

Antimicrobial bio-film from rosemary



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1. INTRODUCTION

Civilization has always needed to preserve goods. The concepts of better protection and the preservation of goods has led to the development of plastic packaging. The usage of plastic packaging undeniably holds many advantages. It is lightweight, secure, durable, versatile and recyclable. However, there are also disadvantages to the usage of such materials. It is not always recycled, nor is it perfectly safe to use. Plastic plays a major role in environmental pollution and certain plastics may even pose health risks to consumers. Due to these concerns, the scientific world has oriented itself towards finding a suitable replacement for plastic packaging. These replacements are usually organic compounds since organic compounds require a much smaller period for biodegradation. Alternatives such as these could prove to be good replacements of plastics. Plastic, as previously mentioned can also be harmful to consumers as they contain toxins. However, using organic alternatives could solve this problem as well.

Our project aims to create a bio-plastic that accomplishes the goals of being environmentally friendly, sustainable and safe to use for consumers. Our project, more specifically, is aimed at the production of a bio-plastic intended for food storage. Also, we would emphasize the consumer purposes by aiming to give our product antimicrobial properties, which would certainly be a very useful quality in food packaging.



2. THEORETICAL PART

2.1. Starch

The base product of our bio-film (bio-plastic) is starch. Starch has been an inexhaustible subject of research for many decades. It is an inexpensive, readily available material. Based on biodegradability, availability, renewability, non-toxicity, and affordability, starch is one of the most promising of all the potential biopolymer materials. The use of starch in packaging promotes sustainability and addresses the negative impact non-biodegradable plastics pose on the environment. Thus, starch has attracted a great deal of interest as a potential alternative to conventional plastics for packaging applications.

Starch is a *polysaccharide*, a chain of many glucose molecules. It is the main carbohydrate storage in roots and seeds.

There are two types of glucose chains in starch. One is a simple chain called *amylose*, and the other is a complex branched form called *amylopectin*.

In the starch grains in a plant, amylopectin makes up the bulk of the material, between 50 to 80 percent by weight, made up of several million amylopectin molecules per starch grain. The rest is a much larger number of the smaller amylose chains, made up of 500 to 20,000 glucose units in each chain. Amylopectin molecules are made of several million glucose units.

Amylopectin forms branched structures with about 30 glucose units in a chain between branches. This makes the molecule somewhat striped in appearance, with the knotted branch points all in a row, and the smooth chains separating them. These molecules are so large that this striped appearance shows up under a light microscope, forming what appear to be "growth rings" in the starch grain.

Amylose chains tend to curl up into *helices* (spirals) with the hydrophobic parts inside. This allows them to trap oils and fats inside the helix, as well as aroma molecules.

Starch occurs as semi crystalline structures, granules (large particles), composed of amylose and amylopectin.

Because starches are so good at absorbing water and bulking up, they are important in the "mouth feel" of many food products, and are used as fat substitutes.

2.1.2 Gelatinization of Starch

Starch has a very important property which is used to create the bio – film. This property is the ability of starch to gelatinize. It is through this process that bio-plastics from starch can be created.

Starch gelatinization is a process of breaking down the intermolecular bonds of starch molecules in the presence of water and heat, allowing the hydrogen bonding sites (the hydroxyl hydrogen and oxygen) to engage more water. This irreversibly dissolves the starch granule in water. Water acts as a plasticizer.

Three main processes happen to the starch granule: granule swelling, crystallite or double helical melting, and amylose leaching.

- During heating, water is first absorbed in the amorphous space of starch, which leads to a swelling phenomenon.
- Water then enters via amorphous regions the tightly bound areas of double helical structures of amylopectin. At ambient temperatures these crystalline regions do not allow water to enter. Heat causes such regions to become diffuse, the amylose chains begin to dissolve, to separate into an amorphous form and the number and size of crystalline regions decreases. Under the microscope in polarized light starch loses its birefringence and its extinction cross.
- Penetration of water thus increases the randomness in the starch granule structure, and causes swelling; eventually amylose molecules leach into the surrounding water and the granule structure disintegrates.

The gelatinization temperature of starch depends upon plant type and the amount of water present, pH, types and concentration of salt, sugar, fat and protein in the recipe, as well as starch derivatization technology are used. Some types of unmodified native starches start swelling at 55 °C, other types at 85 °C. The gelatinization temperature of modified starch depends on, for example, the degree of cross-linking, acid treatment, or acetylation.

Gel temperature can also be modified by genetic manipulation of starch synthase genes. Gelatinization temperature also depends on the amount of damaged starch granules; these will swell faster. Damaged starch can be produced, for example, during the wheat milling process, or when drying the starch cake in a starch plant. High amylose starches require more energy to break up bonds to gelatinize into starch molecules.

2.1.3 Starch retrogradation

Gelatinized starch, when cooled for a long enough period (hours or days), will thicken (or gel) and rearrange itself again to a more crystalline structure; this process is called retrogradation. During cooling, starch molecules gradually aggregate to form a gel. The following molecular associations can occur: amylose-amylose, amylose-amylopectin, and amylopectin-amylopectin. A mild association amongst chains come together with water still embedded in the molecule network.

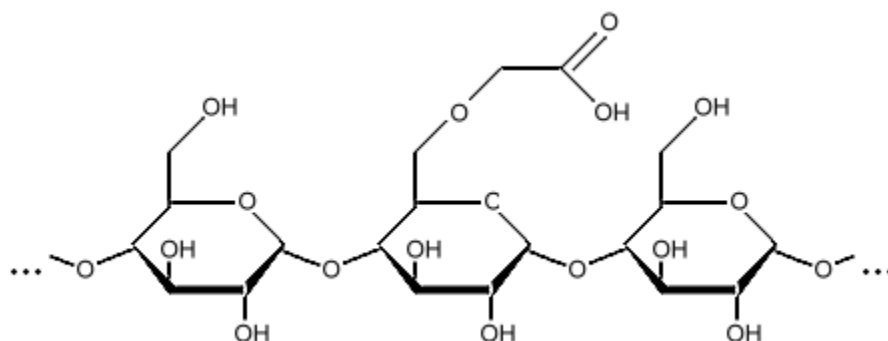
Due to strong associations of hydrogen bonding, longer amylose molecules (and starch which has a higher amylose content) will form a stiff gel. Amylopectin molecules with longer branched structure (which makes them more similar to amylose), increases the tendency to form strong gels. High amylopectin starches will have a stable gel, but will be softer than high amylose gels.

Basically, once gelatinized starch undergoes retrogradation, its structure crystallizes. A stiff gel is formed. This gel is important because it can carry a resemblance to plastic.

Starch modifications

Starches can be modified in several ways to change their function. They can be cross-linked, where the chains get stuck together into a mesh. They can be heated to break the long chains down into simpler molecules like dextrin, polydextrin, and malto-dextrin. These are simply short starches.

Since starch's functional properties change when starch is modified it is important to consider possible modifications which may allow for the production of better bio-plastics.



2.1.4 Carboxymethyl starch

Starches can have a hydrogen replaced by something else, such as a carboxymethyl group, making *carboxymethyl starch*.

Adding the carboxymethyl group makes the starch less prone to damage by heat and bacteria. Carboxymethyl starch is used as an additive in oil drilling mud, and is used in the goo that makes ultrasound examinations messy. Carboxymethyl starch is also called a *starch ether*.

Carboxymethyl groups make the starch more hydrophilic (water loving), and aid in cross-linking. This makes carboxymethyl starch useful in aspirin and other tablets to make them disintegrate quickly.

Longer carbon chains can also be added, such as carboxyethyl groups, or carboxypropyl groups.

Adding bulky functional groups like carboxymethyl and carboxyethyl groups reduces the tendency of the starch to recrystallize. When the starch stays as a gel, the product is softer, and we say it is "fresh". When the starch regains its crystalline form, the product becomes firmer, and we say it is "stale". The technical term for this is *starch retrogradation*.

Starches can be *esterified* by modifications with an acid. An ester is the result of reacting an alcohol with an acid. The starch loses a hydroxyl group, and the acid loses a hydrogen. These combine to form water as the other product of the reaction.

Using acetic acid (CH_3COOH), starch acetates are formed, which are used as film-forming polymers for pharmaceutical products, and as the polymer in biodegradable packing foam "peanuts". Starch acetates have a lower tendency to create gels than unmodified starch.

Acids can also break the long chains into shorter molecules, much like heat does, to form polydextrins, malto-dextrin, or dextrin. Enzymes are also used to do the same thing.

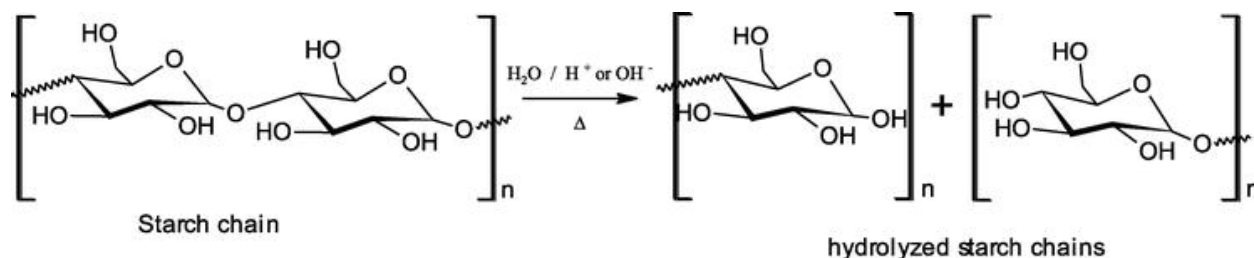
2.2. Reactions of starch

The reactions of starch that allow for such modifications are described in the following:

The reactivity of starch is dependent on the hydroxyl functions of the constituent α -D-glucan polymers. Thus, starch is able to undergo the following reactions.

2.2.1 Hydrolysis

Hydrolysis is an addition reaction and simply involves the addition of a water molecule across a bond resulting in the cleavage of that bond and formation of the cleavage products, usually with hydroxyl group or alcohol functionality. Hydrolysis of starch can be achieved by chemical or enzymatic process. Chemical process of hydrolysis usually employs heating starch in the presence of water or dilute hydrochloric acid. Hydrolysis is also used to remove fatty substances associated with native starches. Hydrolysis under acidic condition is called roasting, resulting in acid modified starch. Treatment of starch with sodium or potassium hydroxide results in alkaline modified starch. Hot aqueous alkaline solutions can be used, and this improves the reducing value of that starch.



Hydrolysis of α -(1 \rightarrow 4) glycosidic bond.

The products of starch hydrolysis include dextrin or maltodextrin, maltose and glucose. Dextrins are mixtures of polymers of d-glucose units linked by α -(1 \rightarrow 4) or α -(1 \rightarrow 6) glycosidic bonds. The percentage of products obtained depends on the conditions used for the reaction such as duration and strength/amount of reagents used. Enzymic hydrolysis uses the enzyme malto-amylase to achieve hydrolysis and this is the process that usually occurs in starch digestion in the gastrointestinal tract. Dextrins are white, yellow, or brown water-soluble powder which yield optically active solutions of low viscosity. Most of them can be detected with iodine solution, giving a red coloration. White and yellow dextrins from starch roasted with little or no acid are called British gum. The properties of dextrinized starch is dependent upon the reaction conditions

(moisture, temperature, pH, reaction time) and the products characteristics vary in its content of reducing sugar, cold water solubility, viscosity, color and stability.

Hydrolytic processes have been used in the food industry to produce starch derivatives with better functional properties and processing applications. Acid and alkali steeping are the two most widely used methods for starch isolation in the food industry, with numerous modifications.

2.2.2 Esterification reaction

The condensation of an alcohol and carboxylic acid usually under acidic condition, to produce an ester and water, is called esterification. Basically, the reaction is between the carboxylic acid group and the alcohol group with the elimination of a water molecule. When the acid anhydride is used, an alkaline condition is preferred in the reaction.



Esterification reaction of carboxylic acids and alcohols.

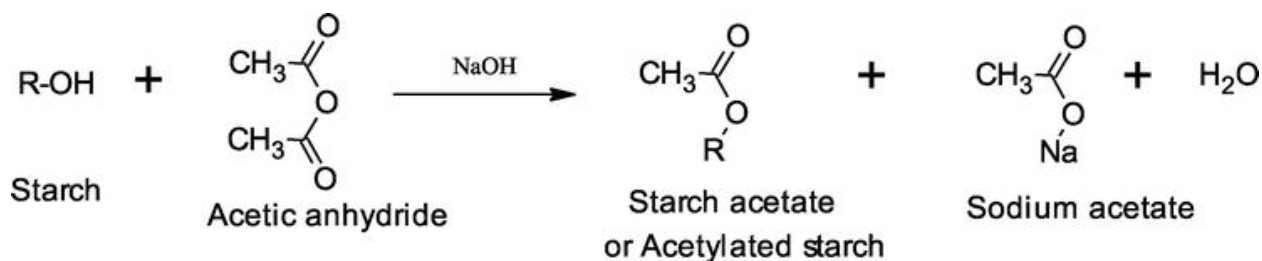
The reaction is usually reversible and the forward reaction is favored under low pH and excess of alcohol while the reverse is favored under high pH. Removal of one of the products during the reaction will also favor the forward reaction.

For starch, the reaction is between the carboxylic acid group (—COOH) of fatty acids or —COCl of fatty acid chlorides and the alcohol group (—OH) of the glucose units. Esterification is generally used to introduce more lipophilic groups into the starch molecule making it more lipophilic and for producing crosslink starch when polyfunctional compounds or multifunctional reagents capable of esterification or etherification are used. Esterification weakens the inter-molecular bonding that holds the granules together and hence alter the granule shape and sizes as well as other functional properties of the starch. The degree of substitution (DS) is dependent on the concentration of reagent used, the type of reagent used, the catalyst and the duration of reaction.

2.2.3 Acetylation of starch

Starch, as previously mentioned can be esterified with a carboxylic acid such as acetic acid. We use this method of esterification (with acetic acid) to modify starch and in turn, our final end product, the bio-plastic. This reaction is called acetylation.

Starch can be acetylated by reacting it with acetic anhydride or acetic acid to produce acetylated starch. The DS of the hydroxyl group with acetate group is dependent on the reaction conditions. Acetylated corn starch of DS 0.05, 0.07 and 0.08 have been obtained using 4, 6 and 8% (starch d.w.) acetic anhydride respectively and aqueous sodium hydroxide as catalyst.



Acetylation of starch with acetic anhydride

The introduction of the bulkier acetyl group compares with hydroxyl group causes steric hindrance to the alignment of the linear chains. This allows for easy water percolation between chains thus increasing the granule swelling power and solubility resulting in lower gelatinization temperature. The steric hindrance of less polar acetyl group also reduces the amount of inter-molecular hydrogen bond formation, and weakens the granule structure, preventing molecular re-association and realignment required for retrogradation. However, depending on the DS and the interplay between the weakened granular structure as result of interruption of the inter- and intra-molecular bonds, and reduced bonding with water molecules as a result of the hydrophobicity of the acetyl groups, the viscosity of the final product can be enhanced.

Acetylation improves paste clarity and freeze-thaw stability of starch. Starch acetates of low DS are commonly used in the food industry for quality consistency, and as texture and stability enhancers. The Food and Drug Administration (FDA) maximum DS of acetylated starches for food application is 0.1. Starch acetate of high DS exhibit high degree of hydrophobicity and thermoplasticity and are soluble in organic solvents like chloroform and acetone, and are mostly used in non-food applications. At 0.0275 DS, corn starch exhibit lower paste gelling, which is practically lost at 0.05 DS. Most commercial starch acetates have <0.05 DS.

2.3 Plasticizers

Plasticizers are additives that decrease the plasticity or decrease the viscosity of a material. These are the substances which are added in order to alter physical properties of materials. These are either liquids with low volatility or solids. They decrease the attraction between polymer chains to make them more flexible.

Despite the numerous advantages of starch, it does not possess thermoplastic properties and cannot be used directly as packaging material. In addition, its end products have poor dimensional stability and mechanical properties. Native starch-based materials are reported in many investigations to be very brittle with many surface cracks and are difficult to handle. However, these drawbacks can be resolved by the addition of plasticizers to pure starch to improve its workability and suppress film brittleness. The ultimate role of plasticizers is to enhance the flexibility and processability of starch by reducing the strong intermolecular interactions between starch molecules. As a result, the mobility of polymeric chains increases, which improves the flexibility, extensibility and ductility of plasticized films. On the contrary, the addition of plasticizers decreases film mechanical resistance. The type and concentration of plasticizer employed has significant effect on the physical, thermal, mechanical and barrier properties of films. The most common plasticizer utilized for starch-based films are polyols such as glycerol and sorbitol, amongst many others.

In the production of our bio-films we use both sorbitol and glycerol as plasticizers to increase the flexibility of our products.

2.3.1 Sorbitol

Sorbitol is a sugar alcohol with a sweet taste which the human body metabolizes slowly. It can be obtained by reduction of glucose, which changes the converted aldehyde group ($-CHO$) to a primary alcohol group ($-C(OH)H_2$). Most sorbitol is made from potato starch, but it is also found in nature, for example in apples, pears, peaches, and prunes. It is converted to fructose by sorbitol-6-phosphate 2-dehydrogenase. Sorbitol is an isomer of mannitol, another sugar alcohol; the two differ only in the orientation of the hydroxyl group on carbon 2. While similar, the two sugar alcohols have very different sources in nature, melting points, and uses.

2.3.2 Glycerol

Glycerol is a simple polyol compound (an organic compound containing multiple hydroxyl groups). It is a colorless, odorless, viscous liquid that is sweet-tasting and non-toxic. The glycerol backbone is found in lipids known as glycerides. Due to having antimicrobial and antiviral properties it is widely used in FDA approved wound and burn treatments. It can also be used as an effective marker to measure liver disease. It is also widely used as a sweetener in the food industry and as a humectant in pharmaceutical formulations. Owing to the presence of three hydroxyl groups, glycerol is miscible with water and is hygroscopic in nature.

Both of these compounds act as plasticizers, weakening the intra-molecular bonds of starch granules. They allow for an increase in flexibility of the bio-plastic that would otherwise be too brittle to use for packaging.

2.4 Rosemary

An integral part of our product is the addition of rosemary.

Rosemary is an aromatic evergreen shrub with leaves similar to hemlock needles. It is native to the Mediterranean and Asia, but is reasonably hardy in cool climates. It can withstand droughts, surviving a severe lack of water for lengthy periods. In some parts of the world, it is considered a potentially invasive species. The seeds are often difficult to start, with a low germination rate and relatively slow growth, but the plant can live as long as 30 years.

The plant flowers in spring and summer in temperate climates, but the plants can be in constant bloom in warm climates; flowers are white, pink, purple or deep blue. Rosemary also has a tendency to flower outside its normal flowering season; it has been known to flower as late as early December, and as early as mid-February (in the northern hemisphere).

Rosemary contains a number of phytochemicals, including rosmarinic acid, camphor, caffeic acid, ursolic acid, betulinic acid, carnosic acid, and carnosol. Rosemary essential oil contains 10–20% camphor.

2.4.1 Antimicrobial Properties of Rosemary

Natural extracts of the *Lamiaceae* family, such as rosemary, have been studied because of its bioactive properties. Several studies have reported that rosemary extracts show biological bioactivities such as hepatoprotective, antifungal, insecticide, antioxidant and antibacterial.

The inhibitory effect of rosemary is the result of the action of rosmarinic acid, rosmaridiphenol, carnosol, epirosmanol, carnosic acid, rosmanol and isorosmanol. They interact with the cell membrane, causing changes in genetic material and nutrients, altering the transport of electrons, leakage of cellular components and production changes in fatty acid. In addition, it also produced an interaction with the membrane of proteins that produced the loss of membrane functionality and its structure.

Carnosic acid is a phenolic diterpene, (diterpenes are a class of chemical compounds composed of two terpene units, often with the molecular formula $C_{20}H_{32}$) endowed with antioxidative and antimicrobial properties. It is increasingly used within food, nutritional health and cosmetics industries. Carnosic acid is used as a preservative or antioxidant in food and nonfood products (e.g. toothpaste, mouthwash and chewing gum -in which it has an antimicrobial effect on the microbes responsible for bad breath- or skin care products).

Carnosol is a phenolic diterpene found in the herbs rosemary (*Rosmarinus officinalis*) and Mountain desert sage (*Salvia pachyphylla*). It has been studied in-vitro for anti-cancer effects in various cancer cell types.

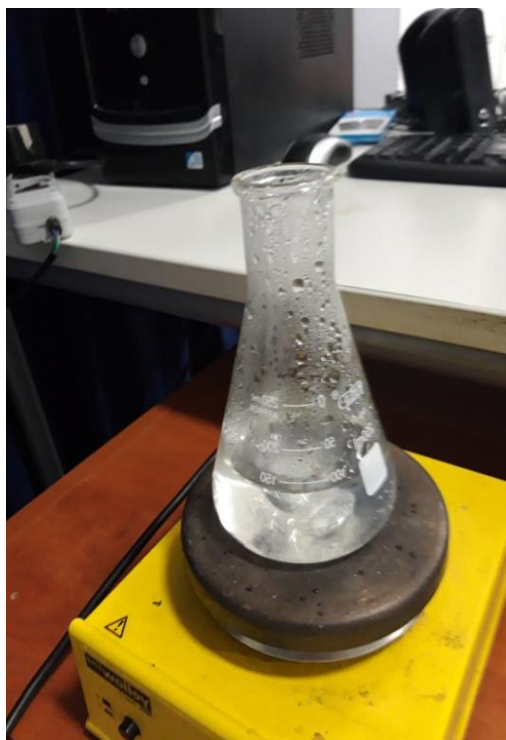
Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid commonly found in plants belonging to the Boraginaceae and the subfamily Nepetoideae of the Lamiaceae family. The compound has a number of important biological activities, e.g. antiviral, antibacterial, antiinflammatory, anticancer and antioxidant.

The antimicrobial herb rosemary, with all of its constituents is added to the bio-plastic to give it antimicrobial properties as well.

3. PROCEDURE FOR CREATING THE BIO-FILMS

The process of the creation of the bio-films will be elaborated in the following.

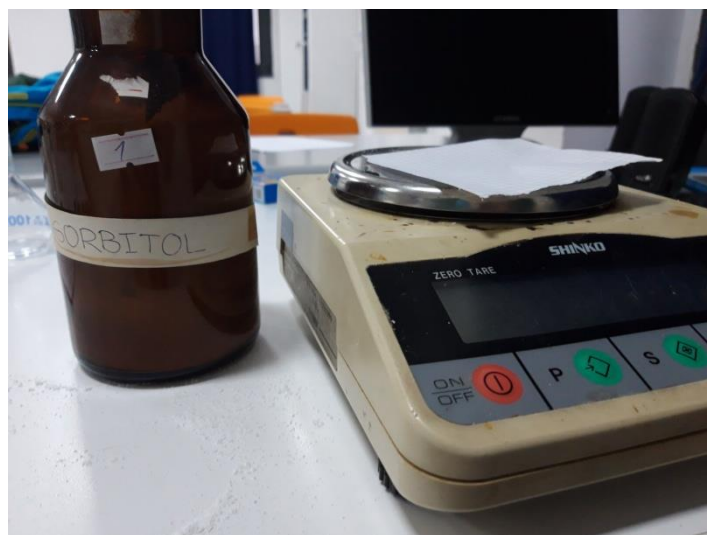
Firstly, starch is placed in an aqueous solution above a magnetic stirrer. The starch solution is then stirred with the help of a magnetic stir bar. Afterward, this solution is placed in a beaker atop a butane gas Bunsen burner. Additional water is then also added. Minced rosemary leaves along with acetic acid are then added and the entire mixture is heated. Sodium hydroxide is then added to the mixture as well to neutralize the solution. When this mixture reaches a high enough temperature, as a final ingredient sorbitol is added acting as a plasticizer. Once the mixture becomes viscous it is taken out of its container and placed on a surface where it proceeds to cool and harden, leading to the desired bio-film. Since acetylation modifies starch, biofilms with different concentrations of acetic acid were produced to determine the optimal concentration of acetic acid needed to achieve the best results. In half the cases, glycerol was used as a replacement for sorbitol. We also produced a separate bio-film with sorbitol by adding more water. Adding more water can cause functional changes to the final product.



4. MATERIALS

The materials used for the creation of a single bio-film are listed below:

- Minced rosemary leaves 0,5g
- Starch 20g
- Water
- Sodium hydroxide (NaOH) 4ml
- Sorbitol 5ml
- Glycerol 5ml
- Acetic acid (CH_3COOH) 10ml
- Beaker
- Butane gas Bunsen burner



5. METHODS

20g of starch are placed in a 90ml aqueous solution above a magnetic stirrer. The solution is then stirred with the help of a magnetic stir bar. The starch solution is then placed in a beaker seated atop a butane gas Bunsen burner. 30 ml of water along with 0,5g of minced rosemary leaves and 10 ml of acetic acid (CH_3COOH) are added to the beaker. The Bunsen burner is turned on heating the contents in the beaker. 4ml of sodium hydroxide (NaOH) is then also added to the mixture as well as 5ml of sorbitol when the temperature gets higher. The mixture is stirred until it becomes viscous. This should occur when the temperature is between 55°C and 85°C . When this happens, the mixture is extracted from the beaker and laid out to cool and harden at room temperature. A total of six biofilms were created using different concentrations of acetic acid and different plasticizers, while the rest of the ingredients remained the same. In three cases sorbitol was replaced with glycerol. The first bio – film was created using 99% concentrated acetic acid, while the second and third with 90% and 80% acetic acid, respectively. The additional three biofilms were created similarly; every one of these bio-films used the same amount of ingredients as the previous bio-films however, in these cases the sorbitol was replaced with glycerol. A separate bio film was also created by adding 60 ml of water in the beaker instead of 30ml.



6. RESULTS

A) The three films we obtained using glycerol show the expected and wanted physical and chemical properties. The properties are listed below:

Acetic acid 99% Bio - Film: This bio - film shows total flexibility, similar to a plastic bag. Although it is flexible, it is resistant and can withstand a large pressure and force. Mass: 8,06g

Acetic acid 90% Bio – Film: This bio - film is also extremely flexible, resembling plastic. Its texture is softer than the one from plastic, but it is tough and can endure a fair amount of force and pressure. Mass: 5,97g

Acetic acid 80% Bio – Film: Even with a lower concentration of acetic acids it still displays useful qualities such as the previous ones. It is still flexible, although slightly less than the other 2 films produced with glycerol. Mass: 8,14g

B) The properties of the three films obtained with sorbitol are listed below:

Acetic acid 99% Bio - Film: The first film with the highest concentration of acetic acid is tough but less flexible than the ones with glycerol. Mass: 1,46g

Acetic acid 90% Bio – Film: This film shows a bit smaller flexibility than the ones with glycerol. It is tough and can withstand a fair amount of pressure. This film mimics some harder plastic types. Mass: 5,57g

Acetic acid 80% Bio – Film: This film is similar to the ones made with glycerol, it is very flexible and durable. Mass: 2,96g

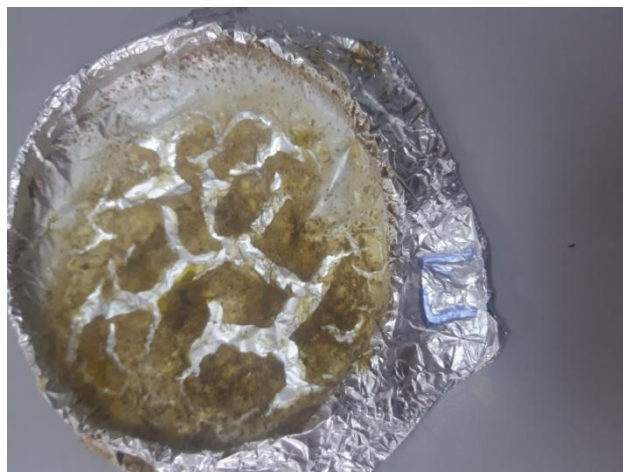
The additional bio – film created with sorbitol presented very interesting qualities. It shows smaller flexibility than the rest of the films, but it is strong and can withstand a good amount of force. It is thicker and mimics some harder plastic types. Mass: 7,24g

The bio-films before cooling



The bio-films after cooling

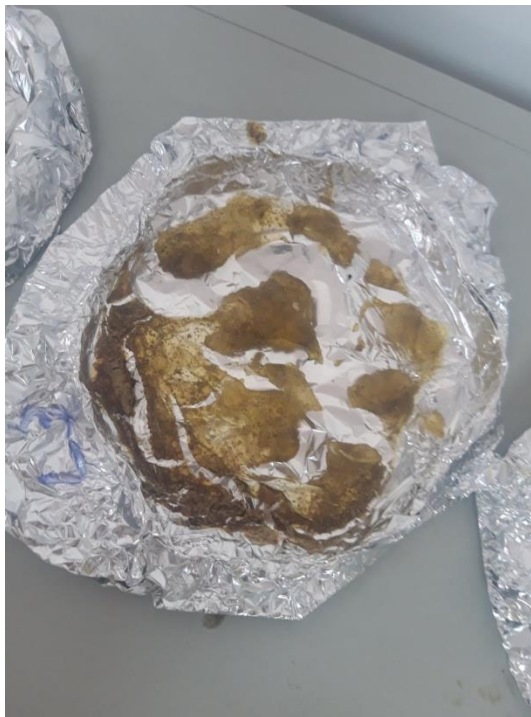
The bio-film for which we used glycerol with 99% concentrated acetic acid



The bio-film for which we used glycerol with 90% concentrated acetic acid



The bio-film for which we used glycerol with 80% concentrated acetic acid



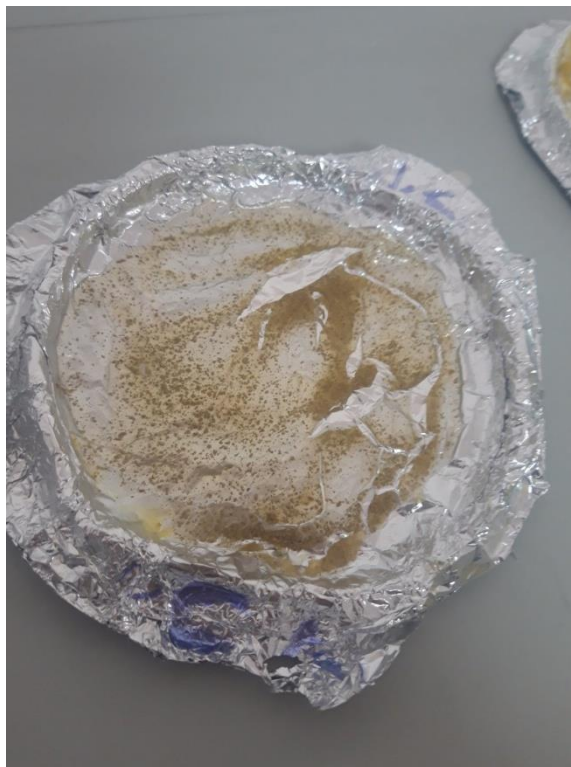
The bio-film for which we used sorbitol with 99% concentrated acetic acid

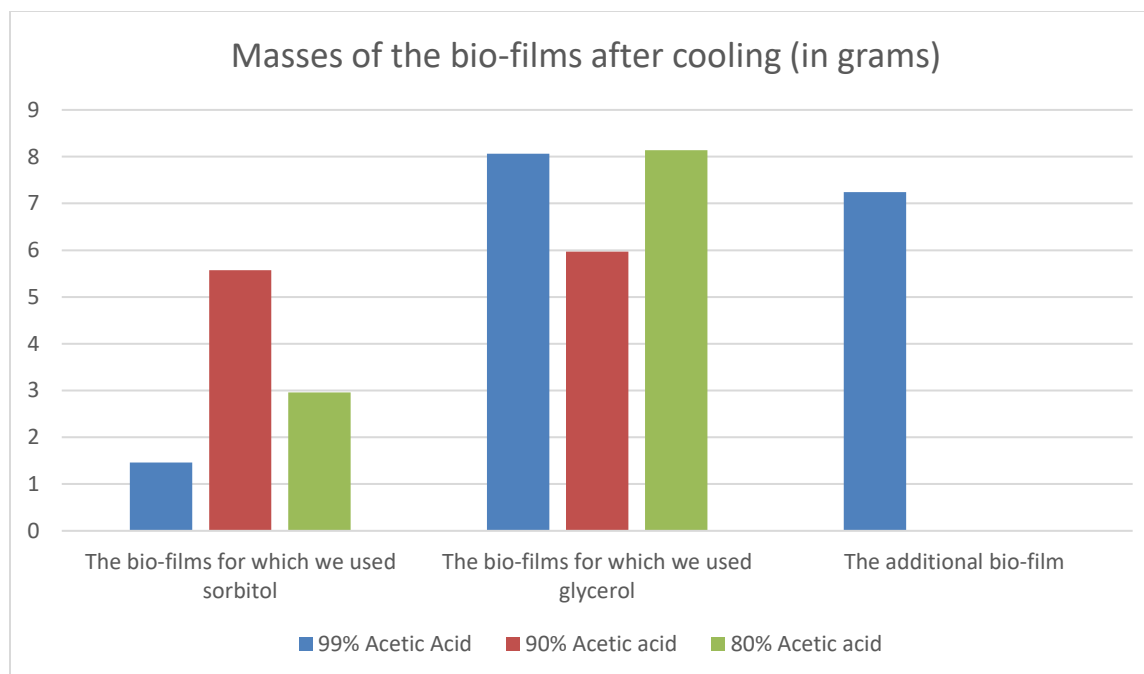


The bio-film for which we used sorbitol with 90% concentrated acetic acid



The bio-film for which we used sorbitol with 80% concentrated acetic acid





6.1. – Graph showing the masses of the bio-films after cooling

7. DISCUSSION

We concluded that the higher concentration of acetic acid makes the glycerol films more flexible, with great texture. This is convenient for both the production of fragile and delicate things, as well as larger everyday products.

The trend observed with the films using sorbitol, is the opposite of the one seen using glycerol. Namely, in this case, the films produced with smaller concentrations of acetic acid are more flexible.

The films made with glycerol are generally more flexible, while the ones made with sorbitol are firmer, and exhibit more similarities to common plastics.

The additional film with sorbitol presents a certain resilience that mimics some harder plastic types. It is rigid and therefore it could show promise in the field of packaging.





It is also important to reiterate, that all the above-mentioned bio-films, contain antimicrobial properties, due to the addition of the herb rosemary.

From our procedures, we have successfully obtained the desired results. We were able to produce biodegradable plastics with inherent antimicrobial properties, using starch and rosemary.

8. CONCLUSION

Through our experiments we have shown that a bio-plastic with antimicrobial properties, can be created successfully. The use of bio-plastics in consumer goods is essential. The polymers currently used in the food industry are made from non-renewable synthetic materials, which, despite having excellent functional properties, are causing serious environmental problems due to the generation of high amounts of non-degradable solid waste in the environment. Successfully implementing widespread commercial use of bio-plastic, will play a fundamental role in controlling the interactions between food and the environment while protecting and maintaining product quality, beyond its basic function.

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