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Encapsulation of *Lactobacillus acidophilus*PTCC 1643 with composite wall of xanthan gum, soy protein isolate and fructooligosaccharide: investigation microcapsule characteristics

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ABSTRACT

Encapsulation is an effective method to increase the viability of probiotic bacteria.The aim of this research is to determine the optimal formulation for microencapsulation of *Lactobacillus acidophilus* PTCC 1643 with soy protein isolate, xanthan gum and fructooligosaccharide as wall materials by freeze drying method.For this purpose, 19 treatments with Combine design were prepared by Design expert software.Encapsulation efficiency, particle size, zeta potential, and probiotic viability, FTIR and SEM tests were performed on the samples.The optimal sample was selected based on the lowest zeta potential and particle size, and the characteristics of microcapsules were determined by the aforementioned tests.The results showed that probiotic viability increased and decreased with increasing concentration of soy protein isolate and xanthan gum, respectively.The particle size of the samples also increased with increasing the concentration of soy protein isolate.In general, according to the protective effect of xanthan gum, soy protein isolate and fructooligosaccharide on probiotics, it can be concluded that these compounds can be used as a wall for coating to enrich food products.

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1. Introduction

In recent decades, the development of functional foods such as probiotic products has increased. Probiotics are defined as live microorganisms that can have a positive effect on the host if used adequately. Probiotics have benefits such as maintaining the natural intestinal microflora, strengthening the immune system and reducing blood cholesterol [1, 2]. The most common probiotics included in functional foods are Lactobacillus species, which are known as non-pathogenic microorganisms in the gut and play an important role in preventing the colonization of pathogens and regulating the host's immune response. *Lactobacillus acidophilus*It shows antimicrobial effect through the production of organic acids and bacteriocin [3]. Lactobacillus is resistant to bile acid and has an antibiotic effect on intestinal pathogens [4, 5]. Microencapsulation is one of the most common techniques used to increase the viability of probiotics. Encapsulation traps the active agent (core material) inside another compound (wall material) and produces particles in the nanometer or micrometer range. The wall or covering materials used in micro-coating methods should be edible, safe and biodegradable. These compounds can be carbohydrates, proteins, resins and lipids [6,7].

Xanthan gum is a heteropolysaccharide composed of D-glucose, D-mannose and D-glucuronic acid units. Xanthan gum from aerobic fermentation of culture medium*Xanthamonas campestris* Pure is produced [8]. This hydrocolloid has become one of the most successful hydrocolloids due to its high performance, especially in environments such as acid, high salt, and high shear stress [9].

In dietary intermediate proteins, soy protein isolate[1] (SPI) is an important food protein with great potential as a (nano) carrier of insoluble biological agents due to its surface hydrophobic nature. SPI is known for its functional properties, non-toxic nature, low cost, easy availability and high nutritional value. It is used in the food industry [6]. Also, this compound is widely used in many food products such as processed meat, nutritious drinks, infant formula and substitute dairy products due to its emulsifying and gelling capabilities [10]. The main protein components in SPI are glycinin and β-conglycinin. Glycinin is composed of an acidic subunit and a subunit linked by a single disulfide bridge. β-conglycinin also consists of three α subunits, α and β is formed [11].

A fructooligosaccharide is a fructose oligomer linked to glucose or fructose molecules containing up to ten sugar moieties. Fructo-oligosaccharides are highly soluble in water and low-calorie carbohydrates. Fructo-oligosaccharide dosage levels in the range of 2 to 50% (w/w) are recommended for different food formulations. Fructooligosaccharides are one of the short chain carbohydrates that are currently part of the category of prebiotics. These carbohydrates with prebiotic properties show a high resistance to digestion and absorption by the digestive system, which causes a decrease in calorie content [12, 13].

In recent years, many researches have been conducted on the encapsulation of probiotics by different researchers, which can be the encapsulation of Lactobacillus acidophilus with the wall of rice husk protein and maltodextrin [14], the encapsulation of Lactobacillus acidophilus with the wall of whey protein isolate and lactose [15], Undercover*Lactobacillus acidophilus* with alginate wall and whey protein isolate [16] and lining*Lactobacillus plantarum* with soy protein wall [17].

The purpose of this research is bacterial encapsulation*Lactobacillus acidophilus*PTCC 1643 with xanthan gum composite wall, soy protein isolate (SPI) and fructo-oligosaccharide and determining the optimal sample based on zeta potential, particle size and encapsulation efficiency tests.

2- Materials and methods

2-1- Materials

Xanthan gum (11138662),Soy protein isolate (SPI) (S1674-500G) and fructooligosaccharide (MFCD00677049) from Sigma Aldrich, MRS culture mediumMan, Rogosa, Sharpe) Agar and MRS.Man, Rogosa, Sharpe)Broth from Merck, Germany, and the components of PBS buffer

[1] .Soy protein isolate

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(Phosphate Buffered Saline) (0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 grams of KH_2AFTER_4, 8 grams of NaCl, pH = 7.2) and other chemicals were from Merck, Germany. Probiotic strain*Lactobacillus acidophilus*PTCC 1643 was purchased from the collection of industrial microorganisms of Iran.
The equipment used includes English Astell, Behdad Iranian 37°C incubator, refrigerated centrifuge, Sartrious 0.001 gram precision scale, Reax Control vortex, Heidolph, Schwabach, Germany, DLS device (Model SZ-100, Horiba Ltd., Kyoto, Japan), scanning electron microscope (JSM-840 SEM, Tokyo, Japan) and spectrophotometer Bruker, Karlsruhe, Germany (Model Equinox 55 LS 101) were used.

2-2-preparation of probiotic bacteria

To prepare inoculum, 0.5 g of freeze-dried powder of cells*L. acidophilus* It was added to 5 ml of MRS broth and incubated anaerobically at 37°C for 72 hours. Then the solution was cultured in 95 ml of MRS broth and under the same conditions until reaching 12 log CFU/ml of*L. acidophilus* It was incubated for 72 hours. The biomass was harvested using a centrifuge at 4000 x rpm for 10 minutes at 4°C. The supernatant was separated and after washing the cells, they were centrifuged twice with saline solution without sterile buffer. Then, again, saline suspension was prepared and prepared for microencapsulation until reaching a solution containing 12 log CFU/ml of bacteria. Optical density at 625 nm using a spectrophotometer (UV-1800PC, Shimadzu Corp., Kyoto, Japan) gave the number of cells in log Cfu/mL12. The microbial count was measured as Cfu/mL and the average data was converted to logcfu/mL[18].

2-3- Encapsulation of probiotic bacteria

Encapsulation of bacteria was performed according to the method of Maleki et al. [15]. First, the wall materials including SPI, xanthan gum and fructooligosaccharide were mixed and prepared in different proportions in sterile phosphate buffer saline (PBS) after dissolution according to table (1). So, the concentration of the initial xanthan solution before mixing was 0.2% (w/v) in sterile saline phosphate buffer (PBS) and the concentration of SPI was 0.5% (w/v) in sterile saline phosphate buffer (PBS) and dissolution in pH 10 was performed. The prepared primary solutions were kept in the refrigerator at 4°C for 24 hours in order to completely rehydrate. Then, mixing was done with the proportions in the table (1) using a magnetic stirrer. Next, fructooligosaccharide powder was added to the resulting mixture according to the percentage determined in table (1) and stirred for 10 minutes using a magnetic stirrer.
In sterile conditions, 10 ml of bacterial suspension (bacterial concentration 10 $Cfu/ml^{9}7.5\times$) was prepared. Then, SPI and fructooligosaccharide were added to 40 ml of gum solution and mixed with a magnetic stirrer for 10 minutes. After this step, 2% sterile Tween 80 and 2% glycerol steril were added to the resulting suspension and mixed with a magnetic stirrer. It was mixed for 10 minutes. Before placing the prepared solution in the freeze dryer, the sample was frozen in a freezer at -30 degrees Celsius. Then, it was put into a freeze dryer under vacuum conditions at -80 degrees Celsius. The freeze-drying process lasted 48 hours. Dry microcapsules were collected and stored in sterilized aluminum containers at 4°C.

2-4- Efficiency of bacterial encapsulation

To determine the efficiency of microcoating, first, 1 g of the powder was added to 10 ml of sterile trisodium-citrate 2% w/v and mixed for 10 minutes using a Vortex mixer (Reax Control, Heidolph, Schwabach, Germany). After centrifugation at 5000 rpm at 37°C for 10 minutes, successive dilutions of the supernatant were prepared and cultured using MRS agar medium and incubated at 37°C for 48 hours. The efficiency of microcoating was calculated using the following equation [19]:

$$EE\% = \left(\frac{N}{N_0}\right) \times 100$$

in which, N live released bacteria and N_0 The initial amount of bacteria is added.

5-2- Survival*Lactobacillus acidophilus*

Viability is to determine the viability of probiotic bacteria during the expiration time of the product

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and entering the consumer's body and turning it into the established microbial flora of the intestine, which is used to strengthen the individual's immune system. For this purpose, the dilution of 0.1 g of*L. acidophilus*Covered inside was prepared and cultured in MRS agar culture medium and then incubated at 37°C for 5 days and finally the number of bacteria (Cfu/g) Log was counted [15].

6-2- Particle size and zeta potential

The particle size of the powder samples was measured using a DLS apparatus using a nanoparticle analyzer (Model SZ-100, Horiba Ltd., Kyoto, Japan) with a He-Ne laser at 25 °C. 2 ml of the sample was diluted twice with distilled water and the measurements were performed at the scattering angle of 173 degrees and the wavelength of 633 nm. Mean hydrodynamic particle size (meanz) based on intensity weighting using the Stokes-Einstein equation for the particle size distribution is given below. ζ potential was measured at a constant temperature of 25°C using a folded capillary electrophoresis cell. The results were obtained from software with tools (Horiba) [20].

2-7- FTIR spectroscopy

FT-IR analysis was used to study the chemical structure of microcapsules. FT-IR analysis of cm^{-1}4000 to cm^{-1}400 with a resolution of cm^{-1}4 and scan speed s^{-1}40, with 32 scans per sample, was performed using a spectrophotometer Bruker, Karlsruhe, Germany (Model Equinox 55 LS 101). KBr pellets were prepared with a ratio of sample to KBr (1:10 w/w) [21].

2-8- Scanning electron microscope

Scanning electron microscope (SEM) was used to evaluate the surface morphology and microstructural properties of the encapsulated powder produced by freeze drying. The samples were covered with a thin layer of conductive gold and then examined using a scanning electron microscope (JSM-840 SEM, Tokyo, Japan) with a voltage of 20 kV [22].

3- Experimental design and statistical analysis

In this research, combine design was used for 19 treatments according to table (1) using 12Design expert software. Analysis of variance and regression were used to check the effect of variables and obtain the model. In this study, the first type error level was $\alpha = 0.05$. Also, Excel 2016 software and Minitab 20 software were used to draw graphs and check other tests.

Table 1 The matrix of mixture design for microcapsules wall components

Run	Xanthan gum (%)	Soy protein isolate(%)	Fructooligosaccharide (%)
1	0	100	1
2	75	25	1.5
3	50	50	2
4	50	50	0
5	100	0	1
6	100	0	1
7	0	100	2
8	75	25	0.5
9	100	0	2
10	50	50	1
11	0	100	1
12	0	100	0
13	50	50	2

14	50	50	1
15	100	0	0
16	50	50	1
17	25	75	0.5
18	25	75	0
19	25	75	1.5

4- Results and discussion

4-1- Covering efficiency

Entrapment rate encapsulation efficiency*L. acidophilus*It shows the inside of the capsules. Based on the results of statistical analysis, the efficiency of bacterial encapsulation*L. acidophilus* Inside the capsules, it changes significantly ($p<0.05$) by changing the percentage of wall material. According to Figure (1), with increasing the level of xanthan gum and decreasing the percentage of SPI, the efficiency of the coating increased. Thus, the highest efficiency of coating was observed at moderate levels of SPI and xanthan gum. Also, with the increase of fructooligosaccharide level, the encapsulation efficiency first increased and then decreased. In general, the highest level of efficiency was observed at the levels of 75% xanthan gum, 25% SPI and 1% fructooligosaccharide. The reason for the increase in coverage is due to the presence of soy protein, xanthan gum, and fructooligosaccharides in the wall matrix, which provides benefits such as creating a protective coating on the bacterial cell wall and replacing part of the place of water molecules in the cells during the drying process. This action helps to protect the destruction of the cell membrane during the freeze-drying process and thus increases the efficiency of the encapsulation [20]. The highest efficiency is when SPI is in medium concentration. This may be due to the formation of a better protective layer on the bacterial cell membrane. Increasing SPI concentration at high levels causes excessive osmotic pressure in the intercellular environment, which results in cell water leaking out and causing plasmolysis and cell death, and finally, the efficiency of the endothelium decreases [15]. Similar results regarding the increase of encapsulation efficiency at medium levels of wall compositions were reported by Maleki et al. [15] for microencapsulation.*Lactobacillus rhamnos* with whey protein isolate wall, inulin and crystalline nanocellulose and Yasmin et al. [23] for encapsulation*Bifidobacterium longum* It was provided with whey protein isolate wall, pectin and alginate.

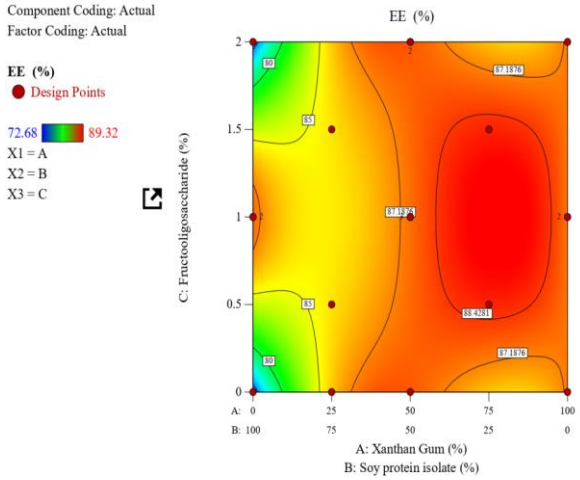

Fig 1 Encapsulation efficiency of microencapsulated *L. acidophilus*

4-2- Survival*Lactobacillus acidophilus*

Bacterial survival*L. acidophilus*It was evaluated at 37°C for 5 days. Figure (2) bacterial viability*L. acidophilus* Shows encapsulated. As can be seen, increasing the concentration of SPI increased the viability of the encapsulated bacteria. In the sample containing xanthan gum and without soy protein isolate, the bacterial viability was the lowest. Nevertheless, xanthan gum had a positive effect on bacterial survival due to its synergistic property with soy protein, which was due to the low solubility of soy isolate and its precipitation in the proportions prepared with xanthan. As a result of encapsulation*L. acidophilus* With SPI, xanthan gum and fructo-oligosaccharide, it led to a significant improvement in the viability of encapsulated bacteria. These findings are in line with the results reported by Huq et al. [24] for

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encapsulation*Lactobacillus rhamnos* with alginate, nanocrystal cellulose and lecithin and Maleki et al. [15] for microencapsulation*Lactobacillus rhamnos* It was crystalline with whey protein isolate, inulin and nanocellulose.

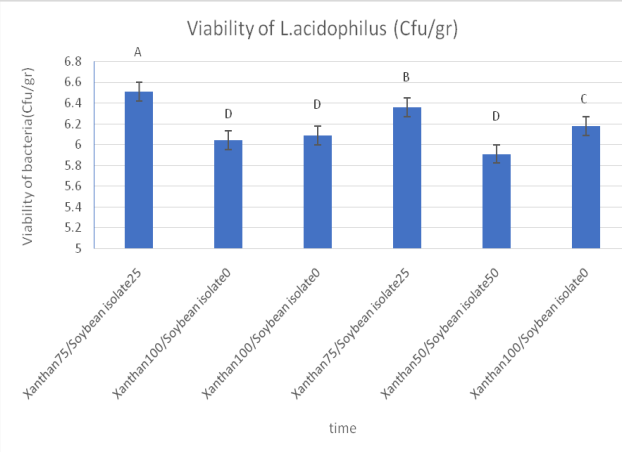

Fig 2 Viability of microencapsulated *L. acidophilus*

4-3- Particle size and zeta potential

Many properties of emulsions depend on particle size distribution and average diameter. Therefore, it is necessary to know the size of emulsion particles and to control and measure the size of the particles in checking the stability and quality of emulsions. The results showed that the change in wall material concentration had a significant effect on the size of microcapsules ($p < 0.05$). According to Figure (3), with increasing SPI percentage, the particle size of prepared capsules increased. This situation can be due to the increase of SPI concentration, the chance of collation between hydrophobic and sulfhydryl groups from one protein group to other protein groups increases [1]. In general, the difference in the particle size of different samples with different concentrations of wall materials may be related to the different properties of gelation and creating a solution of the materials used [20]. The results obtained were in agreement with the findings of Maleki et al. [15].

Zeta potential of capsules is another important factor in powders to evaluate the stability of colloids. The high zeta potential of colloidal particles increases the electrostatic repulsion force and thus increases the physical stability of the system. The balanced range of zeta potential is considered above mv30+ or below mv30- [25]. According to the obtained results, the value of zeta potential for different samples is less than -mv30, which indicates the appropriate colloidal balance of the produced microcapsules, which stability is important in preventing accumulation [26]. Also, based on the results, with increasing SPI concentration, the zeta potential of the samples decreased (Table 2).

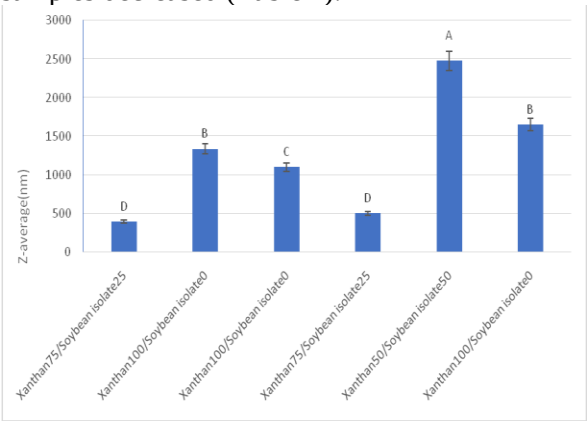

Fig 3 Z average of microencapsulated *L. acidophilus*

Table 2 Zeta potential of microencapsulated *L. acidophilus*

Sample	Zeta potential (mV)
Xanthan75/ Soy Protein Isolate 25	-2.56
Xanthan100/ Soy Protein Isolate 0	-3.33
Xanthan100/ Soy Protein Isolate 0	-12.3
Xanthan75/ Soy Protein Isolate 25	-13.7
Xanthan50/ Soy Protein Isolate 50	-10.6
Xanthan100/ Soy Protein Isolate 0	-7.11

The lowest zeta potential corresponding to the sample with pure xanthan gum wall

Was. While the combination of xanthan gum with other matrix wall materials such as SPI and fructooligosaccharide caused a synergistic effect and increased zeta potential.

4-4- FTIR spectroscopy

FTIR spectroscopy is used to evaluate the interaction between sample compounds [27]. FTIR sample of soy protein isolate, xanthan gum and encapsulated Lactobacillus acidophilus sample with xanthan gum wall, SPI and fructooligosaccharide are shown in Figure (4). As can be seen, the FTIR absorption pattern of the sample in the range of cm^{-1} 500 to cm^{-1} 4000 was shown. In the spectrometry related to SPI in Figure 4-A, an index peak in cm^{-1} 3417 is observed to be related to OH and N-H groups on soy protein strands [7]. Peak index in cm^{-1}2931 associated with C-H stretching bond and peak in cm^{-1} 1649 is probably due to the structure of the first type of amide (C=O vibrations), which is the

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most important spectrum in identifying the secondary structure of the protein [19]. Another absorption peak in cm^{-1}1530 is caused by the presence of N-H groups related to the second type of amide. The peak can be seen in cm^{-1} 1239 indicates the presence of C-N stretch bond in third type amide [14]. The peak in cm^{-1}1038 is due to hydrogen bonding in SPI [1].

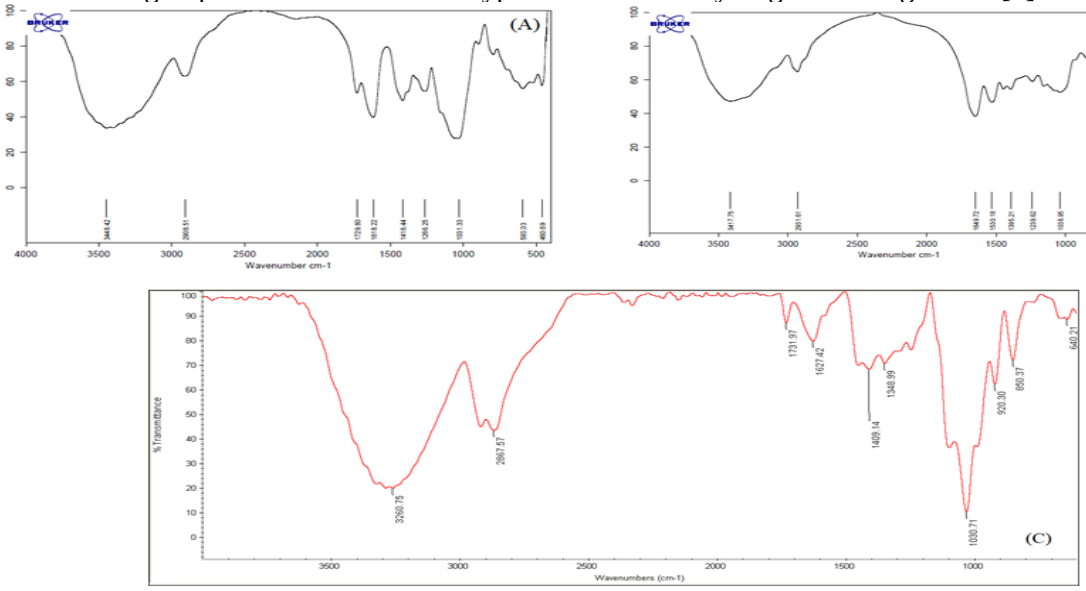


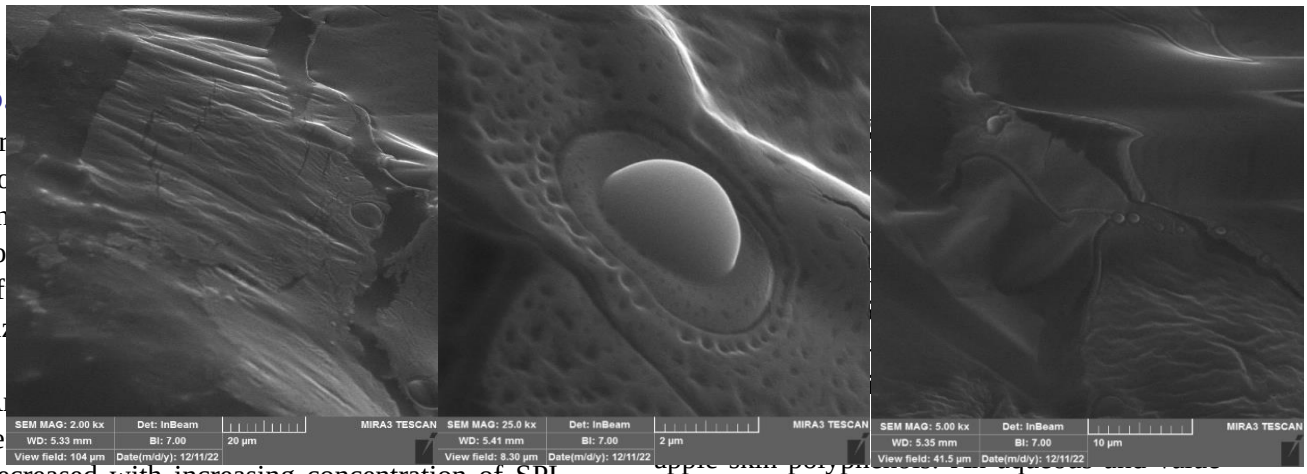
Fig 4 FTIR spectra of the optimum sample

The spectrum related to xanthan gum shows the index peak in cm^{-1} 3448, which indicates the presence of O-H stretching bond and another peak cm^{-1} 2908 is due to C-H stretching bond (Figure 4-B) [20]. Another significant peak in cm^{-1} 1729 is related to C=O vibrational stretching bond. Peak in cm^{-1} 1618 and cm^{-1} 1416 is due to symmetric and asymmetric vibrational bonds -COO-. Peak index in cm^{-1} 1031 also represents C-O-C stretch bond[23].

The peak corresponding to the optimal sample containing SPI and xanthan gum shows a slight change in the peaks in the absorption region. Absorption peak in cm^{-1} 2867 is attributed to symmetric and asymmetric C-H groups [1]. Available peaks in cm^{-1}910 to cm^{-1}1750 is related to the presence of carboxylic acid groups in the cell wall of gram-positive bacteria [15]. Visible absorption peak in cm^{-1}1348 is related to C-N stretching bond and N-H bending bond [15]. Peak in cm^{-1} 1030 indicating the presence of =CH and =CH vibration bonds$_2$is [14]. The peaks at 850 to 1000 are due to the presence of monosaccharide C-H bonds [16].

4-5- Scanning electron microscope

Scanning electron microscope images of the encapsulated sample*L. acidophilus*with xanthan gum wall, SPI and fructooligosaccharide are given in figure (5). According to the pictures, a smooth and uniform surface without cracks and cracks is observed on the surface of the microcapsules, which indicates less fragility of the system wall. This helps to resist the mechanical forces associated with expansion and inflation during spray drying. In addition, it can provide better protection of probiotic microorganisms by reducing air permeability. Indentations are also observed on the surface of the sample, which may be related to the low temperature in drying and the combination of materials used in the wall for encapsulation [20]. Also, these irregularities may be caused by the presence of probiotics inside the capsules. Similar results were reported by Rajam and Anandharamakrishnan [20] who stated that the surface without cracks and splits for the encapsulated sample.*Lactobacillus plantarum* observed with fructooligosaccharide wall. In addition, Motalebi Moghanjougi et al. in [28] similar results for microencapsulation*Lactobacillus acidophilus* And*Bifidobacterium* reported with pectin wall and sodium alginate. In addition, these results are in line with the findings of Cabuk and Harsa [29] and Amiri et al. [30] for the overlap *L. acidophilus* It was with pullulan and whey protein.



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decreased with increasing concentration of SPI and xanthan gum, respectively. Also, the coating efficiency increased as a result of the increase in the percentage of xanthan gum, but the increase in SPI caused the coating efficiency to decrease. The particle size of the samples also increased with increasing SPI concentration. FTIR and SEM tests were performed to study the microstructure and morphology of the optimal sample. In general, based on the results of this study, it can be seen that the combination of SPI, xanthan gum, and fructooligosaccharide has the necessary potential for encapsulating probiotics as wall materials that can be used to enrich food products.

6- Resources

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درون پوشانی *لاکتوباسیلوس اسیدوفیلوس* PTCC 1643با دیواره کامپوزیتی صمغ زانتان، ایزوله پروتئین سویا و فروکتوالیگوساکارید: بررسی ویژگی‌های میکروکپسول‌ها

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چکیده

درون پوشانی روشی موثر برای افزایش زنده مانی باکتری‌های پروبیوتیک می‌باشد. هدف از این پژوهش تعیین فرمولاسیون بهینه برای میکروکپسولاسیون *لاکتوباسیلوس اسیدوفیلوس* PTCC 1643 با ایزوله پروتئین سویا، صمغ زانتان و فروکتوالیگوساکارید به عنوان مواد دیواره با روش خشک کردن انجمادی می‌باشد. بدین منظور ۱۹ تیمار با طرح Combine توسط نرم افزار Design expertتهیه شدند. آزمون‌های کارایی درون پوشانی، اندازه ذرات، پتانسیل زتا، و زنده مانی پروبیوتیک، FTIRو SEM بر روی نمونه‌ها انجام شد. نمونه بهینه بر اساس کمترین پتانسیل زتا و اندازه ذرات انتخاب گردید و ویژگی‌های میکروکپسول، با آزمون‌های فوق‌الذکر تعیین گردید. نتایج نشان داد زنده مانی پروبیوتیک با افزایش غلظت ایزوله پروتئین سویا و صمغ زانتان به ترتیب افزایش و کاهش یافت. اندازه ذرات نمونه‌ها نیز با افزایش غلظت ایزوله پروتئین سویا افزایش یافت. به طور کلی با توجه به اثر محافظتی صمغ زانتان، ایزوله پروتئین سویا و فروکتوالیگوساکارید بر پروبیوتیک‌ها می‌توان نتیجه گرفت که این ترکیبات به عنوان دیواره برای درون پوشانی می‌توانند برای غنی سازی محصولات غذایی مورد استفاده قرار گیرند.