The use of Vitally Active Cellulose Membranes for the Reduction of Pathogenic Bacterial Count in White Cheese

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Abstract

   This study was designed to evaluate the effects of cellulose membranes produced by *Acetobacter xylinum* bacteria, after enrichment of the growth media with Alzahdy palm dates syrup to enhance cellulose production for reducing the contamination of locally-produced white cheese with pathogenic bacteria. Cellulose was vitally activated by incubation with both probiotics *Lactobacillus acidophilus* and *Lactobacillus plantarum* and the effectiveness of the produced cellulose membranes was measured by studying six characteristics: elongation, tensile strength, membrane rupture, permeability to oxygen, permeability to water vapor, and thickness (mm). The produced membranes showed remarkable functionality and characteristics for all studied tests. The results indicate that the cellulose membranes showed high antibacterial activity after incubation with *Lactobacillus acidophilus* and *Lactobacillus plantarum* for 24 and 48 hours against five different pathogenic bacteria, namely *E. coli, S. aureus, Pseudomonas sp., B. cereus* and *S. typhymurim*. Moreover, a positive result was obtained by reducing the number of these pathogenic bacteria after treating the white cheese with the produced cellulose membranes.

   Keywords: cellulose membrane, pathogenic bacteria, white cheese, probiotics.

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استعمال الاغلفه السليلوزيه الفعاله حيويا في خفض عدد الممرضات البكتيريه في الجبن الابيض

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الخلاصه

   صممت هذه الدراسة لتقييم تأثير الغشاء السليلوزي المنتج من قبل بكتريا *Acetobacter xylinum* , بعد اغناء الوسط الزرعي بعصير تمر الزهدي لتحسين انتاج السيليلوز , لتقليل تلوث الجبن الابيض المحلي بالممرضات البكتيريه. تم تنشيط الغشاء السيليلوزي حيويا عن طريق حضنه مع المعززات الحيويه *Lactobacillus acidophilus* and *Lactobacillus plantarum* وتم قياس كفاءة ونوعيه الغشاء من خلال دراسه الخواص السته التي شملت: الاستطاله, الشد السطحي, تمزق الغشاء, النفاذيه للاوكسجين , النفاذيه لبخار الماء والسمك, واظهر الغشاء المنتج خصائص وفعاليه عاليه لجميع الفحوصات المدروسه. بينت النتائج ان الاغشيه السليلوزيه اظهرت فعاليه ضد البكتريا بعد حضنها مع *Lactobacillus acidophilus* and *Lactobacillus plantarum* لمدة 24 و 48 ساعه ضد خمسه انواع من الممرضات البكتيريه *E. coli, S. aureus, Pseudomonas* sp*., B. cereus* and *S. typhymurim*

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بالاضافه الى ذلك تم الحصول على نتائج موجبه عن طريق تقليل اعداد الممرضات البكتيريه في الجبن .
الابيض بعد معاملته بالغشاء السليلوزي المنتج.

Introduction

The main component of plant cell walls is cellulose, that is generally used as a crude compound for board, paper, or rayon fibers in manufacturing industries. Recently, wood is the major source of cellulose because of its increased content of this material. However, polysaccharides, such as lignin and hemicellulose, are still reported as contaminants to cellulose collected from wood pulp. Therefore, it is necessary to seek for other replacement sources of cellulose with higher purity [1].

One of the main problems that are encountering public health worldwide is foodborne or food poisoning diseases (FBD). According to the World Health Organization (WHO), as a result of consuming contaminated food, about sixty- hundred million people around the world, or 1 out of 10 persons, become ill each year [2]. Among the microorganisms causing FBDs are bacteria, which have many virulence factors that confer the ability to cause diseases. Toxins that can be produced in food are among these factors, which can disturb the digestive tract when colonized by the pathogen bacteria [3].

Gram-negative psychrotrophs, coliforms, lactic acid bacteria, yeast, and molds are among the food contaminating microbes that can cause spoilage of dairy products [4]. Moreover, many bacteria that are found in a dairy product are of public health concern, such as *Listeria monocytogenes*, *Salmonella* spp., *Yersinia enterocolitica*, *Campylobacter jejuni*, and *Escherichia coli*, along with pathogenic strains of *Staphylococcus aureus* and enterotoxigenic strains of *Bacillus* spp., *Clostridium* spp., and enterococci [5 ,6].

Various derivatives of milk, such as butter, yoghurt, and cheese are vital foods for humans. Nevertheless, they also provide an excellent medium for the growth of many kinds of microorganisms [7]. The stabilized curd of milk solids is cheese that is produced by the coagulation of casein and entrapment of milk fat [8]. It is a ready- to- eat dairy product, which is a rich source of proteins, vitamins, calcium, and phosphorus. However, various factors, including handling, packaging material ,and environment can cause microbial contamination of cheese [9].

White cheese, that is called locally "raw cheese", is one of the traditional fresh cheese types that are generally prepared from unpasteurized milk in different areas of Iraq. The short preparation and production time from raw milk can increase contamination by bacteria. Research dealing with food-borne disease occurrence, including that of dairy products, and food surveillance indicate newly prepared cheese as a good source of pathogens [10 ,11]. In Iraq, as an agricultural country, waste recovery and membrane filtration for food industries are among the challenging applications. Although the cost of manufactured membrane in the last years is much lower than its current cost, it is still relatively high compared to that of agriculture products. However, membrane manufacturing is still costly even with advanced research, due to substances that need to be imported, such as chemicals and polymers [12]. For these reasons, there is a need to search for simple methods that provide less costly membranes. To produce membranes of microfiltration range that exclude microorganisms, alternative methods should be developed [13].

However, various bacteria, rather than plants, can produce cellulose as an alternative source, although plant cellulose is the most important source. There has been a growing interest in bacterial cellulose as a new type of natural polymers [14, 15]. Cellulose produced by bacteria do not have similar microstructure and mechanical properties to that produced by plants, such as higher mechanical strength, finer web-like network, higher crystallinity, higher polymerization degree, as well as higher water-absorbing capacity [16].

This study aimed to investigate the antibacterial activity of cellulose membrane produced by *A. xylinum* by reducing bacterial contamination of white cheese after incubation with probiotics.

Materials and Methods

Bacterial cells preparation

Acetobacter xylinum bacteria that were used for producing cellulose was obtained and grown as described by a previous work [17].

Preparation of Alzahdy palm dates syrup

Alzahdy plam dates were obtained from local markets in Baghdad city, Iraq. The syrup of Alzahdy date was prepared according to an earlier published procedure [18], with minor changes. Stone-free

dates (200 mg) were mixed with 500 ml of boiled distilled water and left for 24 hrs. Then, they were mixed in a blender for 1 min at low speed, and for more 3 min at a higher speed. Through a double layer of cloth, the homogenized extract was filtered. After that, the syrup was diluted by distilled water to Brix 8 and distributed into 300 ml flasks, each with 100 ml of syrup. After that, the flasks were sterilized by autoclave at 121 C^o for 15 min.

Cellulose production

The flasks containing date syrup, as a production edium, were inoculated with activated *A. xylinum* at 5%, so that each 1 ml contained 10^6 CFU /ml, and incubated at 30 C^o for 7 days [13].

Cellulose extraction

According to an earlier published method [19], the flask contents were filtered after the last day of incubations and then washed two to three times with distilled water to remove any sugar. Later, sodium hydroxide (0.5 M) was added to remove bacterial cells and the remaining contents were incubated in water bath at 90 C^o for 1 hour. The mixture was washed three time with distilled water, dried at 70 C^o, and kept to be used as cellulose membranes in next steps.

Activation of probiotics

Two isolates of *Lactobacillus* bacteria were used in this study, namely *Lactobacillus acidophilus*, as capsules, and *Lactobacillus plantarum*, as pills. The contents of both the capsules and pills were dissolved in MRS broth (De Man, Rogosa and Sharpe broth)and the flasks were incubated at 37 ^{o}C for 48 hours. After that, they were inoculated with skimmed milk broth (14%) and incubated at 37 C^o until coagulation [20].

Production of vitally activated cellulose membranes

Sterilized flasks containing 100 ml of MRS broth were inoculated separately with 10% of previously activated *Lactobacillus acidophilus* and *Lactobacillus plantarum* (10^6 cell), with duplicate for each treatment. Under sterile conditions, cellulose membranes were added to the flasks and incubated at 37 C^o at different periods (24, 48 hrs). Later, the cellulose membranes were collected and dried in oven at 40 C^o. Finally, they were kept in sterile containers for studying their characteristics and using them to produce disks (by using casting knife) to be used in inhibition of some pathogenic bacteria [20].

Studying the characteristics of cellulose membranes

The characteristics of the produced cellulose membranes were investigated according to a previously described method [21]. The thickness of cellulose membranes was measured by using a Vernier caliper device at sensitivity degree close to 0.01 mm and the thickness medium of six randomly selected positions in the membrane was calculated. The permeability of the membranes to water vapor was estimated. The amount of permeable water was calculated through the reduction in the weight of distilled water and moisture to 50% at 23 C^o for a minimum period of 12 hrs. The permeability to oxygen was estimated by using Oxygen Permeability Tester Device. Membrane rupture test t was performed by using Rupture Tester Device. Determination of tensile strength and elongation of membranes was achieved using a special device depending on computerized data analysis. The latter four tests were performed in the laboratories of the Ministry of Industry and Minerals, Baghdad, Iraq.

Studying the antibacterial activity of the cellulose membranes

Preparation of pathogenic bacteria: pathogenic bacterial isolates *(E. coli, S. aureus, Pseudomonas sp., B. cereus,* and *S. typhymurim*) were obtained from the laboratories of Science College/ Baghdad University, Baghdad, Iraq.

Estimation of antibacterial activity: the inhibition activity of cellulose membranes that were vitally activated with *Lactobacillus acidophilus* and *Lactobacillus plantarum* for 24 and 48 hours against some pathogenic bacteria was estimated according to an earlier published method [22]. Sterilized nutrient agar petri dishes were streaked with pathogenic bacteria by using L-shape spreader. Disks of vitally activated cellulose membranes, which were previously prepared , were inserted on the surface of the agar. Later, the petri dishes were incubated at 37C^0 for 24 hrs. After incubation, the diameter of the zone of inhibition was calculated in millimeters.

Using cellulose membranes in reducing bacterial numbers in white cheese

Manufacturing of cheese: the milk was obtained from The Dairy factory/ Collage of Agriculture, University of Baghdad**.** Cheese was made by the sour cheesing method [17], by firstly slow pasteurization of the milk at 63 C^o for half an hour. This was followed by cooling to 5 C^o and the

addition of acid with agitation and increasing the temperature with continuous agitation until obtaining complete cheese curd. Finally, the product was left for 15 minutes to separate the whey and the cheese was pressed in a cooling room for 24 hours.

Treatment of cheese with cellulose membranes: the manufactured cheese was divided into 10 parts, with 500gm for each treatment. Control cheese samples, i.e. without cellulose membranes were termed as C, while cheese samples treated with probiotic- incubated cellulose membranes were termed as T, after removing the whey. . To each treatment, 1 ml of previously mentioned pathogenic bacteria (10^6 cell/ml) was added and finally preserved at the refrigerator temperature for 14 days. Microscopic examination was performed at different periods (0, 2, 4, 6, 8, 10, 12 and 14 days). All cells other than those of the tested microorganisms and the possibly unstained and, therefore, uncountable bacteria in the cheese, were disregarded.

Results and Discussion

A remarkable attention has been given to *Acetobacter xylinum* as cellulose producing bacteria, due to its increased applications in many industries. Cellulose production was shown to be enhanced by adding many natural and synthetic substances according to many previous studies. The authors of an earlier study [21] added coconut water and pineapple juice, while in another study [23], chitosan and glycerol were added. In our study, and according to a previous report [17], dates syrup, precisely Alzahdy syrup, was used. High quantity of cellulose was produced, which was later treated with probiotics to enhance its functionality and membrane characteristics, as shown in Table-1. However, both types of the used probiotics were shown to have closely similar effects on the quality of the cellulose membrane.

Table 1-The characteristics of vitally active cellulose membranes

Bacteria Type	Elongation	Tensile strength	Membrane rupture	permeability to oxygen	permeability to water vapor	Thickness (mm)
Lb. acidophilus	42.9	80.3	818	55.920	2490.545	3.5
Lb. plantarum	43.7	81.5	820	55.221	2485.320	3.6

Antimicrobial activity appears as the length of the inhibition zone around the tested compound. Due to the compound antimicrobial activity, clear zone around the sample is formed, which indicate no microbe's growth. The increased diameter of the inhibition zone indicates that the compound is highly inhibiting the microbial growth.

One of the important applications of cellulose membranes is the antibacterial activity, which was shown to be highly enhanced after treatment with probiotic bacteria. The metabolic compounds produced by probiotics such as *Lactobacillus acidophilus* and *Lactobacillus plantarum* were shown to play a significant role in activating or increasing of either membrane quality or antibacterial activity. In a different study [23], nanoparticles were shown to play such role of probiotics, also enhancing the antibacterial activity. The results in Table-2 indicate the antibacterial effects of *Lactobacillus plantarum*-treated cellulose membrane after 24 and 48 hours of incubation against five different pathogenic bacteria: *E. coli, S. aureus, Pseudomonas sp., B. cereus,* and *S. typhymurim*. A high antibacterial activity was shown against all tested bacteria, while the highest zone of inhibition was recorded after 48 hours against *B. cereus.*

Table 2-The antibacterial activity of vitally active cellulose membranes after treatment with *Lactobacillus plantarum* at 24 and 48 hours

Treatment period/ hrs.	Pathogenic Bacteria/ Inhibition diameter (mm)				
	E. coli	*S. aureus*	*S. typhymurim*	*B. cereus*	*Pseudomonas* sp.
24	13	15	14	15	12
48	17	18	14	20	14

The results in Table-3 indicate the antibacterial activity of vitally active cellulose membranes after treatment with *Lactobacillus acidophilus* at 24 and 48 hours of incubation against the same tested pathogenic bacteria. The results indicate large zones of inhibition against all tested bacteria at both periods of incubation, where the highest antibacterial activity was, again, recorded against *B. cereus* after 48 hours of incubations. However, cellulose membrane was shown to cause higher diameter of zone of inhibition against gram negative bacteria than gram positive bacteria. One of the suggested mechanisms is that the bacterial cells are negatively charged surface [24]. Our cellulose membrane are positively charged, so it became simple to bind to the surface of negatively charged bacteria, which will affect and destroy the proteins in the cell membrane and inhibit the growth of bacteria. In addition, the active metabolic compounds provided by probiotic bacteria also play a role in enhancing the antibacterial activity of cellulose membranes.

Table 3- The antibacterial activity of vitally active cellulose membranes after treatment with *Lactobacillus acidophilus* at 24 and 48 hours.

Treatment period/ hrs.	Pathogenic Bacteria/ Inhibition diameter (mm)				
	E. coli	*S. aureus*	*S. typhymurim*	*B. cereus*	*Pseudomonas* sp.
24	11	13	12	17	11
48	12	15	13	19	13

Locally produced white chees in Iraq was recoded with high percentage of bacterial contamination. Thus, this study was designed to find a solution for such problems in the field of cheese production. Following our above described data that reflect the highly positive antibacterial activity of cellulose membrane , the results in Table-4 reflect the obvious reduction in the bacterial count after treating the cheese with the cellulose membranes, in compassion with controls witch represent the untreated cheese, at several incubation periods (0, 2, 4, 6, 8, 10, 12 and 14 days). The table showed an increased reduction in the number of bacterial cells after treatment with cellulose membrane against both gram positive and negative bacteria in comparison with untreated cheese. This may give the possibility of the suggestion of cellulose membrane as an approach to preserve cheese and reduce its contamination with pathogenic bacteria.

Table 4-Bacterial count (CFU/ ml) in white cheese during incubation periods (0, 2, 4, 6, 8, 10, 12 and 14 days) for both untreated and treated cheese with cellulose membrane

Time/ Day		Pathogenic Bacteria (Log CFU/ml)				
		E. coli	*S. aureus*	*S. typhymurim*	*B. cereus*	*Pseudomonas sp.*
Zero		6.301	6.301	6.301	6.301	6.301
2	T	6.748	6.633	6.939	6.949	6.991
	C	7.361	7.255	7.505	7.301	7.919
4	T	5.949	5.929	6.505	6.633	6.732
	C	7.880	7.716	7.792	7.812	7.992
6	T	5.707	5.361	5.698	5.477	5.000
	C	7.982	7.908	7.963	7.939	8.301
8	T	4.602	4.963	5.973	5.954	5.898
	C	8.716	7.986	8.342	8.342	8.968
10	T	4.973	4.176	5.255	5.812	5.857
	C	8.880	8.397	8.633	8.707	8.477
12	T	4.518	3.857	4.079	5.361	5.633
	C	8.977	8.939	8.880	8.892	9.716
14	T	3.623	2.301	4.740	3.602	4.778
	C	8.301	8.986	8.944	8.991	10.959

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