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Review

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A Review on Production of Exopolysaccharide and Biofilm in Probiotics Like Lactobacilli and Methods of Analysis

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Abstract: Synthesis of exopolysaccharides (EPSs) by lactic acid bacteria is well known and the EPS produced by Lactobacillus has been highly regarded in recent years because of its unique physical and chemical application in the food and pharmaceutical industry. One of the capabilities of probiotics is the use of EPS to form a biofilm produced in tense environments. In this paper after a short description about EPS, the reason for production in bacterial cells, and its biosynthesis pathways; the capability of Lactobacilli for EPS and biofilm formation are reviewed. The chemical composition of EPS, its role in the bacterial life cycle as well as applications for humankind have been studied. Then the important components in biofilm formation are described and variable influencing on biofilm formation (surface, bacterial cell surface, contact time and environmental characteristics) are reviewed. The relationship between EPS and extracellular polymeric precursors as well as the relationship between biofilm formation and EPS production are mentioned. Finally, methods for quantification of carbohydrate (enzymatic, physical, chemical methods), biofilm formation and EPS extraction (Tallon and Bajpai methods) are reviewed and advantages of methods are compared. EPSs produced by probiotics is important due to the application as a thickening agent, emulsifier, heavy metal eliminator, and drug delivery carrier. Also, it has been considered for its anti-cancer, anti-viral, and cholesterol-lowering properties. So forming biofilm by some probiotics in simple and mixed culture are discussed, the relationship between EPS and biofilm production are discussed. When probiotics produce biofilm, they can be more tolerated in the processing of food production and in the gastrointestinal tract. So the efficacy of probiotic transfer may increase by a self-protection potency without any required encapsulation processing, solvent residue, time and energy consumption, etc. Also, identification and measurement methods are reviewed and compared.

Keywords: Probiotic; Lactic acid producing bacteria; Exopolysaccharide; Capsule; Biofilm formation.

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1. Introduction

Probiotics that confer health beneficial impacts to the host, when administered in adequate amounts. Some of them can also, can be considered as a suitable way for bioremoval of pollutants including toxins, heavy metal (Pb^{2+}, Cr^{2+}/Cr^{3+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+} etc.), residues, etc. from water and foodstuff [1-6]. Antimicrobial activities of synbiotic extract could

differ in their antagonistic activities against diarrhoeal causing organism which could be due to the metabolite secreted by the lactic acid bacteriocin especially the type of organic acids and added inulin as a prebiotic and for food preservation [7].

2. Exopolysacchride

Exopolysaccharides (EPSs) of lactic acid bacteria (LAB) adhere closely to the bacterial surface with covalent bonds which may be released into the surrounding medium or attached loosely to bacterial cells. EPS as high molecular weight polymers made up of sugar substitutes are divided into two groups: hemopolysaccharides and heteropolysaccharides. The EPS produced by lactobacilli has been highly regarded in recent years because of its unique physical and chemical properties in the food industry as a viscosity, jelly, thickener, emulsifier, heavy metals removal.

In the pharmaceutical industry, as agents for the transfer of drugs and in the field of therapeutic anticancer, antiviral, anti-inflammatory, and the property of lowering blood cholesterol. One of the capabilities of probiotics is the use of EPS to form a biofilm that is produced by a number of its isolates in tense environments [8, 9].

3. EPSS biosynthesis pathways

Two separate mechanisms have been identified for biosynthesis of EPS. Homopolysaccharides are synthesized via extracellular mechanisms by enzymes secreted to the cell exterior, while heteropolysaccharides are produced via more complex mechanisms. Precursors of EPSs are firstly produced in cytoplasm and then the other stages of the biosynthesis take place outside the cells. Synthesis of EPSs by bacteria employs a broad spectrum of enzymes that are not specific and unique to the production of EPSs.

Nucleotide diphosphate sugars as an active form of monosaccharides, provide various types of active monosaccharides for microbial cells via epimerization, dehydrogenation, and decarboxylation reactions. Isoprenoidglycosyl lipid carriers have a role in their synthesis [8]. Enzymes involved in the synthesis of EPSs can be divided into four groups: 1) enzymes involved in internal carbohydrate metabolism, 2) enzymes involved in producing nucleotide sugars and in converting them into each other, 3) glycosyltransferases that shape repeating units and attach them to glycosyl lipid carriers, and 4) enzymes involved in polymerization and translocation of carbohydrates [9].

4. EPSS - producing labs

Most EPS-producing LABs belong to the *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus* Sps. Some strains of Bifidobacteria have also exhibited the ability to produce these polymers.

4.1. Lactobacilli.

These are Gram-positive catalase-negative bacteria of fermentative metabolism because they lack respiratory chains. Some are microaerophiles and others obligate anaerobes. There are more than 125 strains in the *Lacobacillus* Sp. [10].

Lactobacilli are commonly found in human and animal intestines, form a protective barrier against pathogenic bacteria, and exhibit antagonistic activities against the

gastrointestinal tract diseases caused by bacteria of the *Listeria*, *Salmonella*, *Shigella*, and *Klebsiella* Sps. [11]. They also play a role in protecting the female urinary-genital tract with their antimicrobial activities against some pathogens like *Proteus vulgaris* [12].

Although Gram-positive bacteria also produce EPSs, the main producer of EPSs are LABs. EPS producing LABs are isolated from dairy and non-dairy products under various conditions by adding various types of sugar [13].

5. Chemical composition of EPSS produced by labs

Researchers reached the general consensus that the EPSs present in lactic acid producing bacteria were polysaccharides consisting of repeating (branched) units that included α and β bonds and were secreted in various types although their monomeric constituents were clearly similar to each other. For example, *L. acidophilus* LMG 9433 and *L. rhamnosus* C83 have lost their rhamnose, or *L. sake* 0-1 only has glucose and rhamnose. The use of complicated compounds in culture media causes production of various types of EPSs. This indicates that the types of EPSs vary under different conditions [14].

6. Various EPSS in bacteria producing them

The gene producing EPSs in mesophilic Lactobacilli is a plasmid that may be lost due to its instability, but thermophilic Lactobacilli have a gene cluster for EPS production which they delete under unstable conditions or transmit as mobile elements. Various types of heteropolysaccharides with different molecular weights and component sugars are secreted by mesophilic and thermophilic Lactobacilli. In general, thermophilic LABs produce more heteropolysaccharides compared to mesophilic LABs [13].

7. The role played by microbial EPSS

In the natural environment of microbial life, EPSs play an important role in protecting microbial cells against water loss and drying, phagocytosis, phages, antibiotics, toxic compounds like toxic metallic ions, sulfur dioxide and ethanol, protozoan predators, and osmotic stress, and in helping microbial cells to adhere to solid surfaces, form biofilms, and recognize cells (through binding to a lectin). The important point is that bacteria do not use EPSs as a food source because most bacteria producing EPSs lack the ability to catabolize them. Capsular EPSs and lipopolysaccharide O-antigen play a role in the response of the host immune system to pathogenic bacteria like *Streptococcus agalactiae* [14, 15].

7.1. EPSs applications.

The important microbial EPSs in industry are dextran, gellan, xanthan, pullulan, alginate, and glucan produced by yeast. New microbial biopolymers can satisfy the unmet needs in the industry [14]. Bacterial EPSs are used to remove heavy metals [14, 16].

EPSs also has various applications in pharmaceutical industries like coating material for drugs [17]. In medicine, they are utilized for their mentioned health-promoting effects. They are used in the food industry for fermentation in food processing. EPSs are utilized in the food industry to improve rheological properties, provide consistency, increase viscosity, and enhance the taste of food materials [18]. They are also used in the food industry to produce lower fat and higher quality dairy products [13].

The high-molecular-weight homopolysaccharide dextran produced by LABs has antiviral properties and enhances the immune system of aquatic organisms. The EPS produced by *Leuconostoc mesenteroides* RTF10, *Lactobacillus sakei* MN1 is utilized as fish feed [19].

8. Biofilm

Formation of bacterial biofilms is in fact stages in the life of bacteria that start with their attachment to a surface [20]. Biofilms are complex bacterial communities attached to surfaces that are created by the extracellular matrix that the bacteria produce. This matrix consists of EPSs, nucleic acids and proteins. Bacterial biofilms can protect bacteria against environmental stresses, the immune response of the host, antimicrobial agents and antibiotics [21, 22]. Bacterial biofilms were discovered in the late 17th century.

Using a rudimentary microscope, Antony van Leeuwenhoek showed the presence of small particles (called animalcules) on the surfaces of teeth. A group of bacteria joined to each other and adhered to a surface were then studied in 1934. In general, biofilm construction includes initial attachment of bacteria to a surface, production of extracellular polymers the most important of which are the EPSs, formation of a microcolony and growth and maturity and, finally, dispersion of some of the bacteria [23-25]. Fig 1. presents the process of the formation of a biofilm.

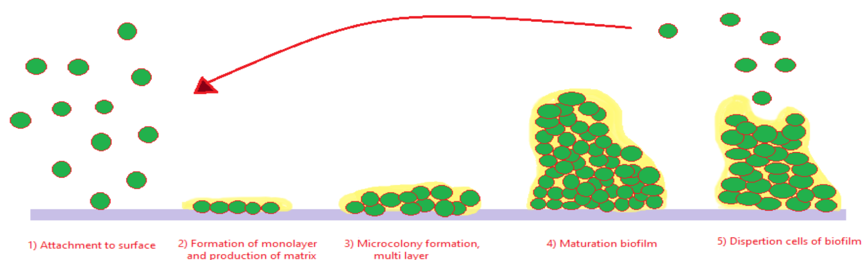

Figure 1. The process of biofilm formation.

8.1. The important components in biofilm formation.

Biofilm construction depends on the interaction between the four main components of bacterial cells, surface attachment, the surrounding environment, and the contact time.

8.1.1. Effects of the attachment surface.

Surface features like roughness, cleanliness, and wettability (determined by hydrophobicity) are among the factors influencing adhesion of bacteria to surfaces [23].

8.1.2. Bacterial cell surface properties.

Cell physical and chemical properties including electrical charge of bacterial cell surface, which is related to the presence of acid groups like carboxyl, phosphate and basic groups such as the amines present on cell surface, play a role in the attachment of bacterial cells to a surface. Cell surface appendages like pili, flagella, and surface polysaccharides also play an important role in bacterial attachment [23].

8.1.3. Environmental characteristics.

These characteristics, which are among the effective factors for bacterial attachment, include the hydrodynamic conditions of the environment, the physical and chemical properties

of the environment including the pH of the surface, nutrients, ionic power, and temperature [25].

8.1.4. Contact time.

Contact time between bacteria and the surface is an important factor for the establishment of an irreversible attachment. It has been proved that increases in contact time improve the strength of the attachment to the surface Factors influencing biofilm maturity include dissolved oxygen concentration, carbon source, osmotic pressure, and pH of the environment. Biofilm formation is a complex process controlled by various bacterial genes including those related to expression of quorum-sensing signals [26].

8.2. Biofilm construction by probiotic Lactobacilli.

One of the useful abilities of probiotic Lactobacilli is forming biofilms for protecting themselves against environmental stresses and for helping their colonization and population maintenance [27].

Probiotics form complex communities known as biofilms and have several useful properties for developing microbial populations under biotic or abiotic condition [20]. It must be mentioned that biofilm formation in the digestive system requires effective attachment to epithelial cells, effective attachment time, and stabilization of the bacteria on epithelial cells.

These cells then prevent the competitive attachment of pathogenic bacteria and forming biofilm, also they can stimulate the host immune system [28].

Studies have been conducted so far on biofilms of probiotic Lactobacilli like *L. rhamnosus, L. plantarum, L. fermentum,* and *L. reuteri*. One of the components that participate in adhesion and biofilm formation is S-layer. It being a protective sheath against hostile environmental agents and having an important role in the establishment of *Lactobacillus acidophilus* in the gastrointestinal tract. The Stability of this part of microbial cells was examined in different conditions [29, 30].

Another area of interest in research on probiotics is using them for coating bacteria. For example, there is a hypothesis stating that this coating makes it possible to add probiotics to some food materials, allows the probiotics to remain stable over time, prevents them from reacting with food constituents, and enables them to resist gastric pH when attached to the intestines. These are achieved because small amounts of a specific probiotic are coated with polymeric materials to protect them against factors like heat, moisture, freezing, and gastric pH. Therefore, these coated probiotic bacteria survive and reach the main location where attach to the intestines [24].

In recent years, use of probiotic plankton cells for coating has been limited and utilization of fourth generation probiotics (that is, making use of their biofilm shape with a double-layer protective coating) is a new and attractive area that has attracted the interest of researchers in recent years [31].

8.3. The relationship between EPS and extracellular polymeric precursors.

Extracellular polymeric precursors, which are produced by LABs to construct biofilms, include a set of glycoproteins, nucleic acids, phospholipids, and polysaccharides, especially EPSs [32]. One of the components existing in the outer layer of bacteria, besides EPSs is S-layer that protect bacteria against some environmental risk [31].

8.4. Biosynthetic pathways of extracellular polymeric precursors in probiotic Lactobacilli and factors influencing their production.

Synthesis of extracellular polymeric precursors serves several functions: it causes microbial attachment to the solid surface, the formation of a microcolony, and maturity of the biofilm structure in addition to making the biofilm resistant to environmental stresses and to disinfectants. In some cases, the matrix of extracellular polymeric precursors enables the bacteria to obtain a series of their required food materials. Production of extracellular polymeric precursors for attachment of microorganisms is a complex process influenced by various factors; in addition, the processes involved in biofilm development are different in the various species [33].

8.4.1. Extracellular polymeric precursors.

In general, the matrix containing the polymeric precursors is 0.1-1μm thick. In some bacterial species its thickness is 10-430 nm and the matrix is not very valuable. The chemical structures of the polymeric materials secreted by bacterial cells into the environment are varied [20, 21]. Constituents of extracellular precursors differ even within a bacterial species. Most external microbial layers include neutral carbohydrates (hexane the most and pentane the least prevalent) and uric acid. The most common extracellular carbohydrate constituents are acetate, pyruvate, fumarate, and succinate esters. The presence of polypeptides in the matrix of extracellular polymeric precursors is specific to a limited number of Gram-positive species. Polysaccharides and proteins are the most widely studied constituents present in the layer of extracellular polymeric precursors [34]. The structures of the polysaccharides produced by microbial cells are very different with respect to the types of bonds. This is observed in the case of microbial cells belonging to the Streptococcus, Leuconostoc, and Sinorhizobium Sp. Microbial EPSs have been compared with respect to being homopolysaccharides or heteropolysaccharides. Homopolysacchar-ides only contain one type of monosaccharide (D-glucose or L-fructose). Homopolysaccharides are divided into the following three groups:

A-D-glucans: These are produced by *Leuconostoc mesenteroides*. The constituents among the D-glucosyl units mostly include α (1→6) bonds. The branches are mainly in the form of α (1→3) and less frequently in the forms of α (1→2) and α (1→4).

B-D-glucans: These are mostly produced by the Pediococcus and Streptococcus genera. The D-glucosyl units are connected by β (1→3) bonds and the branches by β (1→2) bonds.

Fructans: These are mainly produced by the species *Streptococcus salivarius*. The fructosyl units are connected by β (2→6) bonds.

A number of LABs produce heteropolysaccharides. These molecules are formed from repeating monosaccharide units such as D-glucose, D-galactose, L-fructose, L-rhamnose, D-glucuronic acid, L-guluronic acid, and D-mannuronic acid.

The types of both bonds between monosaccharide units and chain branches determine the type of the heteropolysaccharide. The most frequently found heteropolysaccharides include pyruvate, succinate, and fumarate sub-units. Bacterial alginate is an insoluble heteropolysaccharide with D-mannuronosyl and L-guluronosyl at its two ends. Alginate is mostly produced by *Pseudomonas aeruginosa* and *Azotobactervinelandii*. The secreted extracellular proteins have molecular weights of 10-200 kDa. These constituents include 40-60 percent of hydrophobic amino acids [32].

8.5. Physiological study of extracellular polymeric precursors.

Synthesis of extracellular biopolymers by microbial cells depends on the presence of carbon and nitrogen in the culture medium. Most extracellular polymers of microorganisms are produced using carbohydrates as the carbon source and ammonium salts and amino acids as the nitrogen source. In general, production of extracellular polymeric precursors increases under conditions where the largest amounts of glucose are present in the environment. Synthesis of extracellular constituents in *Acetobact-erxylinum* takes place with access to fructose, sucrose, and starch at concentrations of 25-100 g/L. The lowest production level of precursors is observed under conditions where lactose and xylose are present. Carbohydrates like xylose, ribose, sucrose, lactose, glucose, fructose, and mannose are the precursors required for the production of these extracellular precursors. Moreover, low nitrogen content in the culture medium greatly increases synthesis of extracellular biopolymers [32].

8.6. Molecular features related to synthesis of extracellular polymeric precursors.

The mechanisms regulating production of extracellular polymeric precursors have not been completely determined yet. Shaping the production process of the precursors requires enzymes. Each enzyme, produced by the gene related to the synthesis of extracellular polymeric precursors, is responsible for carrying out a separate part of the process. Tang *et al.* (1990) showed that a region of the *Xanthomonascampestri*s genome (the rpf gene cluster) produces both the extracellular polymeric molecules and the enzymes that control their production (and are involved in the transmission process). Under abnormal conditions, microorganisms undergo changes in taxonomy and produce a collection of extracellular materials. These changes are observed in *Pseudomonas aeruginosa* [32, 35].

8.7. The relationship between biofilm formation and EPS production.

One of the abilities of probiotics is using EPSs as one of the important constituents of extracellular polymeric precursors utilized in biofilm construction.

Salas-Jara *et al.* (2016) extracted the EPSs from *L. fermentum* CO-979 and investigated its biofilm construction. They also used *L. casei*Shirota as the control and made some changes in the Tallon method to extract the EPSs. After studying the biofilms of *L. fermentum*UCO-979 and *L. casei*Shirota, they found that stronger biofilms were formed in the isolates with the passage of time. Finally, they measured the amounts of extracted EPSs at different times and noticed that there was a relationship between the quantities of extracted EPSs and biofilm construction [24].

Results obtained by Verhoeven *et al.* (2007) indicated that glucose was the initial raw material for producing EPSs, it was the most important part of the biofilm matrix, and the efficiency of producing EPS production and biofilm construction changed with changes in the quantity of glucose [15].

Branda *et al.* (2005) stated that the presence of EPSs played a vital role in biofilm expansion [19]. Moreover, Lebeer *et al.* (2007) cultured *L. rhamnosus* GG in media containing various compounds to show a linear relationship between biofilm construction and EPS production [17]. They extracted the EPSs in the stationary phase and studied their biofilms at the same time. It was found that stronger biofilms were formed in the culture media in which more quantities of both capsular EPSs and EPSs released into the medium were produced. They concluded environmental factors and culture media played a substantial role in biofilm

construction and, consequently, in the production of EPSs in *L. rhamnosus* GG, and also noticed that the effect of EPSs on biofilm formation was dependent on the culture media. For example, the largest amounts of EPSs were produced in the AOAC culture media whereas the best biofilm shapes were observed in the mTSB culture medium. Therefore, they reached the conclusion that the size, chemical structure, and location of EPSs and surface features were involved in the attachment of the bacteria.

Vasquez *et al.* (2017) demonstrated that the EPS dextran produced by *L. sakei* MN1 was an anti-microbial attachment factor and prevented biofilm formation in this species, whereas the same EPS did not affect biofilm construction in *L. mesenteroides* RTF10. In addition, OlayaRendueles *et al.* (2013) showed that a polysaccharide named A101 prevented activity and development of biofilms [19, 36].

9. Methods

9.1. Mixed probiotic cultures.

Many bacterial species including pathogenic bacteria become more resistant to extracellular stressful conditions by constructing biofilms consisting of two or more bacterial species. To prepare probiotic cultures, the turbidity of each isolate was adjusted to that of a 0.5 McFarland standard, 100µl of each isolate was poured into each well of the microplate so that there was 200µl of the two bacterial suspensions in each well. The biofilm was then stained with crystal violet (2g of crystal violet dye was dissolved in 10 ml of ethanol absolute, and the solution was passed through Whatman filter paper and was raised to volume using 90 ml of distilled water). After decolorization by acetic acid, absorbance was read at 492 nm using an ELISA plate reader [37].

9.2. Methods for carbohydrate analysis and quantitative assessment of carbohydrates.

Nowadays identifying structures of oligosaccharides has a special role in biological research and is an important factor for making advances in analysis [38].

9.2.1. Enzymatic methods.

Enzyme-based analytical methods have the ability for specialized reactions. These specialized reactions are rapid and accurate and detect even low carbohydrate concentrations. Many enzymatic kits are available and are used for specialized diagnostic tests.

9.2.2. Physical methods.

Various physical methods have also been developed for quantitative assessment of carbohydrates. These methods make changes in the physicochemical characteristics of carbohydrates present in a sample. Polarimetry, IR, refractive index, and density are among these methods.

9.2.3. Chemical methods.

These are based on reactions between sugars and other compounds that result in changes in color or sedimentation. Concentrations of the carbohydrates are then obtained based on measuring density, using spectrophotometric methods, and titration. There are various

methods for measuring concentrations of carbohydrates [39]. Table 1.presents chemical methods for sugar assessment.

9.3. The phenol-sulfuric acid method.

Analysis of oligosaccharides is very complicated because, unlike proteins and nucleic acids, a number of them are branched and use various types of bonds to bind to other substances. The high charge density in oligosaccharides and polysaccharides and the sulfate esters in them add to the difficulty. Hydrolysis of oligosaccharides and polysaccharides in strong acids produces a mixture of monosaccharides. Quantitative chromatography methods are used to identify these monosaccharides in order to determine the general composition of the polysaccharide polymers. NMR spectroscopy provides extensive information on polymers and on configurations of anomeric carbons.

Colorimetric assays for reducing sugars and polysaccharides have been used for a long time. Simple sugars, oligosaccharides, polysaccharides and their derivatives including methyl ethers have (or potentially have) free oxidizing agents that produce a yellow-orange color when treated with phenol and concentrated sulfuric acid. This is a very sensitive reaction and the produced color is stable. The phenol-sulfuric acid method is a suitable method for assessing carbohydrates and their related derivatives [40].

This method was first developed by Dubois *et al.* in 1956 to determine the total concentrations of sugars and their derivatives in which simple sugars (monosaccharides), oligosaccharides, and their derivatives like methyl ethers react with free reducing agents. The color produced in these reactions remains stable for several hours. Therefore, this method determines the total carbohydrate content. It is not a stoichiometric method and requires a standard curve drawn by using known concentrations of a carbohydrate.

The total extracted carbohydrates in the various methods were used in the phenol-sulfuric acid method to draw the standard curve for glucose. In this method, first 250µl of 5% phenol and then 1,250µl of concentrated sulfuric acid were added to 0.5ml of the extracted EPS solution. After 10 minutes the solution was vortexed for 30 seconds and, 20 minutes later, absorbance was read at 490nm. In this experiment, a two-phase system is first formed after the addition of phenol and sulfuric acid. However, a uniform orange color is produced after the solution is vortexed [40]. Here, the control sample consists of 300µl water+250µl 5% phenol+ 1,250µl concentrated sulfuric acid.

9.4. Application of trichloroacetic acid.

Trichloroacetic acid (TCA), which is usually used to precipitate proteins, precipitates them irrespective of their physico-chemical conditions, but it is not able to precipitate unfolded proteins. TCA causes proteins to precipitate by dehydrating the hydration shells around them. Other studies also indicated that TCA changed protein conformations and precipitated them due to its acidic property. However, the exact mechanism of this precipitation is not completely clear. Protein precipitation by TCA is somewhat reversible. In general, protein precipitation takes place in one of the following three classes:

Phase 1: This happens at concentrations less than 5% w/v of TCA. Increases in acid concentration advances protein precipitation.

Phase 2: This happens at concentrations of 5-45%w/v of TCA. The largest amount of precipitation happens in this concentration range, and higher concentrations reduce the quantity of precipitated proteins.
Phase 3: This happens at concentrations higher than 45% w/v of TCA. Protein precipitation markedly decreases at 45% w/v and no precipitation, or very little precipitation, happens at 60%w/v of TCA. The noteworthy point is that the amount of protein precipitation is independent of protein concentration at all concentrations of TCA. Results were obtained using SDS-PAGE and reading absorbance at 290 nm [41, 42].

Table 1. Chemical methods for determination of sugars.

Technique	Description	Absorbance	Advantages	Defects	Color regent
1.Dinitrisalicyclic acid (DNS)	This method estimates the quantity of reducing sugar in the sample	570 nm	Suitable for assessment of simple sugars like glucose, fructose, etc.	Not suitable for assessing complex sugars like various polymers and polysaccharides	DNS changes the yellow color into orange
2.Anthrone	This method uses diluted hydrochloric acid to convert glucose to inert hydroxymethylfurfural	630 nm	Very sensitive for assessment of glucose	Not suitable for assessing complex sugars like various polymers and polysaccharides	Anthrone creates a green color in the environment
3.The phenol-sulfuric acid method	This method uses concentrated sulfuric acid to break down sugars into simpler units and employs phenol as the color reagent	490 nm	Suitable for assessment of all carbohydrates, monosaccharides, disaccharides, and polysaccharides	Since the toxic material phenol is used in this method, care must be exercised in waste disposal	Phenol creates a yellow color in the environment
4.The Nelson-Somogyi method	This method estimates the quantity of reducing sugar in the sample	500 nm	Suitable for assessment of simple sugars like glucose, fructose, etc.	Not suitable for assessing complex sugars like various polymers and polysaccharides	Arsenomolybdic acid creates a blue color in the environment

9.5. Measurement of biofilm formation in microtiterplates.

Microbial biofilms have been studied for several decades. A set of methods have been developed for culturing and studying biofilms but there is no standard method for investigating biofilms of various bacterial species. At first, biofilms were measured by culturing bacteria inside tubes and by examining biofilm formation on the biofilm walls. Nowadays, various methods like test tubes, microplate test, radioactive labeling, microscopic methods, and Congo red agar plate test are used in studying biofilms. However, one of the most widely-used methods is the microplate test. The process of biofilm formation starts with the initial attachment of bacteria to the surface that is influenced by various factors like surface features, and the turbidity caused by the biofilm is directly related to the incubation period [43]. Many studies have pointed to 2 and others to 3-4 wash cycles. In the study conducted on the number of wash cycles, it was concluded that 3 wash cycles were more effective.

The microplate method is a colorimetric one currently used in most microbiology laboratories. It requires a very low volume of culture medium. In the method developed by Shakeri and Mahdavi, a single colony of the agar culture medium of the tested probiotic Lactobacilli was removed and cultured on MRS broth to prepare the primary culture. This culture was incubated for 24h at 37˚C. A microbial suspension with the turbidity adjusted to that of a 0.5 McFarland standard was then prepared, and 200µl of each isolate was poured into each well. The control wells contained only the sterile medium. The wells were made of

polystyrene. The microplate was incubated for 18, 24, and 48h at 37°C under anaerobic conditions (incubation conditions in 5% CO_2).

The contents of the wells were then emptied and the wells were washed three times with sterile physiological serum to remove all planktonic cells. The microplate was given time to dry. In the next stage, 200µl of 2% violet crystal was added to each well and kept in it for 5 min. Violet crystal can stain biofilms and is used to assess them. The wells were then washed with sterile distilled water. A purple halo was observed in each well. For the quantitative analysis of biofilm construction, 33% glacial acetic acid (v/v) was added to the wells, the plate was shaken several times, and absorbance was read at 492 nm using an ELIZA plate reader [44].

To report the results, the OD values of the samples were compared with that of the control (OD_c):

$OD \leq OD_c$	Biofilm was not formed
$OD \leq 2 \times OD_c$	Weak biofilm
$2\times OD_c \leq OD \leq 4 \times OD_c$	Average biofilm
$OD \geq 4 \times OD_c$	Strong biofilm

The same process was followed for the 24 and 48h.

9.6. EPS extraction.

There is a wide variety of methods for studying EPSs instead of a single comprehensive and complete one. This is because of diagnosis, isolation, and determination of the quantity of EPSs produced by microbial strains, the type of the employed culture medium, and also the accuracy level desired in the separation process influence the study methodology.

Some available methods require a very high speed of the centrifuge that may not be available to all researchers although it is an important factor in the separation efficiency of the ESPs produced by the bacteria. Moreover, TCA concentrations strongly influence extraction of the EPSs released into the medium. This type of EPSs has a much greater share than the capsular EPSs.

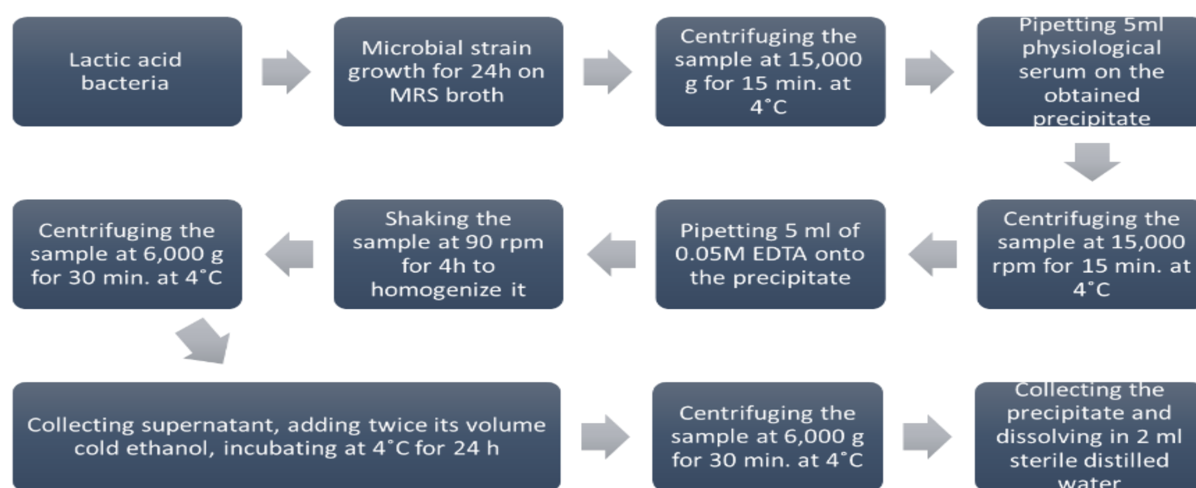

Figure 2. A summary of the Tallon method used to extract capsular EPSs.

Most separation methods of EPSs are time-consuming and tedious and there is the risk of losing polymer during the experiment, especially when the EPS is obtained from complex media. A broad spectrum of different methods has been published each different from the others. Various stages and different temperatures, use of centrifugation at different speeds, and

employment of TCA, Pronase E, or trypsin for removing proteins, are just a few stages utilized in the methods based on precipitation of the EPS with ethanol [45].

According to research by Garcia-Garibay and Marshall (1991), Cerning *et al.* (1994), and Harding *et al.* (2003), precipitating protein with TCA is the most common method widely used for separating EPS from complex culture media. Proteins are precipitated using TCA and removed through centrifugation. The EPSs are then precipitated with ethanol. Precipitating with TCA for removal of unnecessary materials in the environment like proteins and peptides was first carried out by Garcia-Garibay and Marshall in 1991. The use of TCA recovers about 50% of the total produced EPSs. Furthermore, Stingele (1996), Lemoine (1997), De Vuyst *et al.* (1999) showed that precipitation with acetone instead of ethanol reduced the recovered ESPs from the total produced by about 5-10% [14, 46, 47].

9.6.1. The Tallon method.

Tallon *et al.* (2003) purified the EPSs produced by *L. plantarum* EP56 for the first time and studied their biochemical properties [48]. This strain produces both capsular EPS and EPS released into the medium, and exhibits mucoid phenotype and ropy on MRS agar. A CDM culture medium is used to extract EPS from *L. plantarum* EP 56. In addition to basic materials in MRS broth, this culture medium includes a set of vitamins, salts, and minerals that provide more suitable conditions for EPS production [48-50]. Centrifugation was employed to extract EPS released into the medium from the supernatant and to separate the capsular EPSs (linked to the cell surface via covalent bond) from the obtained precipitate. In relation to *L. plantarum* EP 56, capsular polysaccharide has a lower weight than EPS. Grobben *et al.* (1996) suggested that the regulation of EPS biosynthesis methods in *L. bulgaricus*2772 could depend on the carbon source in the culture medium. Growth of this strain in a culture medium containing fructose prevents activation of enzymes that produce EPSs [51].
In general, Tallon *et al.* studied the amounts of total EPS produced by *L. plantarum* EP56 at different temperatures on a enriched medium culture with various sugars like galactose, lactose, fructose, and sucrose. They concluded that more polysaccharide was produced at 25°C in the presence of lactose, and at the same temperature in the presence of glucose the largest amount of EPS was produced by these bacteria.

9.6.1.1. The method for extracting capsular EPS (EPS attached to the wall) by using the Tallon method.

A single colony from the MRS agar culture medium was removed first using an inoculation loop, cultured on MRS broth, and incubated at 37°C for 18, 24, and 48h under anaerobic conditions. Ten ml of the microbial suspension with its turbidity adjusted to that of a 0.5 McFarland standard was then centrifuged at 15,000g for 15 min. at 4°C. Five ml physiological serum was pipetted onto the obtained precipitate, and the solution was centrifuged again at 15,000g for 15 min. at 4°C. The precipitate was then made viscous by pipetting 5ml of 0.05M EDTA on it and was put on a shaker at low speed at 4°C for 4h. It was then centrifuged at 6,000 g for 30 min. at 4°C. Two volume of cold ethanol was added to the supernatant and precipitation of the EPS bounded was carried out for 24 h at 4°C. To separate the precipitated EPS, centrifugation was used at 6,000 g for 30 min. at 4°C. In this stage, the transparent supernatant was discarded and the precipitate containing capsular EPS was dissolved in 2 ml of sterile distilled water to be used for quantitative assessment of capsular

EPS. To become certain of the results, all experiments were repeated three times [49]. Fig 2 shows the stages in extracting capsular EPS.

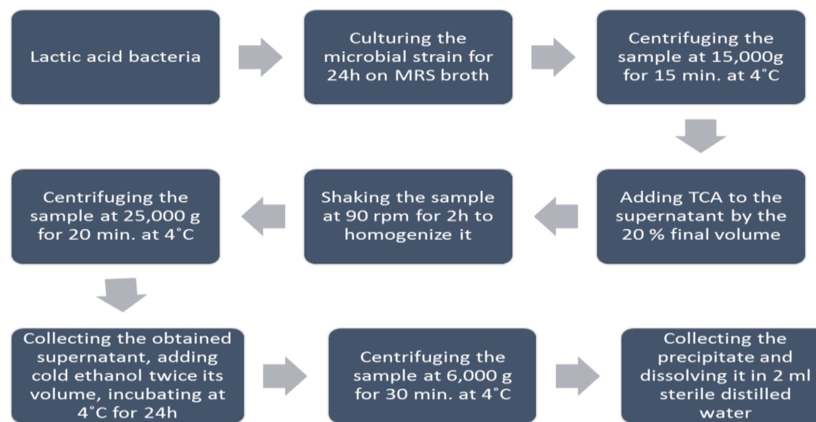

Figure 3. A summary of the Tallon method used for extracting EPS released into the medium.

9.6.1.2. The method for extracting EPS released into the medium by using the Tallon method.

To separate released EPS, first a single colony was removed from the MRS agar using an inoculation loop, cultured on MRS broth, and incubated at 37˚C for 18, 24, and 48h under anaerobic conditions. Ten ml of the microbial suspension with its turbidity adjusted to that of a 0.5 McFarland standard was then centrifuged at 15,000g for 15 min. at 4°C. The supernatant was treated with TCA with the final concentration of 20% and incubated at 4°C for 2h. The precipitated proteins were separated by centrifugation at 25,000 g for 20 min. at 4°C. Cold ethanol was added twice the volume of the supernatant and kept at 4°C for 24 h for the EPSs to separate from the supernatant and precipitate. Centrifugation was then carried out at 6000g for 30 min. at 4°C, and the obtained precipitate was dissolved in 2ml water to be used for quantitative assessment of the ESPs produced by intended strain. Fig3 presents summary of stages in EPS extraction using the Tallon method [48].

9.6.2. Extraction of the EPSs released into the medium using the method Bajpai.

Wang *et al.* (2011) reported that EPS synthesis is a known property of LABs that protects them against adverse environments like drought, toxic materials, and environmental stresses [52].
Bajpai *et al.* (2016) extracted EPS released into the medium by LABs cultured on broth MRS enriched with glucose (10% w/v). They reported that separation and purification of the EPSs was a time-consuming and expensive method. Nevertheless, extraction of EPSs from LABs attracted interest in the past because they were used as preservatives in the food and pharmaceutical industries and as natural agents for providing natural viscosity and consistency [53].

9.6.2.1. The Bajpai method for extracting EPSs released into the medium

Lactic acid bacterium was cultured at 37ºC for 18~24 hours in MRS modified medium supplemented with 10% glucose. After centrifugation (8,000 ×g for 20 min at 4ºC) of culture, the supernatant was collected and added with a final concentration of 14% trichloroacetic acid to denature the protein content. The culture was further left for homogenization in a shaker (90 rpm) for 30-40 min followed by centrifugation at 8,000 ×g for 20 min at 4ºC. The supernatant was then added to cold absolute ethanol (two-fold volume of supernatant) at 4ºC for 24 hours,

followed by centrifuge-ation at 8000 ×g at 4℃ for 20 minutes. These steps resulted in the isolation of crude precipitate. Finally, the precipitate was dissolved in deionized water and dialyzed using Spectra/Por molecularporous tubular dialysis membrane for 24~48 hours. The precipitate was then lyophilized in an IIShin freeze dryer (Korea). The freeze-dried lyophilized powder of lactic acid bacterium was considered to be purified exopolysaccharides. The purified exopolysaccharide was stored at -80ºC for further analysis [53]. Fig 4 presents a summary of the various stages in the extraction method.

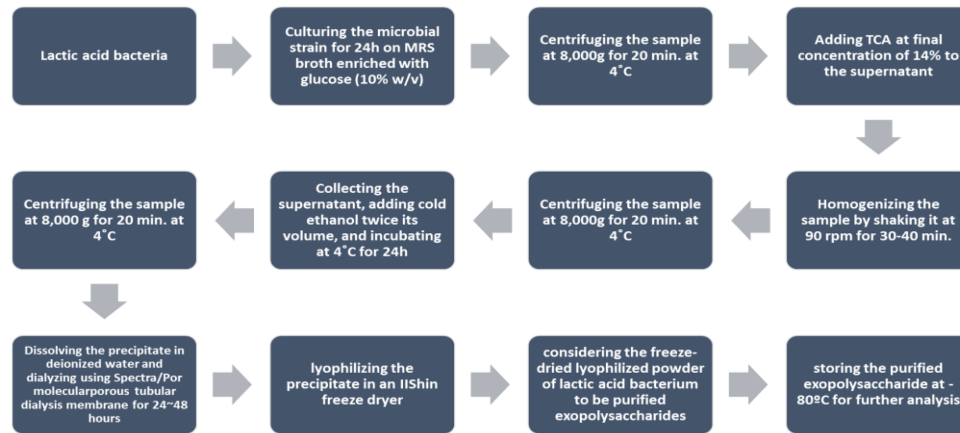

Figure 4. A summary of the method similar to that introduced by Bajpai for extracting EPSs released into the medium.

9.7. Comparison of biofilm formation in microtiterplate Single culture.

The ability to construct biofilms is one of the properties of LABs enhancing their resistance to external stresses. As shown in the research, LABs make biofilms of various strengths depending on the type of culture medium and on culturing conditions [45, 24]. In research by Sala-Jara *et al.* (2016), glucose (2% w/v) was added to MRS broth to study biofilms made by *L. fermentum* UCO-979 and *L. casei* Shirota. It was found that *L. fermentum* formed stronger biofilms than *L. casei* Shirota. In the article by Salas-Jara *et al.* (2016), it was stated that biofilm formation by LABs was one of their useful abilities [24].

Some researchers believe that lack of sufficient glucose in a medium causes stress and expression of the genes responsible for preserving life like biofilm construction, which leads to the formation of stronger biofilms. For example, Lebeer *et al.* (2007) showed that omission of glucose from MRS broth increased the capacity for biofilm construction in *L. rhamnosus* LGG, whereas the same method yielded the opposite result for *L. casei* Shirota and a very weak biofilm was formed [15].

In another study, among Lactobacillus strains isolated from dairy products, *L. acidophilus* formed weak biofilms on MRS broth after 24h, whereas *L. casei* made average biofilms after 24 h. Among 4 strains of *L. fermentum* on MRS broth under identical conditions, two strains made strong biofilms and the others average biofilms [54]. Tahmourespour and Kermanshahi (2011) examined the effect of *Lactobacillus acidophilus* DSM 20079 as a probiotic strain on the adhesion of selected streptococcal strains on the surfaces. It was shown that because of bacterial interactions and colonization of adhesion sites with probiotic strain before the presence of streptococci, adhesion reduction of streptococci was observed, so using of probiotics can be an effective way on decreasing cariogenic potential of oral streptococci [55].

9.8. Mixed cultures.

Burmolle *et al.* (2006) stated that most biofilms found in nature included various species coalitions gathered together for one purpose and influenced by interactions like cooperation and interaction or antagonism and competition. Nevertheless, there have been few reports of interactions and relationships between the species in biofilms. Burmolle *et al.* (2006) showed that *Shewanella japonica*, *Microbacterium phyllosphaerae*, *Dokdonia donghaensis*, and *Acinetobacter Iwoffii* constructed stronger biofilms when they were together in microplates due to their synergistic interactions compared to their single cultures. They stated that the synergistic interactions caused formation of stronger biofilms compared to the situation in which they exhibited competitive interactions [37]. Aoudia *et al.* (2016) reported that the probiotic power of *L. fermentum* increased when it formed biofilms together with other Lactobacilli. Mixed cultures of probiotics are used to treat urinary tract infections [56].

Different strains of a single species that formed weak to average biofilms singly, and various species of a single genus that made biofilms with various strengths singly, were combined and their biofilms were studied to investigate biofilms of isolates and interactions that probiotic bacteria have with each other.

In some isolates, mixed cultures make stronger biofilms, whereas some probiotic strains lack this property due to antagonistic interactions between them.

10. Conclusions

In this study, two methods were described to compare these methods and can claim that these two methods were generally suitable for extraction exopolysaccharides from lactobacillus. It must be noted that various factors like culture conditions and type and environmental conditions influence biofilm formation and EPS production, and EPS production is not necessarily the reason for biofilm making by these strains. To prove this claim, further research must be carried out. It is suggested that future research should: Study biofilm construction by isolates on cultures enriched with glucose and Proteose Peptone; Optimize biofilm formation with respect to time, temperature, carbon source, and various pH values; Investigate biofilm construction by combining more than two bacterial species and strains; Examine biofilm making in other LABs, especially in native isolates; Study effects of the S-layer on biofilm formation; Separate and purify EPSs with higher efficiencies on cultures enriched with various sugars and on enriched cultures like CDM at different temperatures; Investigate genes involved in EPS production and biofilm construction; Examine antimicrobial properties of EPSs extracted from native isolates and determine the structures of their monosaccharides.

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Conflicts of Interest

The authors declare no conflict of interest.

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