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Obtaining Edible Pullulan-based Films with Antimicrobial Properties

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Abstract

A nutrient medium was selected for the efficient production of exopolysaccharide (EPS) by *A. pullulans* C7 strain. The production of pullulan polysaccharide was evaluated on nutrient media with traditional carbon sources and cheap substrates that were plant wastes. For maximum EPS accumulation, we proposed an optimized Czapek-Dox medium with glucose as a carbon source, sodium nitrate as a nitrogen source, and C/N=232:1 ratio (EPS yield 12.79±0.64 g/l). Medium with grape pomace 5% (EPS yield was 15.08±0.34 g/l) and medium with topinambour tuber hydrolysate 5% (EPS yield was 14.44±0.21 g/l) was proposed as a cheap substrate. Edible films with antimicrobial activity were obtained on the basis of the isolated polysaccharide. The antibacterial activity of films against *Escherichia coli* 603 and *Staphylococcus aureus* ST228 was shown when essential oils of rosemary (zones of growth inhibition from 8.41±0.71 to 9.98±0.32 mm) and oregano (zones of growth inhibition from 8.09±0.51 to 9.54±0.24 mm) were added to pullulan. The addition of xanthan gum and glycerol to the films increased their strength and elasticity. The infrared spectrum of the pullulan film showed absorption bands characteristic of polysaccharide structures.

1. Introduction

Recently, the food industry has paid close attention to the creation of fundamentally new packaging materials – non-toxic, easily recyclable, capable of providing effective protection of products from microbial damage and exposure to air oxygen, preventing changes in their properties during production and storage [1]. In addition, edible packaging is completely environmentally friendly and can have a number of unique functional properties and performance characteristics due to the inclusion of antimicrobial substances, vitamins, and flavorings [2]. Films are an effective barrier to prevent unwanted mass transfer in food products (e.g., water vapor and oxygen transfer), thereby improving their quality and extending their shelf life. In some cases, the use of food films also provides an opportunity to simplify and/or reduce the size of secondary synthetic packaging [3].

Currently, the main film-forming components for edible packaging are polysaccharides (starches, cellulose ethers, chitosan, pullulan, dextrins, alginates, carrageenan, pectins, gum), proteins (collagen, gelatin, zein, gluten, soya isolates, casein), lipids (beeswax, carnauba wax, etc., paraffin derived from petroleum; acetic glycerides, glycerides) or their combinations [4]. Edible films derived from these classes of chemical compounds differ in properties. Thus, polysaccharide films are hydrophilic and allow to obtain a wide range of composite packaging materials, since various water-soluble additives can be introduced into the film: flavorings, colorings, etc. They are firmly bound to the basic polymer by hydrogen bonds [5].

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For the development and production of edible food coatings, microbial polymers have recently been increasingly used as promising innovative materials due to a range of valuable properties. One of the contenders for the role of such a material is pullulan. Pullulan is a microbial exopolysaccharide (EPS) synthesized by strains of the yeast-like polymorphic fungus *Aureobasidium pullulans*, which are widely found in air, soil, water, wood, and food [6]. Pullulan is a biodegradable, unbranched, and water-soluble polysaccharide, which consists of linear (1→6) linked maltotriose units and a small number of maltotetraose linked (1→4) units [7]. The regular alternation of (1→4) and (1→6) glycosidic bonds leads to two distinctive properties including structural flexibility and high water solubility. These properties combined with its non-toxicity, non-mutagenicity, edibility, odorless and tasteless, resistance to oils, fats and slight temperature changes have made it useful for food, pharmaceutical, and biomedical applications. Films produced from pullulan are transparent, and have high oxygen impermeability as well as excellent mechanical properties. These properties make them an ideal biodegradable and water-soluble material for food packaging [8].

The cost of pullulan depends primarily on the type of raw material. In the production of pullulan by microbiological method, various carbohydrate substrates are used for the cultivation of producers. The use of agroindustrial wastes can be economically favorable with low cost [9]. There are various reports on the production of pullulan from low-cost sources such as sweet potato, soya cake, potato starch waste, deproteinized whey, agro-industrial wastes such as grape skin extract, starch waste, olive oil wastewater and beet molasses, brewery waste, sugarcane waste, carob pods and topinambur [10, 11].

The main objective of the present study was to optimize the nutrient medium for the pullulan producer and, based on this EPS, to obtain an inexpensive and environmentally friendly material with antimicrobial properties recommended for food packaging.

2. Materials and methods

2.1. Object of the study

Aureobasidium pullulans strain C7 (accession number OR864236) isolated from dark chestnut soil (Almaty, Kazakhstan) was used in the present study [12].

2.2. Selection of nutrient medium

For cultivation of *A. pullulans* C7 we used Czapek-Dox medium with the addition of sucrose, glucose, molasses, topinambour tuber hydrolysates, and grape pomace in the amount of 30.0; 40.0; 50.0 g/L. The ratio C/N = 43 was created according to the prescription of Czapek-Dox medium (carbon concentration 21 g/l, nitrogen concentration 0. 490 g/l), C/N = 165 was created according to optimized glucose-peptone medium (carbon concentration 21 g/l, nitrogen concentration 0.127 g/l), C/N = 232 was created according to optimized glucose-peptone medium (carbon concentration 29.5 g/l, nitrogen concentration 0.127 g/l). Fermentation was carried out semi-submerged at 180 rpm and 25 °C for 5 days.

2.3. Polysaccharide isolation

A five-day-old culture of the strain was centrifuged at 10,000 ×g for 15 min followed by pullulan precipitation with 96% ethanol in a 2:1 (v/v) ratio. The resulting precipitate was washed with 96% ethanol and dried to constant weight. The following parameters were calculated: biomass dry weight (X), EPS dry weight (P), substrate consumption (S), the yield coefficient of biomass (P/X), and yield coefficient of substrate (P/S) [13].

2.4. Preparation of films

Edible films were prepared using a 3% aqueous solution of pullulan as a base. Then 1.5% glycerol and 0.1% xanthan gum were added to the solution. The solution was heated in a water bath at 80 °C for 15 min and then cooled to room temperature. Rosemary (2%) and oregano (2%) essential oils were dissolved in 2 ml of 95% ethanol and added to the mixture. After mixing the components, the solutions were cast onto plain silicone substrates and dried at 50 °C for 20 h in a drying oven RE 53 Redline (Germany).

*2.5. Infrared spectroscop*y

Fourier-transform infrared spectroscopy (FTIR) was performed using Carry 660 spectrophotometer (Agilent, USA) in the wavelength range from 800 to 4000 cm^{-1}, equipped with DTIR attachment (disturbed total internal reflection attachment) with a crystal from Germany (Ge ATR crystal), the number of reference air scans was 8, the sample was scanned 24 times.

2.6. Physicochemical properties of antimicrobial films

The thickness of each film sample was measured using an MT-25 hand micrometer with an accuracy of 0.001 mm. Measurements were taken at 3 different points of each film sample and average values were calculated.

The transparency of the film samples was measured according to the method described in the work [14]. A rectangular slice from each film sample was placed in the chamber of a spectrophotometer. The absorbance was measured at 550 nm. The transparency of the films was calculated by dividing the optical density by the thickness (mm).

The solubility of film samples in water was measured by the method proposed by B. Ghanbarzadeh et al. [15]. The film samples were placed in water for 3 min, then removed and dried at 105 °C to constant weight. The difference in weight of the dried film before and after dissolution was calculated.

2.7. Antimicrobial activity of the films

When determining the antimicrobial activity of the films, *Staphylococcus aureus* ST228 and *Escherichia coli* 603 strains were used as test cultures. The test cultures were suspended in a physiological solution to a concentration of 0.5 on the McFarland scale and sown in a continuous lawn on the surface of nutrient agar. Fragments of Pullulan films 5×5 mm in size were spread on the surface of the test culture lawn. They were incubated at 37 °C for 24 h. Then the growth inhibition zone was measured [13].

2.8. Statistical analysis

Statistical data processing was performed using Statistica 10.0 software. Data are presented as values ± standard deviation. The experiments were performed in three repetitions.

3. Results and discussion

3.1. Exopolysaccharide production of A. pullulans C7 depending on nutrient sources

Optimization of nutrient medium for efficient EPS accumulation was started with the selection of carbon and nitrogen sources. Most often, the main carbon sources for EPS production are glucose and sucrose, which was also confirmed by our previous studies [12]. The yield of EPS when *A. pullulans* C7 strain was cultured on nutrient media with glucose and sucrose at 5% concentration was 9.58 ± 0.50 and 10.36 ± 0.47 g/L, respectively.

Along with carbon sources, nitrogen sources, their nature, and concentration have a direct influence on the growth and development of microorganisms, since nitrogen is a part of amino acids and participates in the formation of protein peptide bonds. In this connection, it was of particular interest to study the effect of nitrogen sources and carbon to nitrogen C/N ratio on the growth of *A. pullulans* C7. The dependence of polysaccharide-forming activity on nitrogen source was studied in liquid nutrient media with ammonium sulfate, ammonium nitrate, and sodium nitrate, creating different ratios of carbon and nitrogen nutrition in the medium (Table 1).

Table 1. EPS production by strain *A. pullulans* C7 on medium with different nitrogen sources.

C/N	Nitrogen source (g/l)	Cell dry weight, g/l	EPS dry weight, g/L	Glucose consumption, %
43:1	$(NH_4)_2SO_4$	0.59 ± 0.03	5.66 ± 0.28	75.20
	NH_4NO_3	2.42 ± 0.12	5.74 ± 0.34	96.70
	$NaNO_3$	4.77 ± 0.19	6.51 ± 0.33	98.90
165:1	$(NH_4)_2SO_4$	0.96 ± 0.05	6.02 ± 0.30	57.90
	NH_4NO_3	3.14 ± 0.16	6.21 ± 0.36	79.70
	$NaNO_3$	5.40 ± 0.17	9.32 ± 0.37	91.60
232:1	$(NH_4)_2SO_4$	0.35 ± 0.02	6.32 ± 0.32	66.40
	NH_4NO_3	3.54 ± 0.18	8.09 ± 0.45	68.30
	$NaNO_3$	6.02 ± 0.20	12.79 ± 0.64	74.20

As shown in Table 1, the most effective source of nitrogen, regardless of concentration is sodium nitrate and C:N ratio = 232:1. These conditions stimulate the synthesis of EPS, the yield of which was 12.79 g/L.

3.2. Use of low-cost power sources for culturing A. pullulans C7

The cost of commercial pullulan produced on media with conventional carbon sources is relatively high, limiting its industrial application. To this end, inexpensive carbon sources derived from agro-industrial wastes such as potato starch, sugarcane cake hydrolysates, rice husk hydrolysates, grape cake, palm kernel sweet potato, and peat hydrolysates have been highlighted [16–19].

In the present study, molasses, topinambour tuber hydrolysate, and grape pomace were used as cheap substrates (Table 2). As can be seen from the table, a high yield of EPS was observed on the medium with grape pomace at 5% and topinambour tuber hydrolysate at 5%. The yields were 15.08±0.34 g/l and 14.44±0.21, respectively.

The increase of pullulan production on topinambour tuber hydrolysate and grape pomace is quite natural, as these substrates, along with molasses, have a rich chemical composition. It is known that topinambour tubers contain a complex of polysaccharides (pectins, fiber, cellulose, hemicelluloses), proteins, amino acids, organic and fatty acids, vitamins, and minerals [20]. The chemical composition of grape pomace is similar to that of grapes. They contain sugars, alcohol, nitrogenous, pectin, tannins, fats, fiber, organic acids [21].

3.3. Preparation of Pullulan-based films and their physicochemical properties

Edible films can incorporate active components to enhance the quality and safety of food products [22]. The presence of antioxidant and antimicrobial compounds, especially those derived from natural sources, leads to a new class of active packaging materials. Essential oils of herbs and spices are known to have antimicrobial properties against various pathogens such as *Escherichia coli*, *Campylobacter jejuni*, *Salmonella enterica*, and *Listeria monocytogenes*. In particular, clove and thymol extracts were effective against *Salmonella typhimurium* and *E. coli* [23]. The use of essential oil-based antimicrobial agents is important and can control the microbial population in products, which in turn can ensure product safety and quality.

In the present study, the pure pullulan film was shown to be transparent, smooth, sticky, non-elastic, and glossy. The addition of glycerol and xanthan gum made the films stronger and more elastic and improved water solubility.

The spectral characteristics of pullulan film with the addition of glycerol and xanthan gum were studied (Fig. 1). The IR spectrum showed absorption bands characteristic of polysaccharide structures. The following absorption bands are noted in the IR spectrum of the film: 3354 cm^{-1} (OH-); 2926 cm^{-1} (C-H-); 1649 cm^{-1} (C=C), and 1028 cm^{-1} (C-O). IR spectroscopy data showed that an absorption band at 855 cm^{-1} was also observed, which characterizes the α-configuration of the glycosidic bond.

When 2% of oregano and rosemary essential oils were added, the films lost transparency and an

Table 2. EPS production by *A. pullulans* strain C7 on medium with cheap carbon sources.

Carbon source	Cell dry weight, g/l (X)	EPS dry weight, g/L (P)	Utilized glucose, g/L (S)	P/X, %	P/S, %	E. coeff. X/S, %
Molasses 3%	3.43 ± 0.07	4.23 ± 0.04	31.23 ± 1.24	123.32	13.54	10.98
Molasses 4%	4.23 ± 0.20	4.98 ± 0.51	25.98 ± 1.06	117.73	19.16	16.28
Molasses 5%	4.31 ± 0.85	5.34 ± 0.95	36.22 ± 1.65	123.89	14.74	11.89
Topinambour hydrolysate 3%	6.81 ± 0.05	11.06 ± 0.02	35.11 ± 1.01	162.41	31.50	19.40
Topinambour hydrolysate 4%	7.66 ± 0.17	13.08 ± 0.19	37.03 ± 1.81	170.75	35.32	20.68
Topinambour hydrolysate 5%	8.67 ± 0.19	14.44 ± 0.21	38.65 ± 1.56	166.55	37.36	22.43
Grape pomace 3%	7.34 ± 0.87	12.01 ± 0.97	37.01 ± 1.87	163.62	32.45	19.83
Grape pomace 4%	8.01 ± 0.05	13.53 ± 0.41	35.99 ± 1.99	168.91	37.59	22.25
Grape pomace 5%	9.31 ± 0.62	15.08 ± 0.34	38.84 ± 1.02	161.97	38.82	23.97

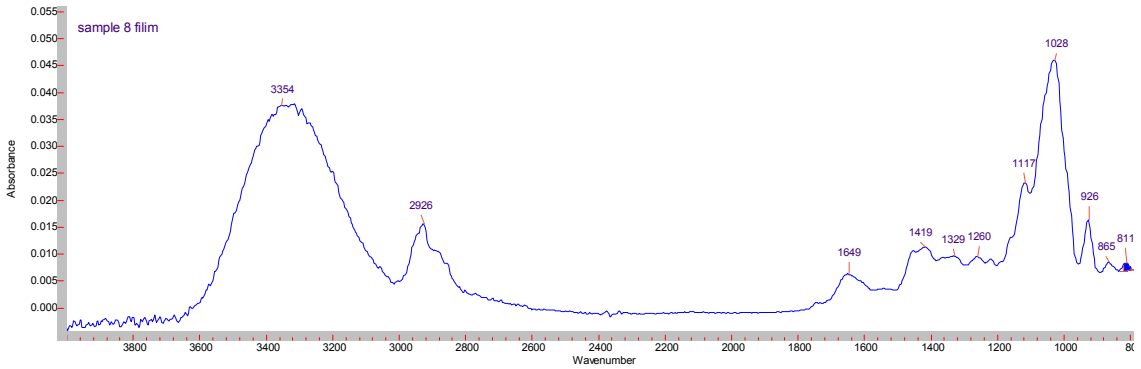

Fig. 1. IR spectrum of pullulan film.

intense odor of oregano and rosemary appeared. These results are in agreement with those reported by Gniewosz M. and Synowiec A. [24]. All the films obtained were not sticky, and easily separated from the substrate. The film with rosemary and oregano oil was more elastic and strong.

The solubility of the film is an important factor in determining its biodegradability [25]. Pullulan is known to be soluble in water and weak alkaline solutions. Its aqueous solutions are stable and have relatively low viscosity compared to other polysaccharides.

As shown in Table 3, water solubility was higher in films made of pure pullulan – 99.00±0.05%. The inclusion of rosemary and oregano essential oils in pullulan films slightly reduced their solubility. These results are in agreement with the studies of M.A. Rojas-Graü and Y. Pranoto [23, 26].

Thickness is one of the important parameters on which the transparency, water vapour permeability, and mechanical properties of films depend. In the present study, the variation of film thickness depending on the composition was observed. As shown in Table 3, the addition of rosemary and oregano essential oils did not affect the film thickness.

The transparency of the film affects the appearance of the packaging and its attractiveness to the consumer. Pure Pullulan film was colorless, shiny, and transparent. The addition of oregano and rosemary essential oils resulted in a cloudy film and yellowish-white coloration, indicating an increase in its optical density. These results are in agreement with previous studies of pullulan [27] and alginate-based edible films [28].

3.4. Antimicrobial activity of edible films

At the next stage of the study, the antimicrobial activity of the obtained films was tested.

It is expected that the developed pullulan films with essential oils as antimicrobial agents will be effective in inhibiting or destroying pathogenic microbes that cause food spoilage. It is known that most infectious diseases are caused by foodborne pathogens such as *E. coli* and *S. aureus*. Some serotypes of *E. coli* can produce enterotoxins that cause diarrhea, abdominal pain, inflammation, ulcers, and other manifestations of food poisoning. Staphylococcus can also produce various enterotoxins, which can cause food poisoning or purulent-inflammatory infections in humans [29]. In this regard, *S. aureus* ST228 and *E. coli* 603 strains were used as test cultures.

As shown in Table 4, essential oils (rosemary and oregano) impart an antimicrobial effect to the films. The zones of growth inhibition of test cultures by the addition of oregano essential oil were 8.41±0.71 and 9.98±0.32 mm, rosemary oil – 8.09±0.51 and 9.54±0.24 mm, respectively. It is known that the antimicrobial effect of rosemary oil is due to the compounds cineole and α-pinene, which cause thickening and destruction of the cell wall of microorganisms [30].

Table 3. Physical properties of pullulan films.

Properties	Pullulan	Pullulan + rosemary	Pullulan + oregano
Solubility, %	99.00 ± 0.05	97.28 ± 0.66	96.32 ± 0.23
Thickness, mm	0.11 ± 0.01	0.12 ± 0.01	0.13 ± 0.01
Transparency	0.73 ± 0.01	0.86 ± 0.02	0.87 ± 0.01

Similar results were obtained by M. Oussalah et al., they revealed, that films of alginate and milk protein containing 1.0% oregano oil were effective against *E. coli*, *S. typhimurium*, *L. monocytogens,* and *S. aureus* which contaminates meat products (beef) [28].

As a result of these experiments, edible pullulan-based films with antimicrobial activity were obtained. An increase in antibacterial activity was shown when essential oils of rosemary and oregano were added to Pullulan.

Table 4. Antimicrobial activity of pullulan films against test cultures

Film composition	Zones of growth inhibition, mm	
	Escherichia coli 603	*Staphylococcus aureus* ST228
Pullulan	0.6 ± 0.03	0.7 ± 0.03
Rosemary oil	7.7 ± 0.3	8.6 ± 0.4
Oregano oil	8.1 ± 0.4	9.1 ± 0.2
Pullulan + rosemary	8.1 ± 0.3	9.5 ± 0.2
Pullulan + oregano	8.4 ± 0.4	9.9 ± 0.3

4. Conclusions

The effect of carbon and nitrogen sources and their ratio on EPS production by *A. pullulans* strain C7 was determined. Medium with grape pomace and medium with topinambour tuber hydrolysate were proposed as cheap substrates. The use of this agro-industrial waste, which has a high nutritional value, as a substrate for EPS accumulation will make the production of pullulan low-cost and contribute to its successful entry into the industrial market. In addition, these wastes are generated in large quantities and pose a serious environmental problem. Therefore, the production of pullulan on their basis will solve the issue of their utilization.

Edible films with antimicrobial activity have been produced from pullulan. The addition of rosemary and oregano essential oils to pullulan gave the films antibacterial activity, while the inclusion of xanthan gum and glycerol increased their strength and elasticity.

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