additional information about the chemical composition by allowing a deeper insight into the chemical structure of the EPS. In addition, it improves knowledge of EPS structure. In this technique, there is no need for staining and therefore it is possible to obtain results from biofilm structures that were not known before (due to missing interactions between the applied stains and the EPS compounds). Thus, there are obvious advantages such as saving time on intensive or complex sample preparation, reduced risk of altering the biofilm or creation of artifacts, the possibility of measuring directly in the aqueous phase, and revealing information in the biofilms‟ native state with spatial resolution in μm Wagner et al. (2009).

13. COMBINED USE OF CLSM AND RM TO INVESTIGATE EPS MATRIX

The combination of these two techniques was applied in order to characterize heterotrophic biofilms. Although CLSM is one of the most understood techniques used to study and characterize biofilm structure, it has some disadvantages that require an additional technique in order to complete the study. These disadvantages include the low specificity of the fluorescent stains due to the variety of macromolecules possible in the EPS matrix. Thus, fluorescent staining cannot perform as a quantitative process – there are some parts of the EPS that cannot be stained, and other parts indicate higher amounts of one type of macromolecule than expected or shown by a chemical analysis of the sample. Therefore, by applying RM these disadvantages may be avoided. By not staining the sample, information about the EPS components of a biofilm matrix can be obtained in situ in a non-destructive way. Different EPS components can be classified chemically based on their specific Raman scattering signals (e.g., polysaccharides, proteins). This is clearly a huge potential of RM. RM reveals changes in the chemical composition of a biofilm matrix that was not detectable by CLSM. These changes might be very small, and the binding characteristics used by fluorescent dyes can be too low for detecting such shifts with CLSM. In addition, in this study, it was clear that changes in the tremendous chemical diversity of the EPS, occurring with increasing biofilm age, were only detected in the Raman spectra. Thus, RM provides extended information which will be useful for the investigation of biofilm removal procedures based on the chemical composition of the biofilm matrix (e.g., in fields of application where physical procedures are not applicable).

14. APPLICATIONS OF EXTRACTION AND QUANTIFICATION AND RECOMMENDATIONS

1) One of the earliest applications began in the 1950s where EPS was produced by axenic cultures and was isolated for serological purposes. The goal was to analyse the changes in the formation and composition of EPS as a function of varying culture conditions Spaeth and Wuertz (2000).
2) Wastewater treatment - Different studies have shown that EPS variation is influenced by growth conditions and growth phase. They have investigated the characteristics of EPS in biological wastewater treatment to determine if EPS can also influence the properties of activated sludge where the most important applications are flocculation, dewatering ability, sorption behaviour, and biological activity. The chemical composition of EPS affects the surface properties of the floc and thus the physical properties of activated sludge. In addition, charged functional groups in EPS play an important role in flocculation. It was found that there is a correlation between EPS composition and surface charge of flocs under both aerobic and anaerobic conditions that were used to analyse the sludge Spaeth and Wuertz (2000).
3) Water systems – In order to protect the security of water systems, there is a need to monitor the accumulation and removal of biofilms. Once a water system has been in operation for any length of time, native bacteria will colonize the surface forming a biofilm. The nature of the biofilm will be a major determinant in the adhesion of biological and chemical threats to the surfaces of the water system Reipa et al. (2006).

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