Bionatura
Latin American Journal of Biotechnology and Life Sciences

Bionatura Issue 2 Vol 8 No 1 2023

Article

Characterization of purified dextran from *Lactobacillus fermentum*

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Available from: http://dx.doi.org/10.21931/RB/CSS/2023.08.02.33

ABSTRACT

The objective of the current study is to characterize dextran purified from *Lactobacillus fermentum* (Lb4) Isolates of *Lactobacillus* spp. were tested for dextran production using mucoidy, ethanol precipitation and spectrophotometric method. Results of the Spectrophotometric method showed that all isolates gave concentrations of dextran ranging from (0.1 to 0.2 mg/ml). Precipitation, purification and characterization of dextran from *L. fermentum* (Lb4) were done. Thin-layer chromatography (TLC), 1H Nuclear Magnetic Resonance (HNMR) Spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Field Emission Scanning Electron Microscopy (FE-SEM), Gas Chromatography-mass spectrometry (GC-MS), Melting Point (MP), Water Solubility index (WSI), and Water Holding Capacity (WHC) had been used for dextran characterization. Characterization proved that pure dextran. Nanosphere structure with a nano-diameter range of about (56.00) nm with molecular weight (73) kDa. According to the melting point test, the purified dextran from L. fermentum had been completely melted at 228°C. The water solubility index (WSI) had been determined (93.35%) and the water holding capacity (WHC) (300%).

Keywords: *Lactobacillus* spp. ; Dextran ; *Lactobacillus fermentum*

INTRODUCTION

Dextran is one of numerous EPS that has gained worldwide recognition for its biodegradability and biocompatibility [9] . Dextran is a bacterial homo-polysaccharide cationic polymer whose main chain is made up of several -glucans linked by -(1-6) glycosidic bonds with varying amounts of branched linkages such as -(1-2), -(1-3), and -(1-4) linked as a single unite or lengthened side chain, the degree of branching depending on the bacterial strain used for production [27,13,14]. The discovery and characterization of new dextrans, as well as their structural properties (molecular weight, linkages, and branches) and potential uses in medical, food, and research fields. Furthermore, extraction sources for dextran-producing bacterial strains are emphasized. Despite being widely known and produced mainly within the food industry, dextran is also known for its suitability for biomedical applications, due to its biodegradability and relative biocompatibility. Incorporating dextran in drug delivery systems takes advantage of its structural integrity in forming hydrogels [8].

Lactobacillus is a gram-positive, nonsporeformer, nonmotile, facultative or microaerophilic bacillus with catalase and oxidase-negative properties. *Lactobacillus*

is considered the oldest and most widely used probiotic [7]. *Lactobacillus* contains many species commonly accepted as healthy and/or included in the safety lists qualitative presumption, including many strains being widely used in food and human nutrition because of their contribution to the processing of fermented foods or their probiotic usage [15]. The most widespread species are those following: *L. acidophilus, L. plantarum, L. casei, L. rhamnosus, L. delbrueckii bulgaricus, L. fermentum, L. reuteri* [2], *L. reuteri, L. cellobiosus, L. casei, L. paracasei, L. curvatus, L. fermentum, L. florum, L. fructivorans, L. kunkeei, L. diolivorans, L. hilgardii, L. plantarum, L. brevis, L. buchneri, L. mali, L. oeni, L. nagelii, L. vini ,L. gasseri, L. jensenii* [29] , at last *L. delbrueckii* subsp. *bulgaricus*, and L. delbrueckii subsp. lactis are the most common thermophilic starters used in the dairy industry [10]. *Lactobacillus* species produce antimicrobial agents, which have a protective or therapeutic impact: reduced gut pH due to stimulation of the lactic acid producing microflora, competition for binding of pathogen-occupied receptor sites, stimulation of immunomodulatory cells, and competition with pathogens for available nutrients at all resulted in positive tolerance to excited pH, NaCl, and bile salts, as well as antimicrobial activity against pathogens [14]. Therefore, this study aimed at producing and characterizing dextran from local probiotics.

MATERIALS AND METHODS

Lactobacillus spp. Isolates

Nine isolates of *Lactobacillus* spp. were isolated from vaginal swabs and infant stool. They were identified using cultural, microscopical, and biochemical tests and the Vitek 2 system using an ANC ID card.

Screening of Lactobacillus spp. Isolates for the Dextran production

Mucoidy Method

By spotting 2ul of bacterial suspension on medium, all isolates of Lactobacillus were inoculated on dextran screening agar (150 g sucrose, 5.0 g bacto_ peptone, 5.0 g yeast extract, 15 g k2HPO4, 0.01 g MnCl2.H2O, 0.01 g NaCl, 0.05 g CaCl2, 50 g agar). Isolates were incubated at 37° C for 24 hours. Visual appearance was used to determine mucoidy colonies, and ropiness was determined by touching them with a sterile loop [19]. Biopolymer dextran producers were identified among the isolates that generated mucoid colonies.

Ethanol Precipitation Method

Discs of sterile filter paper (5 mm) were placed on a dextran screening agar plate and were inoculated with 5 µl of cultural broth from each Lactobacillus isolates to detect the ability of each isolate for biopolymer production [22]. Biopolymer production was assessed based on mucoid colony around these discs. The ability of biopolymer production of isolates was confirmed by mixing mucoid substances in 2 ml of absolute ethanol tubes, forming EPS precipitate, indicating the ability of biopolymer production [6].

Determination of Dextran Concentration by spectrophotometric method

The dextran concentration in the cell-free supernatant of Lactobacillus isolates (9×108 cfu/ml) with absorbance at 600 nm about 0.134 grown in dextran production broth was determined by phenol-sulphuric acid method in a micro-titre plate. To 25 ul of the sample containing dextran in a microtitre plate, (25) ul of 5% (v/v) phenol was added. The mixture was mixed by shaking the plate on a vortex mixer for (30)s. Then the plate was placed in an ice bath and (125) ul of concentrated sulphuric acid was added to each well containing the mixture. The plate was again shaken for 30 seconds to ensure proper mixing of the contents of the wells. Then, the plate was covered and incubated in a water bath at 80°C for 30 min. After cooling to room temperature, the absorbance was determined at 490 nm [22]. A standard graph was plotted using standard dextran (70 kDa).

Dextran production

This process was carried out according to the procedure described by [18] with some modifications, where 250 ml flasks containing (100) ml of dextran production medium (150 g sucrose, 5.0 g peptone, 15.0 g K2HPO4, 0.01 g MnCl2.4H2O, 5.0 g yeast extract, 0.01 g NaCl, and 0.05 g CaCl2 into 1 liter distilled water) were inoculated with 2% of a chosen *L. fermentum* (Lb4) suspension containing (9 x 10^8 cfu / ml) with absorbance at a (600 nm about 0.134), incubated for 24 hours at 30 and 37°C.

Precipitation of Dextran

After 24 hours, the bacterial bacteria culture was precipitated with an equivalent volume of cold ethanol, agitated rapidly, centrifuged at 10,000 rpm for 15 minutes, and the supernatant decanted. Impurities were removed by dissolving precipitated dextran in distilled water. With an equivalent volume of cold ethanol, the dextran slurry was precipitated once more. The redissolving, precipitation, and washing operation was performed three times to remove cell debris. Dextran was precipitated and dried in an oven at 40°C for 45 minutes. On a dry weight basis, the dextran yield was determined [1].

Purification of Dextran Produced by Lactobacillus fermentum

Dextran recovered from precipitation was diluted in distilled water for impurity removal, and the dextran slurry was precipitated with an equal volume of cold ethanol and centrifuged for 10 minutes at 12,000 rpm [1]. The redissolving, precipitation, and washing method was repeated five times to remove cell debris. Purified dextran was dried in an electric oven at 40°C for 45 minutes, and the dry weight of the dried dextran was calculated. The purity of dextran was analyzed by ultraviolet-visible spectrophotometry [17], purified, and standard dextran were dissolved in distilled water to a final concentration of 5% and were analyzed by UV-vis spectrophotometry. The UV absorption spectrum of the sample was recorded in the wavelength range of 200-800 nm.

Characterization of Purified Dextran

Analysis of Dextran by Thin–Layer Chromatography (TLC)

Dextran (0.01 gm) purified from Lactobacillus fermentum was hydrolyzed in 5 % HCl (v/v) and heated for an hour in a water bath at 100ºC. Equal weights (0.01gm) of glucose, fructose, and galactose were dissolved in 1 mL of 1% ethanol. Thin-layer chromatography (TLC) has been performed by using silica. The position and distance of the spots were determined, and the relative flow (Rf) had been calculated as described by [25] :

$$RF = \frac{\text{Distance moved by substance}}{\text{Distance moved by the solvent front}}$$

Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) was performed utilizing (Bruker – Tensor 27 with ATR uint) The instrument measures the amount of infrared radiation reflected or transmitted through a sample and operates in the wave number range of (400 - 4000 cm 1). The outcome is presented as a graphical chart, with the X-axis representing the wave number and the Y-axis representing the percent transmittance.

1H Nuclear Magnetic Resonance (HNMR) Spectroscopy

Purified dextran was analyzed using HNMR Spectroscopy by sending the dextran powder to Tehran, Iran.

Field Emission Scanning Electron Microscopy (FE-SEM)

Field Emission Scanning Electron Microscopy (FE-SEM) was used to characterize purified dextran by sending the powder to Tehran, Iran.

Gas Chromatography-mass spectrometry (GC-MS)

For this analysis, the purified and standard dextran were derivatized with hydroxylamine chloride in pyridine to 2.5% for 30 min at 70ºC, to form the oximes of sugars. Afterward, bis-trimethylsilyl trifluoroucetamide was added to form the trimethylsilylated derivatives for 45 min at 80°C. Identification and quantification were performed by GC-MS equipped with a Column HP 5MS (30 m × 0.25 mm ID × 0.2 um film thickness) with He as the Carrier gas. The injector and detector were set at 275ºC. Samples (1µl) were injected with a split ratio of 1:50 with a temperature program: 80°C for 4 min, then 15°C for 1 min to 270°C and finally 30°C for 1 min to 310°C (2 min). The chromatogram peaks corresponding to sugars and dextran were identified by their retention times. Quantification was performed by determination of the peak area compared with a calibration standard curve [20].

Melting Point

Melting point test was studied using (Melting Point, Digital, Advanced, SMP30). The capillary tube was filled at one end with dextran to a 2-3 mm depth. Then, it was placed in a melting point apparatus and heated. Two temperatures were recorded at this Point: the temperature at which the substance started to liquefy and the temperature at which it became entirely liquefied.

Water Solubility Index (WSI)

The water solubility index (WSI) determines the solubility of a given material in water. The WSI of dextran was determined using the method described by Domzal-Kedzia et al.(2019). 200 mg of the sample was dissolved in 5 ml of Milli-Q water and stirred for 40 min in a water bath at 40°C to obtain a uniform suspension. Then, the sample was centrifuged at 4000g for min, and the supernatant was placed in a petri dish and dried at 105°C for 4h to obtain a dry solid weight. Then, the WSI was calculated based on the following equation :

$$\text{WSI (\%)} = \frac{\text{Dry weight of solids in supernatant}}{\text{weight of dry sample}} \times 100\%$$

Water Holding Capacity (WHC)

The water-holding capacity (WHC) represents the quantity of water material that is apt to retain. The result is the amount of water the polymer can retain in its molecule. The WHC of collected dextran samples was determined as per the method of [17]. 200 mg of the sample was dissolved in 10 ml of Milli-Q water and kept at 40 °C for 10 min. Then, the sample was centrifuged at 14,000g for 30 min, and the supernatant was dumped. The pellet was then put on pre-weighed filter paper to remove the water. The weight of the precipitated sample was noted. The percentage of WHC was calculated according to the following equation:

$$\text{WHC (\%)} = \frac{\text{Total sample weight after water absorption}}{\text{Total dry sample weight}} \times 100\%$$

RESULTS

Screening of Lactobacillus spp. Isolates for the Dextran production

The detection and screening of dextran production for nine isolates of *Lactobacillus* were recorded according to mucoid colonies on the surface of the dextran screening medium. All isolates gave mucoid colonies.

Ethanol precipitation method

The ethanol precipitation method was used to confirm dextran-producing isolates. The preferable method for detecting dextran production is ethanol precipitation, which was carried out on the positive isolates to the mucoidy method and showed that all isolates gave a precipitate but at a variable content of precipitation.

Estimation of dextran concentration by Spectrophotometric method

Dextran concentration was determined using the spectrophotometric method; results showed all isolates that gave a dextran concentration between (0.1 to 0.2 mg/ml) (Table 1).

***Lactobacillus* spp.**	**Dextran Concentration mg/ml**
***Lactobacillus acidophilus* (Lb1)**	**0.2**
***Lactobacillus acidophilus* (Lb2)**	**0.1**
***Lactobacillus acidophilus* (Lb3)**	**0.2**
***Lactobacillus fermenti* (Lb4)**	**0.2**
***Lactobacillus fermenti* (Lb5)**	**0.1**
***Lactobacillus gasseria* (Lb6)**	**0.2**
***Lactobacillus gasseria* (Lb7)**	**0.2**
***Lactobacillus plantarum* (Lb8)**	**0.2**
***Lactobacillus plantarum* (Lb9)**	**0.1**

Table 1. The concentration of dextran produced by probiotics bacteria

Precipitation and purification of Dextran

After the precipitation of dextran, the purification of dextran is produced by Lactobacillus fermentum (Lb4). After purification, the total dry weight of dextran was 0.471 g / L . Purified dextran was characterized by a whitish color with dry chalk powder. The purity of the dextran was confirmed by UV-vis spectrophotometry. The absence of peaks between 260 and 280 nm indicated that the *L. fermentum* (Lb4) dextran was free from nucleic acid and proteins.

Characterization of Purified Dextran

Analysis of Dextran by Thin-Layer Chromatography (TLC)

TLC analyzed dextran contents to determine its components of monosaccharides. The RF values of purified dextran were similar to the RF of standard dextran (Figure 1).

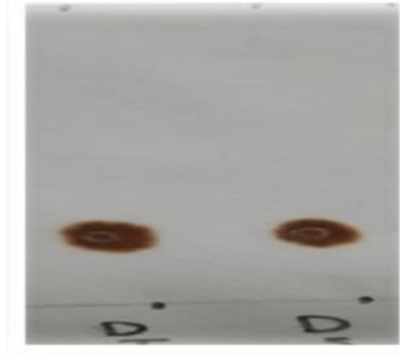

Figure 1. Thin - Layer Chromatography analysis of dextran purified from *Lactobacillus fermentum* (Lb4). • T : Dextran of Lactobacillus fermentum (Lb4) ; Ds : standard dextran .

Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) spectra analysis was used to detect the functional groups of purified dextran from Lactobacillus fermentum (Lb4). The characteristic peaks of dextran indicated that the purified dextran was of the polysaccharide type. The band in the region of (3389 , 3389) cm-1 for standard dextran and purified dextran from L. fermentum (Lb4) respectively was due to V(OH) streaching vibration of the polysaccharide , while the band in the region in (2941 , 2942) cm-1 for standard dextran and purified dextran respectively, was due to C-H streaching vibration, while the band region found in (1145) cm-1 for standard dextran and purified dextran was assigned to valent vibration of C-O-C (glycosidic bond),whereas the region in 1014 cm-1 for each standard dextran and purified dextran was due to the great chain flexibility in dextran around the α-(1,6) glycosidic bond, the band region found in (772 , 774) cm-1 for standard dextran and purified dextran respectively, was due to γ(OH), the absorption peaks at (915) cm-1 for both standard dextran and purified dextran , confirmed α-glycosidic bond, the band region in the 838 cm-1 for each standard dextran and purified dextran , was due to (1-3)α-D-glucan , the band region found in (644) cm-1 of standard dextran and purified dextran from L. fermentum (Lb4) was indicated to the extension of a glycosidic bond (Figure 2 ,3). FTIR spectra analysis of standard dextran and purified dextran from L. fermentum (Lb4) showed dextrans contained both α(1,6) and (1-3) linkages, which confirmed that the purified polysaccharide was dextran.

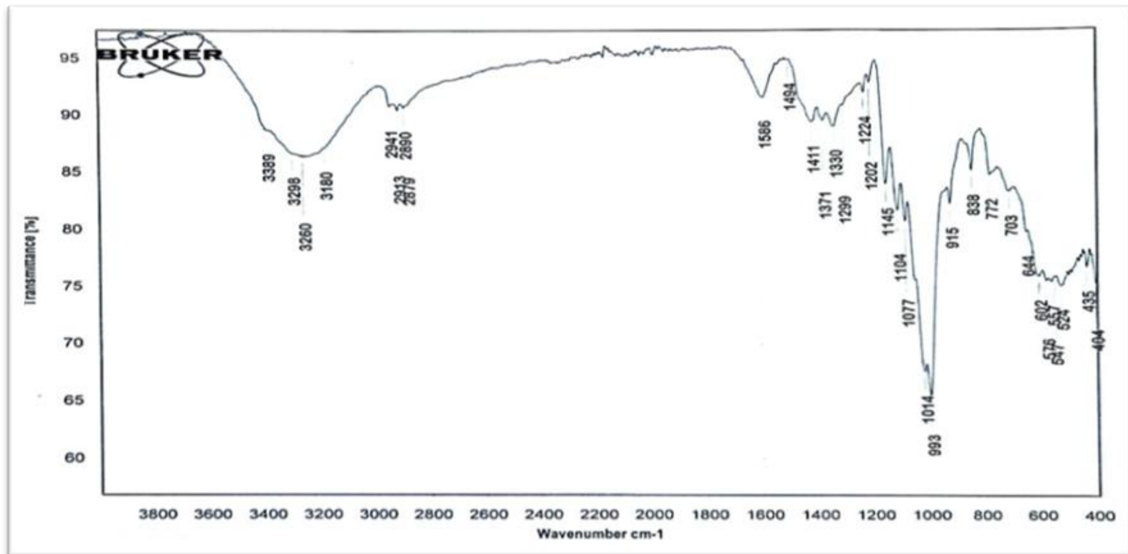

Figure 2. FTIR spectra of Standard dextran.

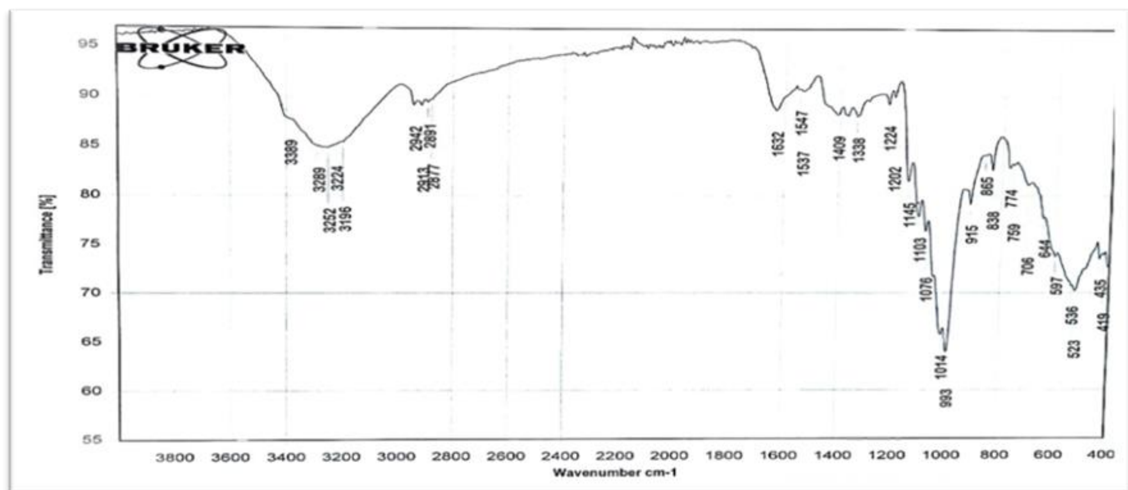

Figure 3. FTIR spectra of dextran purified from *Lactobacillus fermentum* (Lb4).

1H Nuclear Magnetic Resonance (HNMR) Spectroscopy

NMR mainly simplifies the dextran analysis steps and provides information on the polysaccharide structure. The anomeric proton resonances for the 400 MHz 1H NMR spectrum result indicated that purified dextran from *L. fermentum* (Lb4) similar to standard dextran are shown (Figure 4, 5). The 1H NMR spectra contained signals indicated that 3.03 – 3.04 ppm (Ha), 3.05 – 3.10 ppm due to (Hb), 3.36 ppm due to (H_2O), 3.39 – 3.40 ppm (Hd), 3.41 – 3.43 ppm (He), 3.53 – 3.55 ppm (CH_2) of α-(1→3), 3.58 – 3.60 ppm (CH_2) of α-(1→6), 3.61 – 3.64 ppm (HC), 4.35 – 4.36 ppm OH(1), 4.44 – 4.46 ppm OH(3), 4.51 – 4.56 ppm OH(4), 4.63 – 4.64 ppm OH(5) of α-(1→3), 4.76 – 4.77 ppm OH(5) of α-(1→6), 5.41 – 5.55 ppm OH(6) of α-(1→3), 6.20 – 6.21 ppm OH(6) of α-(1→6). The results showed that purified dextran contains α-(1→6) and α-(1→3) bonds.

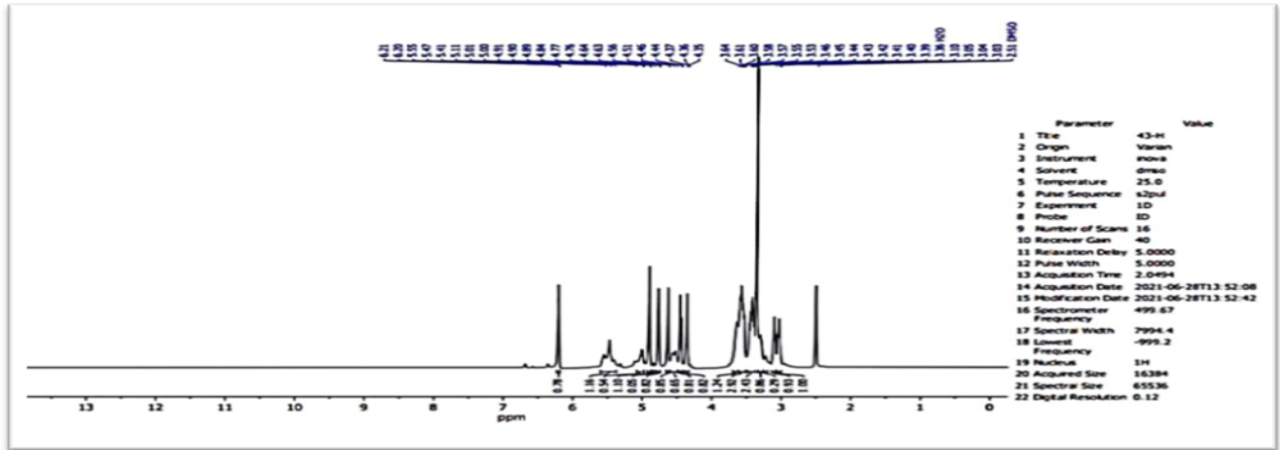

Figure 4. 1H Nuclear magnetic resonance (HNMR) of standard dextran.

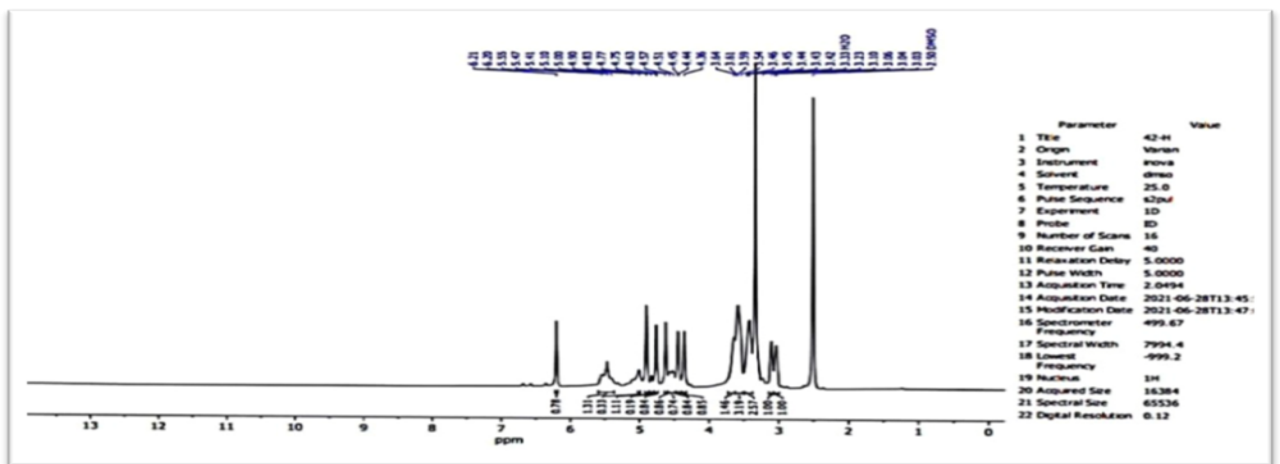

Figure 5. 1H Nuclear magnetic resonance (HNMR) of purified dextran from *Lactobacillus fermentum* (Lb4).

Field Emission Scanning Electron Microscopy (FE-SEM)

The surface micrograph of standard and purified dextran powder from L. fermentum (Lb4) consists of heteromorphic distribution with a nano-diameter range of about (70.51 and 56.00) nm, respectively, so for this reason, the nature of the purified dextran from Lactobacillus is dry, which increased their potential for different applications such as industry, pharmacy and medicine. The pores diameter of dextran purified from Lactobacillus is smaller than the pores diameter of standard dextran. (Figure 6, 7).

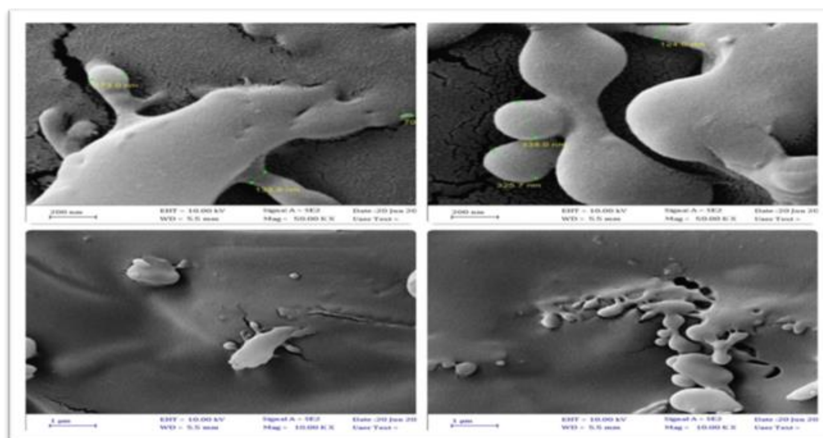

Figure 6. FE-SEM images of standard dextran.

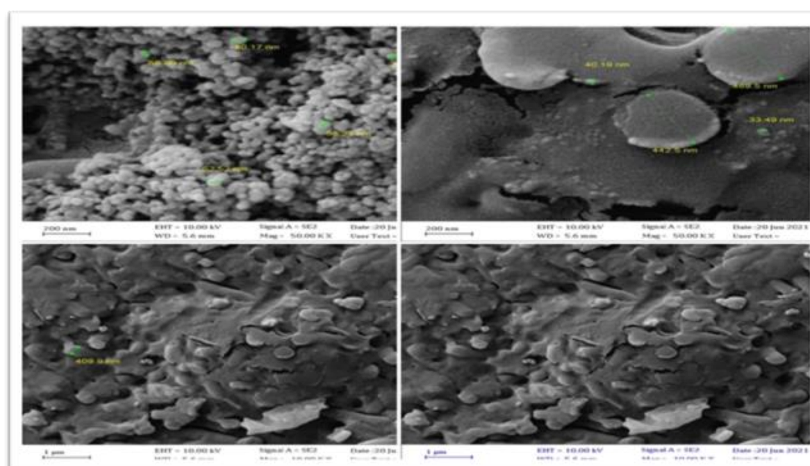

Figure 7. FE-SEM images of dextran purified from *Lactobacillus fermentum* (Lb4).

Gas Chromatography-mass spectrometry (GC-MS)

Gas Chromatography - mass spectrometry was used to analyze the monosaccharide composition of standard and purified dextran from L. fermentum (Lb4). The standard and purified dextran showed the same level of similarity in terms of the break-down sites of the monosaccharides, which include (42, 43, 60, 73) regions as indicated in (Figure 8, 9). Therefore, it was concluded that the molecular weight of dextran purified from L. fermentum (Lb4) was about 73 KDa, close to the molecular weight of standard dextran, 70 KDa. On the other hand, standard and purified dextran have the same level of similarity; the shape is the same crystals, but the crystal structure is different.

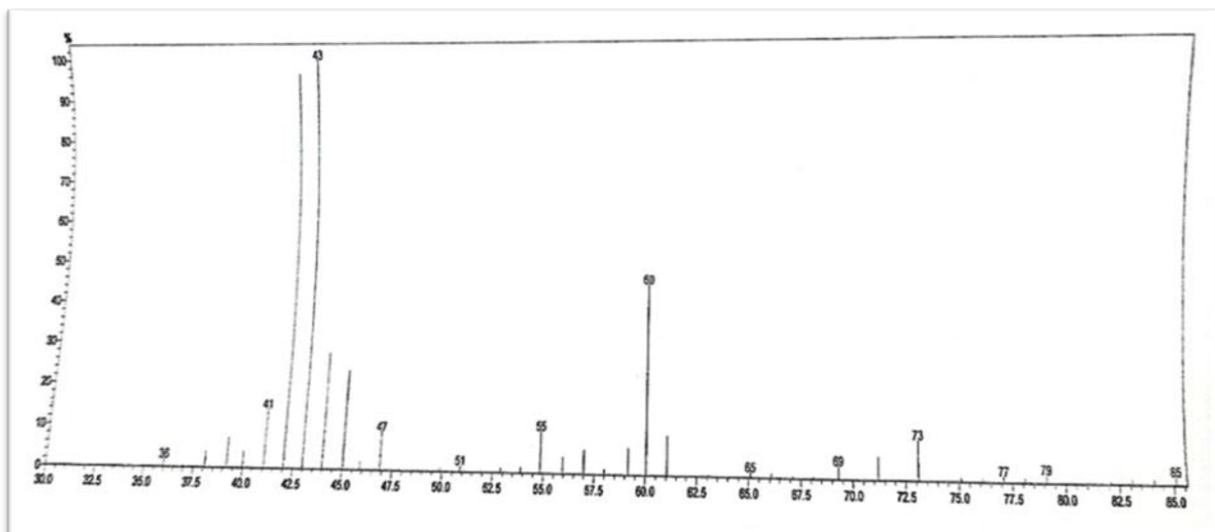

Figure 8. Gas chromatography of standard dextran.

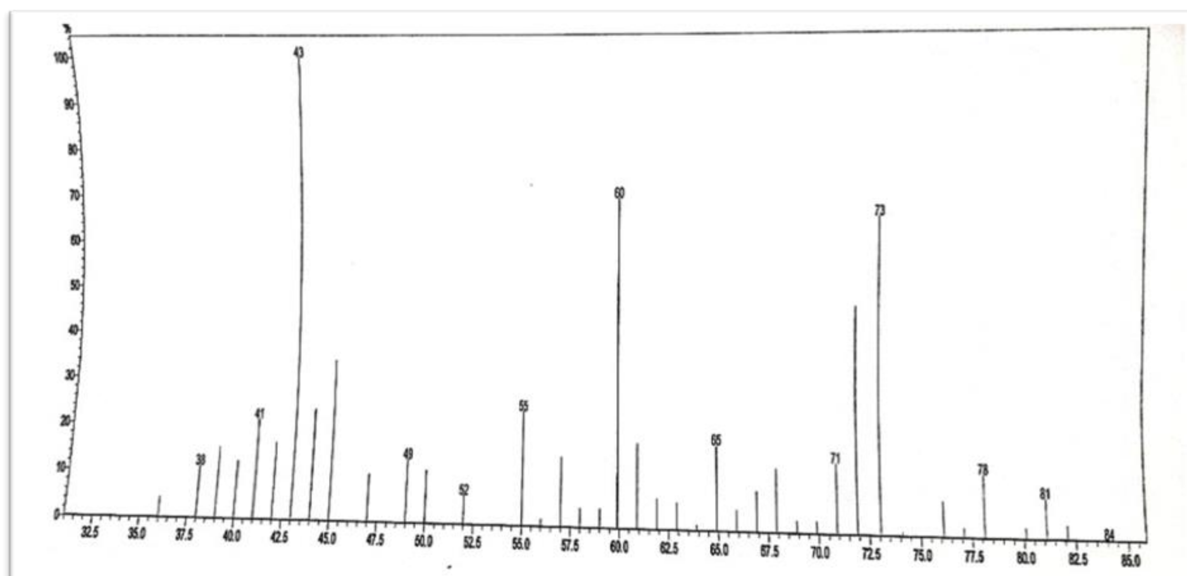

Figure 9. Gas chromatography of purified dextran from *Lactobacillus fermentum* (Lb4).

Melting Point

Melting point analysis was used to detect the temperature at which the dextran began to liquefy. Results showed that the purified dextran from L. fermentum (Lb4) began in liquefy at 225° C, and at 228° C, the dextran completely melted.

Water Solubility Index (WSI) and Water Holding Capacity (WHC)

The water solubility index has been used to determine a substance's degree of solubility in water (WSI). A substance's water-holding capacity (WHC) is the amount of water it can hold. The results showed that the purified dextran from L. fermentum (Lb4) possesses WSI (93.35%) and WHC (300%). Dextran purified from *L. fermentum* (Lb4) WHC requires a Micro-porous structure, which makes it an attractive component for cosmetics manufacturing.

DISCUSSION

Dextran is complex glucan formed by a main chain of D-glucose linked by α-(1→6) bonds with possible branches of D-glucose with α-(1→4), α-(1→3), or α-(1→2) bonds [4], In study by Salman and Kareem, (2020)[26] found the dextran contents that produced by L. gasseri were analyzed by TLC, the RF values of glucose were identical or so close to the hydrolyzed dextran. The Rf value of dextran was 0.33, While the Rf values of glucose, fructose, and galactose were (0.33, 0.24, 0.53) respectively. This result indicated that purified dextran from L. gasseri was composed only of glucose.

In a study done by Vettori *et al*. (2012) [30] found dextran (EPS-F2) exhibited a broad and intense peak at 3406.04 cm−1 due to the stretching vibration of O-H in its EPS chain. Park *et al*. (2022)[23] showed that the band stretched at 2,946 cm−1 corresponds to the bending vibrations of the C-H group. According to a study by Chen *et al*. (2021) [5,] a strong absorption band at 2931.04 cm−1 could be ascribed to the C-H stretching vibration. Gu et al. (2020)[11] showed that the absorption band observed at 1155.16 cm-1 might be attributed to the stretching and bending vibration of the C-O-C bond and glycosidic bridge. As well as a strong absorption peak at 1015,49 cm-1 indicated that this dextran (EPS-F2) contained α-(1→6) glycosidic bonds [18]. Some research by Suner et al. (2018) asserts that if the dextran molecule were linear, it would be soluble because its hydroxy groups (-OH) would be exposed to interact with water molecules. On the other hand, reported that the greater the number of branches, the greater the solubility of the dextran due to the increase in amorphous areas in the molecule that favor water adsorption and retention, Whistler and Balani *et al* . (1973) [32] also found that the main chain of dextran with α-(1→6) bonds adopts a helical shape, which is modified by the presence of branches (α-(1→2), α-(1→3) or α-(1→4)), such that the linear structure of glucan is repeatedly folded.

Khan *et al*. (2007) and Purama *et al*. (2009)[16-24] reported that the minor pore distribution can cause dextran to hold water and thus be used as a texturing agent in the food industry. These minor pores may also be responsible for the compactness of the polymer and the stability of the gel structure when subjected to external forces and the maintenance of the texture properties through storage. Semor *et al*.(2018) and Vuillemin *et al.* (2018)[28-31] reported that the properties of dextran, regarding rheology, depend on its structure and size. On the other hand, Wu *et al*.(2018)[32] used dextran to develop an intrapocket delivery system of minocycline for periodontitis treatment. They applied ion pairing /complexation technique to formulate minocycline-calcium-dextran sulfate complex microparticles with high encapsulation efficiency (97%) and high drug loading) (45%). The solubility and rheological properties of dextran are affected by its molecular weight and branching [12].

CONCLUSION

In conclusion, Lactobacillus fermentum (Lb4) produced biopolymer dextran in sufficient quantity with nano-structure shape, high water solubility and the ability to hold water.

Acknowledgments

The authors thank Mustansiriyah University in Iraq(www.uom ustansiriyah.edu.iq) for supporting the current work.

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Received: May 15, 2023/ Accepted: June 10, 2023 / Published: June 15, 2023
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