Journal of Food Biosciences and Technology,
Islamic Azad University, Science and Research Branch, Vol. 14, No. 2, 59-69, 2024

Effects of Freeze-Drying Process on Decontamination and Quality Characteristics of Conventional Crystal Sugar for Possible Use in Pharmaceutical Products

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Received: 17 July 2023 Accepted: 21 April 2024

ABSTRACT: The increasing demand for pharmaceutical sugar requires higher quality (specifically microbial level) of standards compared to the sugar commonly used in the food industry. While different thermal processes are applied for microbial decontamination of crystal sugar, they usually destruct and degrade its bio-active compounds, that are undesirable. This research was conducted to evaluate the effects of freeze-drying (at – 42^{o}C, and pressure of 0.1 mbar) on crystal sugar contaminated highly with heat resistant bacteria of *G. stearothermophilus* (9×10^{5}) and also most common fungi of *A. niger* (9×10^{4} CFU/g). The results of this study showed that the non-thermal process of freeze-drying could eliminate > 99% of its original *Gs* and *An* microorganisms successfully. Physicochemical tests have indicated that the freeze-dryer process has made 10.4% decrease in crystal size and 23.5% decrease in ash content, that brings the characteristics of sugar crystals closer to international pharmaceutical standards. Furthermore, the microbial load of crystal sugar after freeze-drying process were below the permissible levels identified in European Pharmacopeia. In fact, there is a potential to use freeze-drying technique to decontaminate the conventional crystal sugar and make it appropriate for using (as ingredient) in pharmaceutical products.

Keywords: Hybrid processes, Lyophylization, Pharmaceutical Sugar.

Introduction

Microorganisms are the most important and well-known creatures, while not visible, exist in nature in various forms, and human have spent many years trying to destroy them. The science history has recorded, deaths of millions children and adults due to epidemic microorganisms' that cause diseases such as tuberculosis, plague, cholera, diarrhea, etc. in different regions of the world. Microorganisms cause diseases in different ways such as: breathing, skin crashes and digestion. In gastrointestinal disease tract, except for a few sterile foods, all foods contain one or more types of microorganisms. Some

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microorganisms cause food spoilage and foodborne diseases. Inorder to study the role of microorganisms in food and control them when necessary, is important to isolate them in pure culture and find suitable ways to destroy them (Ray & Bhunia, 2014). Over the years, scientists have proposed different methods to struggle microbial contamination, which the most well-known are thermal methods, such as pasteurization by Louis Pasteur and sterilization by Joseph Lister, which temperatures about 100 degrees and more are used (Jay, 2000). Drying is one of the oldest methods used to preserve food products. However, drying process may slightly or severely affect the properties of food, especially when it is carried out at high temperatures (Harguindeguy et al., 2019).

Since thermal methods have significant effects on food and destroy functional substances such as vitamins, over time, non-thermal methods opened their way to food industry. In recent years, with increasing complexity of food, products offered in modern food industry markets, increasing demand for food safety and quality standards, development and compliancy of effective non-thermal technologies is a priority for food industry and consumers. Freeze-drying or lyophilization is one of processes that has considered in relation to drying food while maintaining its quality. Lyophilization is a process which water is removed from food in the form of ice under low pressure by sublimation. This process has many applications to produce high quality food and pharmaceuticals (Nowak & Jakubczyk, 2020). Among microorganisms *Geobacillus stearothermophilus* (GS), is one of the most resistant bacteria, which used as an indicator to sterilize. This bacterium is rod-shaped, gram-positive and thermophilic, and widely found in soil, hot springs, and ocean sediments, and known as contaminant in food industry. Since this bacterium is an indicator to sterilize, its destruction by non-thermal methods can indicate the removal of all pathogenic bacteria (Burgess *et al*., 2017; Wang *et al*., 2020). *Aspergillus Niger* (AN) fungus is one of other well-known indicators due to its abundance and generality in food industry. *Aspergillus niger* is the cause of black mold disease in fruits and vegetables and is one of the biggest food contaminants in the world (Kalalian et al., 2020). Pharmaceutical sugar is pure sucrose that is produced based on international standards such as IP[1], EP[2], JP[3], USP[4], BP[5]. Pharmaceutical sugar is widely used in pharmaceutical products. Pharmaceutical sucrose used as sweetener, bulking agent and binder in chewable tablets. Pharmaceutical sugar used to remove unpleasant taste and create necessary viscosity in syrups. Pharmaceutical sugar is used to cover some drugs in form of dragees, therefore unpleasant taste and smell of above drug can be hidden (Patil et al., 2021; Porter, 2021). In this study, two microorganisms, *Geobacillus stearothermophilus* and *Aspergillus Niger*, have been used as targets in measuring disinfection power of lyophilization process in production of pharmaceutical sugar.

Materials and Methods

GS bacteria with ATCC number 7953 (PTCC 1713) AN fungus with ATCC number 9142 (PTCC 5010) were obtained from Iran Scientific and Industrial Research Organization. After preparing desired strains of bacteria and fungi, their

[1] Indian Pharmacopeia
[2] European Pharmacopeia
[3] Japanese Pharmacopeia
[4] The United State Pharmacopeia
[5] British Pharmacopeia

required culture media was prepared according to information in the product sheet of ATCC Company. Culture mediums NA (Nutrient agar) and PDA (Potato Dextrose agar) were obtained from Merck Chemical Company.

Analysis tests performed using the following devices:

NMR[1] (Brukers, Ultra shield, 500MHz USA) was employed to show the probable changes in the molecule structure and ICP[2] was used to measure the heavy metals in samples before and after process., (Varian-OES-730-ES). In the case of ICP all the samples must be in aqueous medium and should be filtered through a 0.2 µm filter in order to avoid the presence of micro-precipitates that could damage the equipment. A liquid sample is introduced using a peristaltic pump to ensure constant, stable flow. Commonly, a nebulizer with a high-speed flow of gas (usually argon) to shatter small droplets of liquid into an aerosol was employed. This aerosol is then introduced into a spray chamber that removes the larger droplets. The sample is then injected into a plasma chamber that is approximately 10,000 degrees. This extreme temperature breaks everything in the sample apart into the basic elements. Every element is released at a specific wave length). FTIR (Thermo Nicolet Nexus, 870 FT-IR USA) to investigate probable changes in chemical radicals, SEM (Seron Technology - AIS2300) to show the probable changes in physical appearance of crystals and microorganisms and PSA[3] to measure the crystal size changes (Sympatec Helos/KF) are employed.

First, lyophilized microorganisms transferred from their vials to Nutrient Broth culture medium, and then liquid sample containing *Geobacillus stearothermophilus* cultured on Nutrient agar plates as surface culture and incubated in a 44°C incubator for 48 hours. Liquid samples containing *Aspergillus Niger* cultured on plates containing Potato Dextrose Agar as surface culture and incubated in incubator at 25°C for one week. Then, each of grown samples passaged several times to use in aforementioned experiments (Anon, 2009). The standard equivalent of one McFarland used to prepare bacterial suspension, and the standard equivalent of half McFarland used to prepare fungal suspension. The combination of 0.048 M barium chloride solution and 0.18 M sulfuric acid used to prepare the McFarland standard. One and a half McFarland standards have optical density = 0.01 ± 0.17 and optical density = 0.08-0.13 at the wavelength of 625-620 nm respectively (Habeeb et al., 2007; Andrews, 2006). Bacterial suspension equivalent to McFarland's standard was prepared and sprayed by TLC Atomizer in 10 ml of physiological serum solution and then diluted to 10^6. Three plates cultured from each dilution and then colonies counted. After calculations, it was found that there are 10^5 x 3 CFU/puff of bacteria in each puff of bacterial suspension and 10^4 x 3 cfu/puff of fungus in each puff of fungal suspension.

Pharmaceutical sugar in the world is produced according to ICUMSA45 standards and sent to pharmaceutical factories, and special baby food factories. Pharmaceutical sugar standard based on the latest edition of ICUMSA45 is described in Table 1. Prototype of sugar (A) which considered as the basis for Freeze Dryer (FD) process has the specifications as described in Table 1. In this study, ICUMSA45 standards, 2019 edition, considered as reference for

[1] Nuclear Magnetic Resonance
[2] Inductively Coupled Plasma
[3] Particle Size Analyzer

production of pharmaceutical sugar by freeze dryer process. Also solid food substrate, namely sucrose, used. Sucrose has chosen as basic material for the investigation due to its high consumption in food industry and special uses in the pharmaceutical industry. The sucrose used obtained from the Hekmetan sugar factory. Each 50 grams of sucrose sprayed with 3 puffs of bacterial or fungal suspension by TLC Atomizer and then placed in freezer at - 20 degrees Celsius for 24 hours. Then placed in Siemens freezer for 24 hours at temperature of - 42 degrees Celsius, pressure of 0.1 mbar and condenser temperature of - 58 degrees Celsius and then after 24 hours, microbial tests related to GS and AN was performed according to the method described by Liu *et al*. (2021).

GS bacteria testing has done based on ICUMSA standard method No. GS2/3-49(1998) and AN fungus testing has done based on ICUMSA standard method No. GS2/3-47(1998) (Anon, 2009). 10 grams of sugar and 100 ml of sterile water were added to sterile erlenmeyer and diluted by magnet stirrer. Then the spread culture method was used for culturing in 55°C for 48 h for GS and 25°C for 5 days for AN.

Chemical tests performed based on the ICUMSA reference book according to the following methods:

The principles of determining polarization number GS1/2/3/9-1 (2009), is the measurements of the optical rotation of the sample solution, compared with the optical rotation of the normal solution of sucrose. For determining amount of invert number GS2/9-5 (2007), a solution of the sugar is heated in a boiling water bass with an alkaline copper reagent. The cupric ions are reduced to insoluble cuprous oxide by the reducing sugars present. After cooling the residual cupric ions are titrated with the EDTA using murexide as indicator. For determining amount of sulfur dioxide number GS2/1/7-33 (2009), the color of sulphite/rosanilin complex is measured photometerically at a wavelength of 560 nm, after reaction with formaldehyde. For ash determination test No. GS2/3-17(2002), the specific conductivity of a white sugar solution at a concentration of 28gr/100gr is determined. The equivalent Ash is calculated by the application of a conventional Factor. The Principal of the pH determination test No. GS1/2/3/4/7/8/9-23(2009), is the potentiometric measurements of pH. The electrodes are standardized with buffer solution, rinsed with distilled water and immersed in the sugar solution. The reading is taken after five minutes when the equilibrium potential across the electrodes is judged to have been reached. For color determination test No. GS2/3/9 (2005) and GS2/3/10 (2007), white sugar is dissolved in distilled water to give a 50% sugar solution. The solution is filtered and then the absorbency is measured at a wavelength of 420 nm and then solution color is calculated. Determining percentage of total phenol has done by SINGLETON method.

Results of tests and experimental design of this study analyzed with SPSS version 26 software.

Results and Discussion

Sugar infected with GS bacteria and AN fungus after passage of time and conditions mentioned for freeze dryer process, were tested according to ICUMSA international standard method (Anon, 2009) in terms of contamination after process.

The results of bacterial and fungal tests show that effect of freeze dryer on bacterial and fungal cells is significant and it has been able to reduce the bacterial load up to 10^5 cfu/gr and fungi load up to 10^4 cfu/gr.

Because the contamination created intentionally and the reduction of its load is quite significant (Figure 1) and compared to the ICUMSA45 international standard, sugar obtained from the freeze dryer process completely accepted in terms of its sterility and absence of bacterial and fungal contamination.

Table 1. Values of physicochemical and microbiological variables in control sugar, sugar obtained from freeze-dryer process and standard range

No	Analysis/test	Blank	Freeze Dried	Limit
1	Taste	Natural Sweet	Natural Sweet	Natural
2	Smell	Natural Smell	Natural Smell	Natural
3	Colour of the solution	71.69±3.55	70.12±0.32	45 ICUMSA
4	Moisture content	0.02±0.006	0.027±0.002	0.06% m/m
5	Conductivity ash	0.018±0	0.014±0	0.04% m/m
6	Polarization	99.81±0.036	99.81±0.02	99.7°Z min
7	invert sugar content	0.018±0	0.014±0	0.04% m/m
8	Coliforms	ND	ND	10 cfu/10g
9	Thermophilic Bacteria	ND	ND	
10	Mesophilic Bacteria	<10	<10	100cfu/gr
11	Yeast and Mould	<10	<10	20 cfu/10g
12	Sulphur dioxide (SO2)	<0.5	<0.5	15 mg/kg
13	Arsenic (As)	ND	ND	0.5 mg/kg
14	Lead (Pb)	0.13	9.25	0.5 mg/kg
15	Copper (Cu)	0.55	3.6	1.0 mg/kg
16	Total Phenol	38.07±2.29 (mg/kg)	15.02±2.81 (mg/kg)	
17	PSA	817.24±5.09	732.17±6.22	500-800µm
18	pH	5.08±0.01	5.3±0.01	

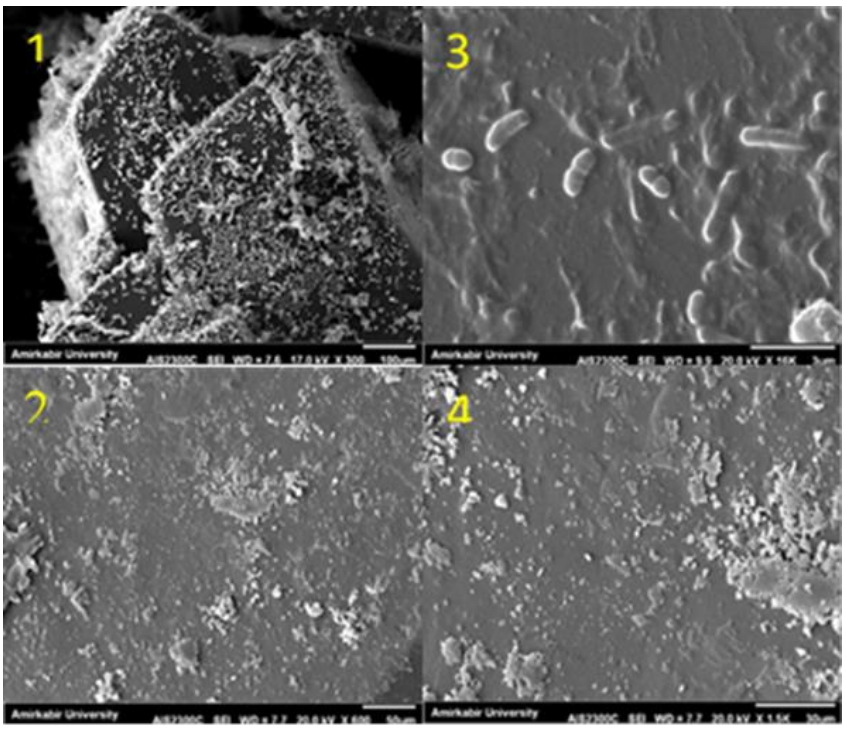

Fig. 1. Sugar: infected with AN Fungus before FD [1], AN Fungus after FD [2], infected with GS bacteria before FD [3], GS bacteria after FD[4].

In investigation of resulting changes of process (Table 2), considering that, amount of polarization(Pol) which is main variable in sugar tests, no significant difference was observed compared to the original sample in relation to changes of Pol. No significant difference observed in investigation of crystal color changes before FD and after FD. The amount of invert did not change significantly after process. Regarding the amount of sulfurous anhydride, since the amount of this substance was zero in the initial sample, there was not observed in the sample after the process. Existence of moisture in the form of crystals during sublimation causes acids in crystal, dehydrated and settle inside the crystal that seems to increase the pH after process and causes significant difference between the averages before and after process (Van Den Berg, 1966).

Regarding ash after freeze dryer process, the amount of ash has significant difference is compared to the original sample and one of the causes is dehydration of salts in crystal. Since the method of measuring ash is conductometry, in this method, soluble salts that can ionized, measured, therefore amount of ash has decreased with dehydration of salts and their structure (Thorat and Suryanarayanan, 2019).

Sucrose itself is a hygroscopic substance. After freeze-dryer process, in addition to sucrose, dehydrated salts are also included in the crystal structure of sucrose, which leads to increase in hygroscopic properties. This probably causes the moisture content of sugar to increase a little after process during transfer as compared to before, that is due to correct transfer and in accordance with principles. This increase is not statistically significant and is still within range of ICUMSA standards (Li et al., 2018). In the conditions of the freeze dryer, due to sublimation action, active site of enzymes changes during removal of moisture in solid form. Sometimes this is accompanied by movement of substrate towards the active site of enzyme that leads to the reduction of phenolic compounds in the sample. One of the enzymes that can be present in crystal is polyphenol oxidase enzyme, some of which enters the process through extract and remains in crystal. During sublimation, pigment of sugar, which is melanoidin, decomposed by enzyme under the conditions mentioned above, and this leads to reduction of phenolic compounds and color of

sugar crystal after the freeze-dryer process, and decreasing color is not statistically significant and is in standard range (Papoutsis et al., 2017; Asadi, 2007). In freeze dryer process, a polymer (rubber) container with the same diameter as the freeze dryer plates was used for freezing. Based on studies, this type of container is made of isoprene polymer or 2-methyl1-3-butadiene. In order to make these containers, in addition to isoprene, salts of elements of groups 4-8 of the Periodic Table or acyl and alkyl compounds of elements of groups 1-3 of the Periodic Table are used, depending on the factory's formulation (Anon, 2007).

If these containers exposed to extreme temperature changes such as high heat or very low cold, the diffusion coefficient of materials increases between 6-7 times. This causes migration of elements and monomers from container to food (Richardson et al., 2012). As seen in the results, samples obtained from freeze dryer have more copper and lead than the control sample. This

increase in amount of heavy metals is probably due to migration of elements from container containing sample during freezing and considered secondary

pollution, which can be eliminated by changing the type of container. During freeze dryer process, pressure resulting from sublimation in removal of solid moisture causes some sugar crystals to break and reduce their size (Levin et al., 2021). Test measuring crystal particles (Figure 2) along with images taken by the electron microscope (SEM) prove this hypothesis (Figure 3).

Table 2. Comparison of the average physicochemical properties in the design of two dependent samples with samples A control sugar and FD sugar obtained from the freeze-dryer process.

Paired Samples Test									
		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	PolA - PolFD	-.006667	.051316	.029627	-.134143	.120809	-.225	2	.843
Pair 2	ColorA - ColorFD	1.573333	3.906153	2.255219	-8.130089	11.276756	.698	2	.558
Pair 3	Ash.A - Ash.FD	.004167	.000058	.000033	.004023	.004310	125.000	2	.000
Pair 4	pH.A - pH.FD	-.213333	.025166	.014530	-.275849	-.150817	-14.683	2	.005
Pair 6	MoistureA - moistureFD	-.007000	.005568	.003215	-.020831	.006831	-2.178	2	.161
Pair 7	TotalphenolA - TotalpheolFD	22.876667	.676782	.390740	21.195448	24.557885	58.547	2	.000
Pair 8	PSA.A - PSA.FD	85.230000	9.362035	5.405173	61.973416	108.486584	15.768	2	.004

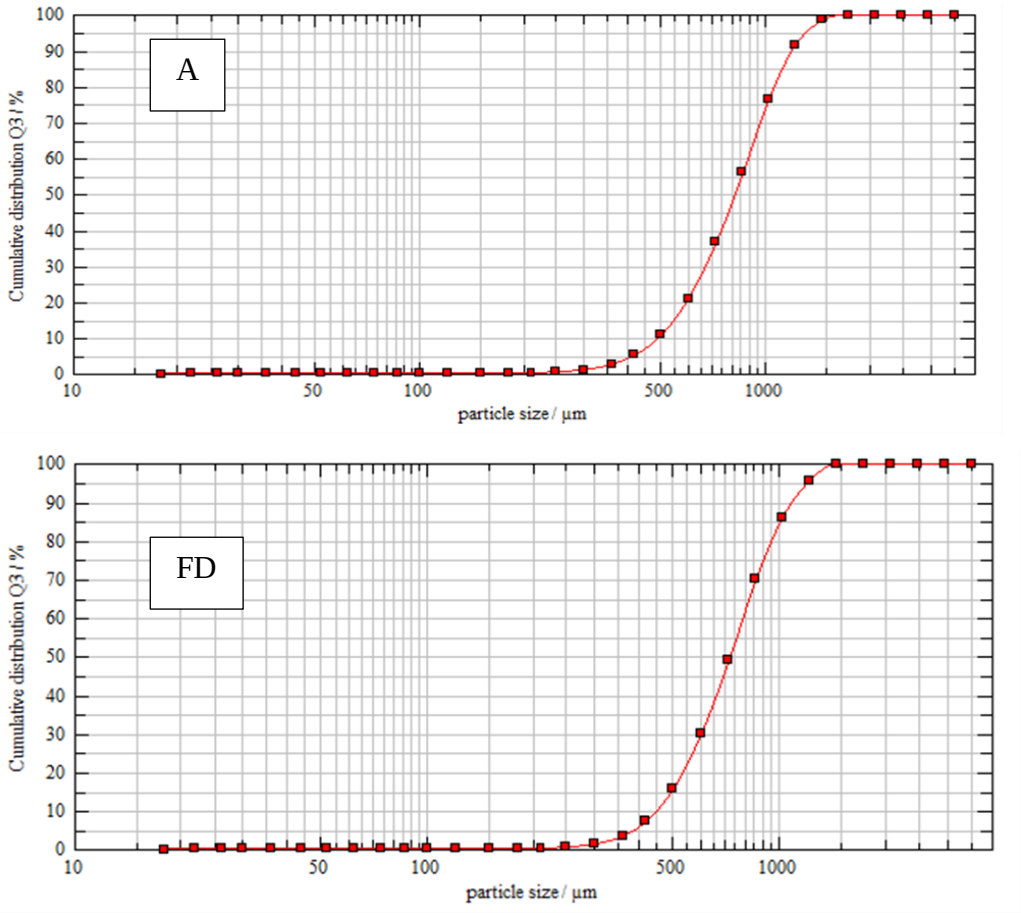

Fig. 2. Particle size distribution of sugar crystals after freeze dryer process.

In order to investigate was structural changes in sucrose molecule, FTIR (Fourier Transform Infrared Spectroscopy) was also performed. Comparing chemical groups of the control sugar (Figure 4 A), with sugar after process (Figure 4 FD), shows no changes were made in chemical groups of sucrose structure, and the main groups of CH and OH are still visible.

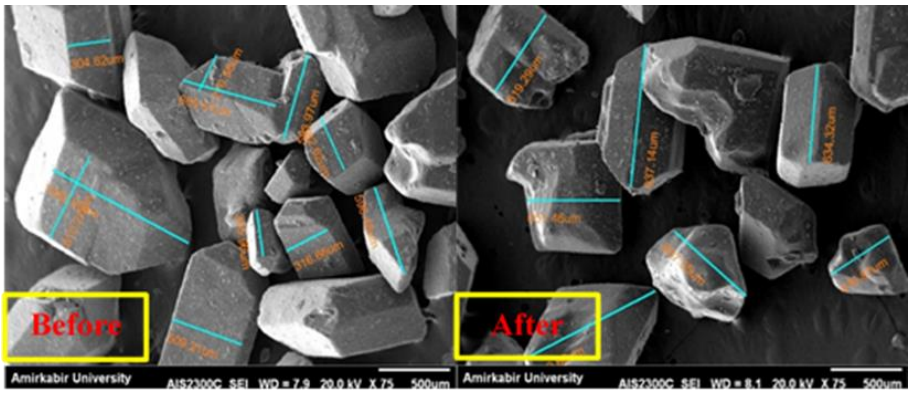

Fig. 3. Change in particle size of sugar crystal before and after FD by SEM**.**

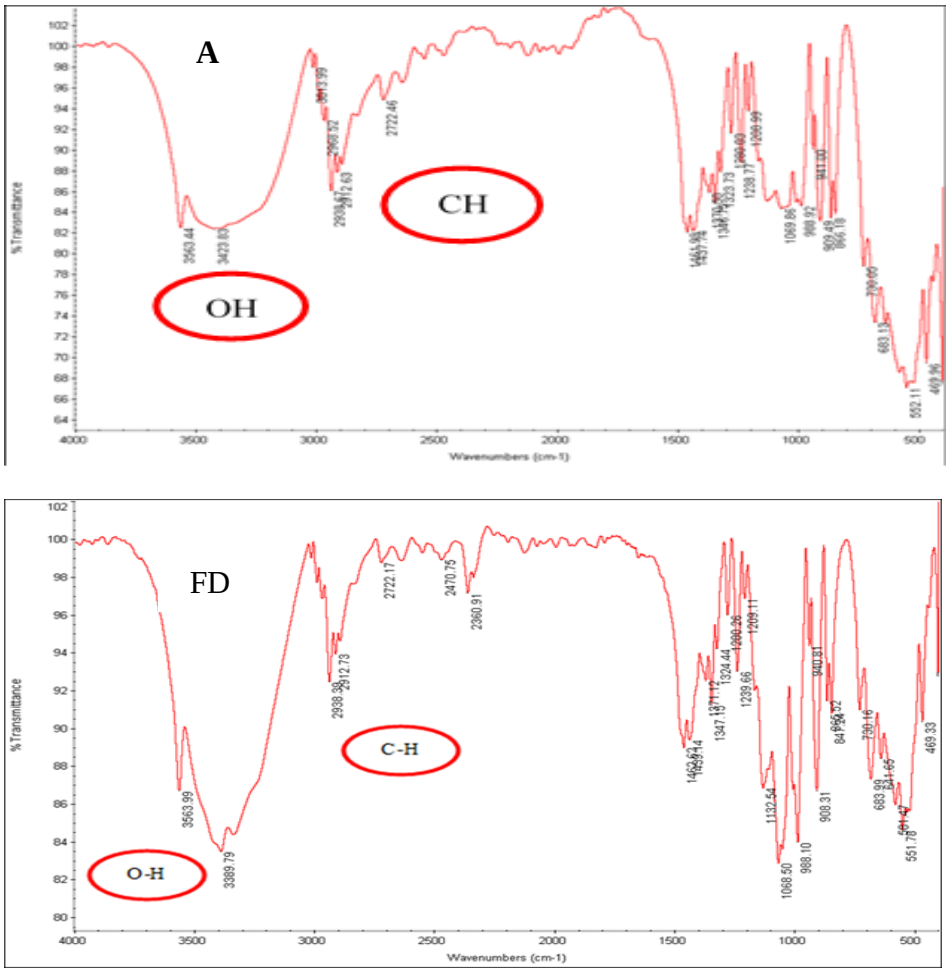

Fig. 4. Fourier transform infrared spectroscopy of sugar before freeze-drying A and after freeze-drying FD.

H-NMR test was also performed to ensure that changes in the structure did not occur. Comparison of H-NMR results (Figure 5) with registered standard diagrams confirms no change in the structure and accuracy of FTIR.

Conclusion

The results of this research show that with FD process, there is a possibility of reducing indicator microorganisms in food contamination, specifically two species of GS bacteria and AN fungus, in sugar produced by sugar factories using the conventional method. It can achieved with regard to pharmaceutical sugar standards in terms of microbial indicators by adding the FD complementary process. According to the available facilities in this research, suitable conditions for the FD process suggests, temperature conditions of -42°C, time of 24 hours and pressure of 0.1 mm bar. One of the most important achievements of this research is that there is no undesirable change on the structure of sugar crystal. Other physical and chemical indicators under application of FD process, percentage of sucrose as the main parameters, color and moisture did not have any significant changes. Physical and chemical parameters of ash, pH, particle size and invert, with degree of changes obtained, are still within the acceptable range of pharmaceutical sugar standards. Changes in some elements, including lead and copper, that probably caused by secondary pollution, are under control with predictable measures. Therefore, it is possible to propose FD process in batch form to sugar factories at the end of the sugar production line with the aim of producing pharmaceutical sugar.

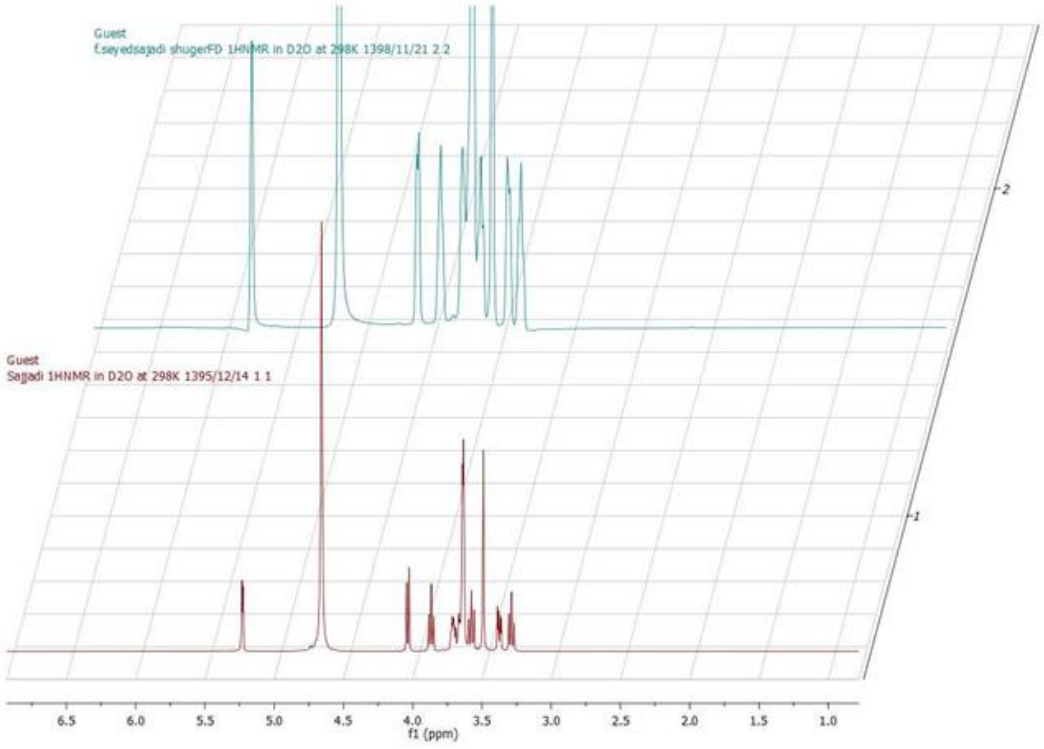

Fig. 5. Comparison structure of sugar before and after Freeze dryer process by NMR.

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