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Fragrant starch-based films with limonene

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Novel fragrant starch-based films with limonene were successfully prepared. Biodegradable materials of natural origin were used and the process was relatively simple and inexpensive. The effect of limonene on physicochemical properties of starch-based films (moisture absorption, solubility in water, wettability, mechanical properties) were compared to glycerol plasticized system. Taking into consideration that the obtained materials could also exhibit bactericidal and fungicidal properties, the studies with *Escherichia coli*, *Candida albicans* and *Aspergillus niger* were performed. Such a material could potentially find application in food packaging (e.g. masking unpleasant odors, hydrophilic starch film would prevent food drying), or in agriculture (e.g. for seed encapsulated tapes).

1. Introduction

Starch belongs to a group of carbohydrates. It consists of two polymers: linear amylose (forming a colloidal solution in water) and branched amylopectin (insoluble in water). The ratio of the two polymers changes depending on the starch origin. Native starch is insoluble in cold water and unstable at higher temperatures. In hot water starch gelatinizes and after cooling forms gel – a result of inter- and intramolecular interactions of amylose and amylopectin molecules. Starch is mostly used in food industry, but also in pharmaceutical, cosmetic and paper industries, as: matrix, binder or filler.[1,2]

In the last decades polysaccharides have been studied as prospective replacers of synthetic polymers in plastic industry. The benefits of applying biopolymer-based films are environmental protection, as well as low cost and availability of the raw material. The films based on polysaccharides could be potentially used for food coating, e.g. fruit, where the film coating acts as a protective barrier for microorganisms and against drying.[3,4]

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A significant advantage of starch-based films is their biodegradability. They are tasteless and odorless.[4] Used for food packaging (approved for food contact)[5] help to reduce humidity of the products as well as keep the flavor. An increase of relative humidity affects the mechanical strength of polysaccharide films, resulting in elongation at break increase.[6,7]

Cyclic terpene hydrocarbons represent attractive substances for broad applications in organic and polymer industry. Nowadays, a special attention is directed to R-(+)-limonene. It is a colorless oil which is sparingly soluble in water and characterized by a sweet orange smell. Limonene is widely used in food and cosmetic industries, and also in production of refrigerant fluids, paints, agrochemicals and cleaning agents.[8-13] R-(+)-limonene is the major component (about 97%) of the orange oil obtained from orange and lemon peels (biomass) which are the waste product of the citrus fruit industry. The main methods of obtaining orange oil from the orange peels are: cold pressing and distillation. Limonene (obtained from the waste orange peels ca.70 000 tons per year) is relatively cheap and easily available raw material for organic syntheses (e.g. for 1,2-epoxylimonene, carvone, carveol, perillyl alcohol and menthol preparation) and for polymers production, especially fragrant ones.[14-21]

The release behavior of limonene from edible films were tested, the polysaccharide matrix used was: chitosan,[22-23] iota-carageenan,[24] methyl cellulose,[25] sodium alginate and pectin.[26]

Until now, there are few reports on the synthesis of starch-based fragrant films. The utilization of terpenes in obtaining starch-based fragrant films but with help of alpha-pinene and eugenol was described in our previous work.[27] In comparison to the limonene, which is obtained from renewable industrial waste - orange peels, alpha-pinene and eugenol are not obtained from industrial waste but from renewable plant material. The studies presented that the obtained starch-based films were characterized by the relatively long time of release the studied fragrant compounds and also by the good absorbance of moisture from air. We did not test in this work antimicrobial properties starch based films with alpha-pinene and eugenol, but taking into account the antimicrobial properties alpha-pinene and eugenol they also can be characterized by antimicrobial properties. The application of limonene obtained from cheap industrial waste (orange peels) seemed to be more promising taking into account price of natural limonene in comparison to alpha-pinene and eugenol. Also from the environmentally point of view it is more beneficial. Thus, the aim of this work was preparing novel fragrant starch-based films with limonene using cast method. The effect of limonene addition on physicochemical properties of obtained films were determined (mechanical properties, water absorption, solubility in water, and wettability). Moreover, bactericidal and fungicidal properties were characterized using *Escherichia coli*, *Candida albicans* and *Aspergillus niger.* Fragrant starch-based films could potentially find application where masking unpleasant odors or providing antibacterial properties are beneficial, i.e. in packaging industry or in agriculture for seeds encapsulation, respectively. Such a type of starch films has not been reported in the literature yet.

2. Results and Discussion

The results of tensile strength test for obtained polysaccharide films were collected in Table 1. Glycerol (G) is a common starch plasticizer interacting with polysaccharide hydroxyl groups and as a consequence increasing flexibility of the final material.[2] Starch-based film plasticized with G (named SG) exhibited the highest elongation at break and the lowest tensile strength (ca. 112% and 1.1 MPa, respectively). The highest value of tensile strength was noted for starch-based system containing limonene (named SL) (ca. 38.2 MPa), however, this film exhibited significant brittleness (low elongation at break and high Young modulus: 3% and 2724 MPa, respectively). In the next step, glycerol plasticized starch-based film containing limonene (named SGL) has been prepared. As a consequence, elongation at break decreased to 69%, while tensile strength increased up to 1.7 MPa, when compared to SG system.

Table 1. Elongation at break, Young's modulus and tensile strength of the starch-based films

Sample	Elongation at break, %	Young's modulus, MPa	Tensile strength, MPa
SG	112 ± 27	34 ± 50	1.08 ± 0.5
SL	3 ± 1	2724 ± 334	38.25 ± 4.5
SGL	69 ± 23	44 ± 7	1.69 ± 0.2

In Table 2 the effect of limonene presence on the solubility in water, wettability and the diffusion coefficient of starch-based films were presented. By the contact angle measurements the attraction between water molecules and the molecules on polymer surface could be evaluated. Surprisingly, the contact angle of starch-based film with limonene was significantly lower than those noted for the other systems containing glycerol (for SG and SGL similar values was determined), i.e. 96°, and ca. 101°, respectively (Table 2). Adding a plasticizer into polysaccharide matrix results in higher movability of the macromolecular chains and increase in intermolecular spacing, moreover some part of glycerol moves into the surface, what could explain the similar values of contact angles for the samples containing GL, i.e. SG and SGL.[24] In a case of SL system without the plasticizer, more intense phase separation could occur, and the lipid phase could migrate into the surface.[24] Thus, lower contact angle value could be a result of low limonene solubility in water.

Table 2. Diffusion coefficients, contact angles and solubility in water for the starch-based films

Sample	Diffusion coefficient D_{eff} x 10^5, mm^2/s	Solubility in water, %	Contact angle, °
SG	4.11	28	102
SL	9.20	2	96
SGL	1.49	32	101

During solubility tests all the samples maintained their integrity, i.e. did not dissolve or break apart. The presence of hydrophobic limonene resulted in significantly reduced solubility in water, when compared to SG system, 2% and 28%, respectively. However, similar solubility values for SGL and SG could be the result of plasticizer migration.

An effective diffusion coefficient (D_{eff}) was calculated to determine water absorption rate. It includes the factors affecting the diffusion of water, such as: (i) material inhomogeneity, (ii) extractable materials that can be removed during water uptake and (iii) dimension of the specimens may vary during the time of the experiment[22]. The absorption rate of the starch-based film plasticized with glycerol and containing limonene was noticeably lower than for SG one, ca. 1.5 and ca. 4.1 mm^2/s, respectively). Thus, the addition of limonene diminished the rate of water uptake. However, for SL system significantly higher value of this parameter was noted (9.2 mm^2/s). As SL film did not contained glycerol, it exhibited significant brittleness that could be attributed to the mechanical damages during diffusion coefficient determination process, resulting in enhanced rate of water absorption. These data strongly correlate with the mechanical tests results.

The moisture absorption of prepared starch-based films in time was presented in Figure 1. The lowest value of this parameter was noted for starch-based system with limonene, i.e. 10% after 72 h. Generally, limonene addition resulted in lowering moisture absorption from ca. 15% to ca. 13% after 72 h, for SG and SGL system, respectively. It could be concluded that the presence of hydrophobic limonene in polysaccharide system effectively reduced hydrophilic character of polysaccharide film beneficially limiting its sensitivity to moisture.

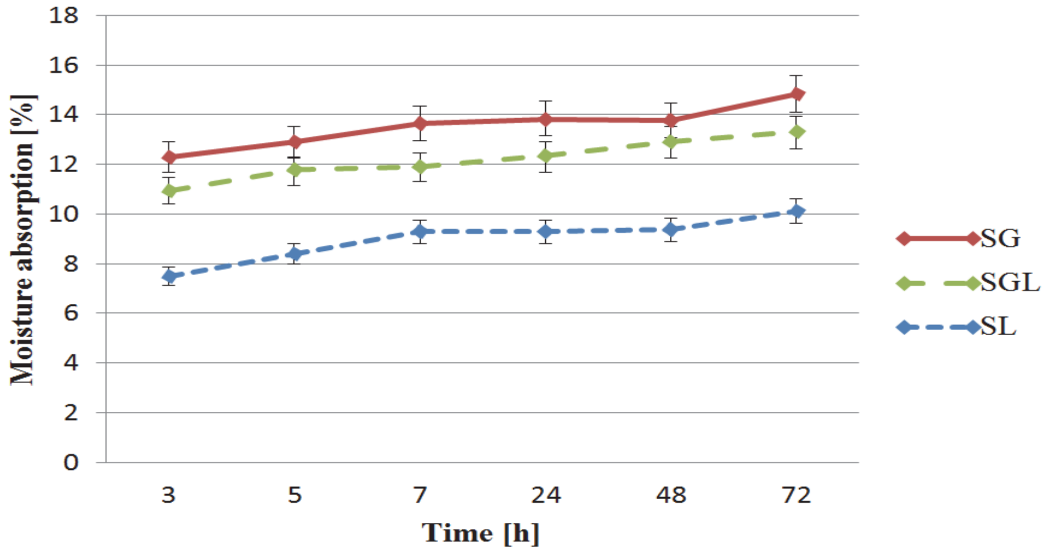

Fig. 1. Moisture absorption of starch-based films

The microbiological tests results were presented in Table 3. The inhibition zones were observed only for pure limonene placed on the Petri dishes with bacteria and yeast culture (Fig. 2A, B). This confirms its antibacterial and antimycotic properties,[22] however the mechanism of bacterial inactivation by limonene is not well known.[28] The lethality of this compound depended on many factors such as pH, heat treatment or type of bacteria strain. Fungi exhibited resistance for pure limonene (Fig. 2C).

Table 3. Hydrolysis zone in biological tests of starch-based films

Sample	**Method preparation (sample discs)**	**Inhibition zone, mm**		
		Bacteria	***Yeast***	***Mode Fungi***
SL	UV	-	-	-
SGL	UV	-	-	-
SL	water	-	-	-
SGL	water	-	-	-
SL	70% ethyl alcohol	-	-	-
SGL	70% ethyl alcohol	-	-	-
Limonene	-	30 ± 0.5	10.3 ± 0.5	-

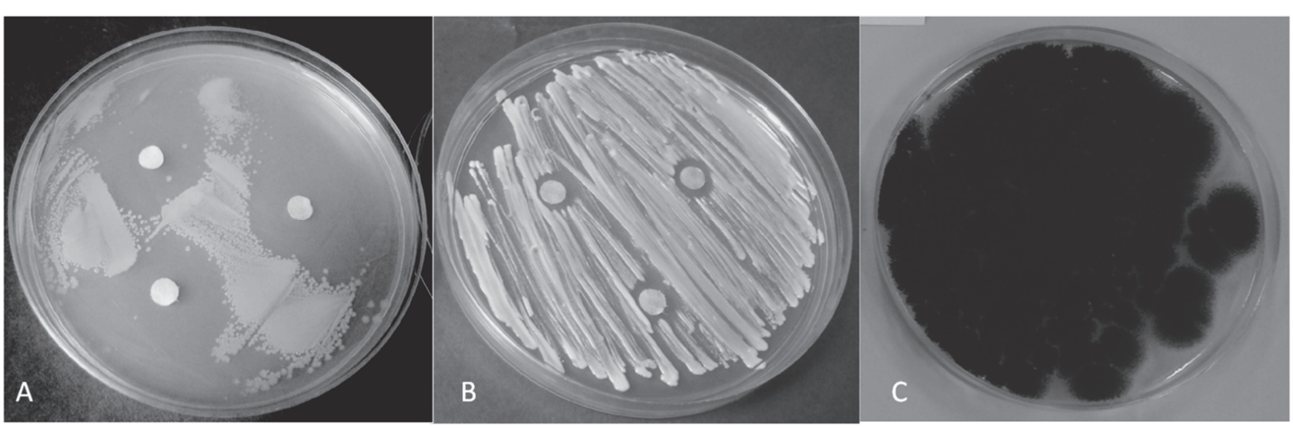

Fig. 2. Limonene inhibition zone for A) *E. Coli, B) C. albicans*, *and C) A. niger*

The inhibition zones were not observed around the SL films discs (Fig. 3). As mentioned above, limonene was entrapped inside the polysaccharide matrix, however some part of the lipid phase could migrate into the surface, thus limonene loss (sterilization) could be attributed to the lack of inhibition zone. That strongly correlated with the contact angle tests results. It is worth to mention that when starch films containing limonene was bent or torn apart an intensive limonene smell could be detected what additionally confirmed this assumption. Moreover, the lack of inhibition zones observed for SL films treated with bacteria and yeast suggested their inertness towards starch.

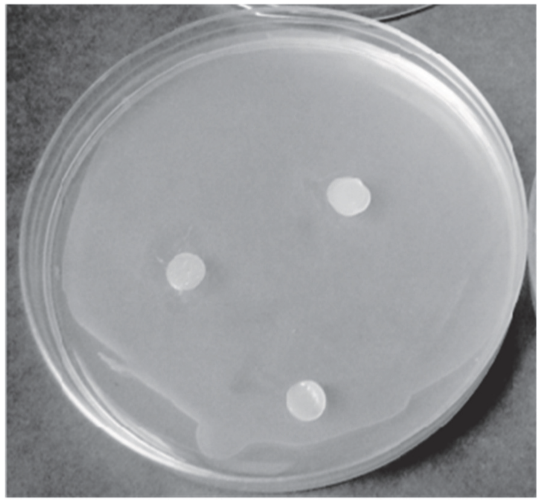

Fig. 3. The exemplary microbiological test result for SL film (*E. coli*)

3. Conclusions

Novel fragrant starch-based films with limonene were successfully prepared. Biodegradable materials of natural origin were used and the process was relatively simple and inexpensive. The presence of limonene considerably affected physicochemical properties of starch-based films. The moisture absorption as well as solubility in water were noticeably reduced (13% and 2%, respectively), when compared to common plasticizer, i.e. glycerol, modified system only (15% and 28%, respectively). Moreover, the tensile strength was significantly improved (ca. 38 MPa, and ca 1.1 MPa for SG, respectively), however because of brittleness - using plasticized addition would be inevitable.

Limonene exhibited antibacterial and antimycotic properties, and entrapped inside polysaccharide matrix released gradually during film handling. Such a material could potentially find application in food packaging area, where polysaccharide film could form an internal layer of a packaging (e.g. masking unpleasant odors, hydrophilic starch film would prevent food drying), or in agriculture (e.g. for seed encapsulated tapes, where limonene released during polysaccharide matrix degradation would protect the seeds against microbial attack).

The comparison the results obtained for starch-based film with limonene with the results obtained for starch-based films with alpha-pinene and eugenol shows that the these results are very similar taking into account physicochemical properties of starch-based films (moisture absorption, solubility in water, wettability, and mechanical properties). Taking into account prices of limonene, alpha-pinene and eugenol, the obtaining of starch-based films with limonene is more beneficial. Also from the environmentally point of view it is beneficial, because for limonene obtaining are used renewable waste orange peels. Only the downside of starch-based films with limonene can be its allergic effect, but it can be solve by using an appropriate concentration of limonene in film, below the harmful level.

4. Experimental

4.1. Materials

For the preparation of the starch/limonene films: potato starch (S) (analytical grade, Nowamyl S.A. Poland), glycerol (G) (pure, Chempur, Poland), and limonene (L), (97%, Aldrich, USA) were used. For the biological tests the following culture medium were used: Agar Sabouraud (AS, pH 5.6±0.2 BTL, Łódź, Poland), Agar Plate Count Agar (PCA, pH 7.0±0.2 BTL Łódź, Poland), and Agar Melt Ekstrakt Agar (MEA, pH 6.0±0.2 MERCK, Germany).

4.2. Preparation of starch-based films

In the 250 ml glass reactor 5 g starch, 100 ml distilled water, 2 g glycerol and/or 2 g limonene were placed. The obtained mixture was stirred at 90°C for starch gelatinization (ca. 30 min). Subsequently, it was poured into Petri dish and dried for 48 hours at 60°C. The obtained films were peeled off and used for further tests. The detailed compositions of prepared starch-based films were presented in Table 4.

Table 4. Compositions of prepared starch-based films

Sample	Starch, g	Limonene, g	Glycerol, g
SG	5	0	2
SL	5	2	0
SGL	5	2	2

4.3. Methods

The mechanical properties of starch-based films were tested using the tensile testing machine (Instron 4026, Instron Corporation). The initial grip separation and cross-head speed were 50 mm and 10 mm/min, respectively. The mechanical tensile data were averaged over ten specimens.

To determine moisture absorption for each film three samples (1.5 cm x 1.5 cm) were prepared and placed for two weeks in a desiccator for drying. Dry samples were weighted and subsequently transferred to climatic chamber (55 ± 2% RH, 25 ± 2°C). The weight of tested samples was controlled for 3, 5, 7, 24, 48 and 72 hours after placing the sample in the climate chamber. Moisture absorption was calculated using the following equation:[3]

$$A_t = \frac{M_t - M_0}{M_0} \cdot 100\%$$

where: A_t – moisture absorption after time t [%], M_0 – mass of the dry sample [g], M_t – mass of sample after time t: 3, 5, 7, 24, 48 and 72 h [g],

Solubility in water was evaluated for each film. Three squares (1.5 cm x 1.5 cm) were cut and placed for two weeks in the desiccator with silica gel for drying. Dry samples were weighted and transferred to the test tubes filed with 50 ml of distilled water. After 24 hours samples were placed back in the dryer for 24 hours (70 °C). Dry samples were weighted before and after 24 hours immersion in water. Solubility was calculated using the following equation[3]:

$$TSM = \frac{M_1 - M_2}{M_1} \cdot 100\%$$

where: TSM - Total Soluble Mater (in water) [%], M_1 – mass of the dry sample [g], M_2 – mass of sample after drying [g]. To study the effect of limonene on the film wettability the Drop Shape Analyzer (Kruss DSA100) was used. One drop of redistilled water (3 ml) was placed on the starch-based film surface and the contact angle was measured using DSA4 software. The initial contact angles (obtained immediately after deposition) in five replications were measured.[19]

To determine the water absorption rate, the effective diffusion coefficient (D_{eff}) at short times was calculated from the slope in the initial linear portion of the $(w_t-w_0)/w_\infty$ vs $t^{0.5}$ following equation:[19]

$$\frac{w_t - w_0}{w_\infty} = \frac{4}{h}\left(\frac{D_{eff}}{\pi}\right)^{0.5} t^{0.5}$$

where:

w_t is the wet weight of films at each time [g], w_0 is the initial weight of dry film [g], w_∞ is the weight of the films when the maximum equilibrium water uptake was reached [g], and *h* is the thickness of the specimen [mm].

Antimicrobial activity of fragrant-starch based films was tested against organisms: *Aspergillus niger* (ZUT collection A2), *Escherichia coli* (strain K12, ACCT 25922) and *Candida albicans* (ZUT collection CaC3). The antibacterial activity of starch-based films with limonene (in a shape of discs, 5 mm diameter) were carried out by disc diffusion test. Before the experiments, microorganisms were pre-cultured at 37 °C (*E. coli, C. albicans*) or 25 °C (*A. niger*), respectively. Test cultures were placed on sterile media: PCA – *E. coli,* AS – *C. albicans,* MEA – *A. niger.* The inocula of microorganisms were adjusted to cell density corresponding to 0.55 in McFarland standard (*bioMérieux*, France), approximately 1.5×10^8 CFU mL^{-1} for *Escherichia coli* and *Candida albicans* and approximately 10^6 - 10^7 spores mL^{-1} for *Aspergillus niger*. Three sterile paper discs (Whatman No.1, diameter 5 mm) were impregnated with 5 µl pure limonene (used as control). The discs of fragrant starch-based films were sterilized by UV or ethyl alcohol. The unsterilized, and washed with water discs were tested as well (Table 5). The discs were placed on the appropriate media. The plates were incubated at time and temperature optimum for microorganisms growth. The inhibition zone around the paper discs were measured with a ruler using Acolyte Camera (UK). All tests were repeated three times.

Table 5. Antimicrobial activity tests

Sample	Method preparation (sample discs)	Bacteria *(E. Coli)*	Yeast *(C. albicans)*	Mode Fungi *(A. niger)*
SL	UV	+	+	+
SGL	UV	+	+	+
SL	water	+	+	+
SGL	water	+	+	+
SL	70% ethyl alcohol	+	+	+
SGL	70% ethyl alcohol	+	+	+
Limonene	-	+	+	+

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