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Full Research Paper

Evaluating the effects of blanching and microwave pre-treatments on variations in some selected physiological factors of artichoke leaves in fluidized bed dryer

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Abstract

In this study, temperatures (40, 50, and 60°C), air velocity (3, 5, and 7 m/s) and pre-treatment (without pre-treatment, blanching, and microwave) were used as variables for investigation of antioxidant activity of dried artichoke leaves. The results revealed that variations in temperature and air velocity of the drying chamber and different pre-treatments significantly affected the free radical scavenging level and total phenol content in this plant. Based on the results obtained, it can be concluded that by increasing the temperature and air velocity and using blanching and microwave pre-treatments, the free radical scavenging level and total phenol content increased. The maximum percentage of free radical scavenging was 72.08% at 60°C and an air velocity of 7 m/s in the drying state by using microwave pre-treatment. The maximum total phenol content was 3.55 mg/g of dry matter at 60°C and an air velocity of 7 m/s in the drying state by using microwave pre-treatment.

Keywords: artichoke, fluidized bed dryer, microwave, blanching

Introduction

Artichoke (*Cynara scolymus L.*) is a perennial and cold-sensitive plant with an average life span of 4 years which its height reaches 2 meters (Dermarderosian *et al.*, 2001). Artichoke has important application in food and pharmaceutical industries owing to having polyphenolic compounds such as caffeic acid and its derivatives such as chlorogenic acid, cynarine, cinnarizine, and other natural antioxidants. Based on studies conducted, artichokes and their chemical compounds have a strong source of polyphenolic compounds (Melillia *et al.*, 2007). Dry leaves of artichoke contain 9 to 11% water and 12 to 15% minerals, and they are rich in potassium and magnesium salts. Many phenol, flavonoid, and acidic compounds are found in artichoke (Graifenberg et al., 1995). Cynarine in the artichoke plant is also used in the treatment of Jaundice (Gebhardt, 1998). The preservation of agricultural products is the only method to reduce the loss of food and medicinal plants. It can be stated that drying is the best method for preserving food and agricultural products, such as medicinal plants. Its important role has been

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proven in many countries (Motevali *et al.*, 2011). Drying increases the time of maintenance of harvested products, improves the quality, improves the market conditions of the farmer to keep the prices fixed in the market, and reduces transportation costs due to the removal of water. In addition, using conventional methods of drying the plants can change the amount of chemical compounds (Soysal *et al.*, 2001). Among drying methods, fluidized bed dryer has many advantages, including a high intensity of drying, high thermal efficiency, uniformity in drying, precise control of temperature in the bed, and also the short time needed for drying due to the high rate of heat and mass transfer (Topuz *et al.*, 2004). In order to increase the efficiency and quality of dried products, it is recommended to use assistance methods, which increase the yield of drying process and also the quality of the dried product. One of these methods is using pre-treatment and type of preparation during the drying process (Gholami *et al.*, 2009). The results of many studies have shown that using pre-treatment such as ultrasound (mechanical pre-treatment), microwave (pulse pre-treatment), osmotic (chemical pre-treatment) and blanching (thermal pre-treatment) can cause high variations in quality of the dried product (Ayoubi *et al*., 2015). Microwave is one of the suitable pre-treatments to increases the diffusion coefficient of moisture in the product and reduces the drying time while maintaining the quality of the product *(Wang et al.*, 2004). Microwave causes oscillation in bipolar molecules, such as water molecules, and the oscillation of water molecules in foods causes friction between molecules and creates heat. As a result, all parts of the food uniformly absorb the microwave energy and heat and reduced the initial moisture content of the substance in a short time (Azarpazhooh *et al.*, 2011). On the other hand, using blanching as pre-treatment damages the membrane resistance of the cell at high temperature and this membrane layer is lost. The moisture can be transferred from the inner part of the product to the external surface. Its outflow speed increases, causing an increase in the product's internal mass transfer coefficient or penetration coefficient. Increasing the mass transfer reduces the drying time and reduces energy utilization during the drying process (Motevali *et al.*, 2017). To examine the effect of drying temperature on some quality characteristics of Artichoke (*Cynara scolymus* L.), GhasemNejad et al. (2013) studied five drying temperature of 40, 50, 60, 70 and 80°C. The results revealed that the maximum content of phenol was obtained at 60°C. The maximum content of flavonoid (5.15 mg/g), percentage of free radical scavenging (144.67%) and leaf caffeic acid (4.91 mg/kg) were obtained in samples dried in shadow. Among the temperature treatments with drying device, with increasing drying temperature, the antioxidant performance level was increased, indicating a change in simple phenolic compounds to antioxidant compounds (Ghasemnezhad *et al.*, 2013). By studying the dry temperatures of the oven (30, 40 and 50°C) on phenol content of leaves of plantain, Zubaira et al. (2011) found that drying temperature had a significant effect on phenolic content and total phenol decreased by increasing the temperature. It was also indicated that increasing the drying temperature in a plantain plant decreased the concentration of biological compounds (Zubair *et al.*, 2011). Examining the effect of different temperatures (55 and 75°C) on polyphenolic materials and percentage of free radical scavenging in two apricot cultivars, Madrau et al. (2009) found an increase in temperature reduced ascorbic acid, epicatechin, quercetin, rutin, chlorogenic and neo-chlorogenic. However, by increasing the temperature, antioxidant capacity increased (Madrau *et al.*, 2009).

In other similar studies on the different plants, the effect of temperature on the change in secondary composition was well reported (Parker, 1999; Rushing *et al.*, 2004; Shabby *et al*., 1995). Thus, this study aims to find a suitable drying method to maintain the maximum antioxidant and total phenol content of artichoke leaves in a fluidized bed dryer. To perform this process, temperature and velocity of different air were tested. The effect of using blanching and microwave pre-treatments on the

antioxidant and phenolic properties of artichoke leaves was also examined.

Materials and methods

Sample preparation

The newly-harvested leaves of the artichoke were prepared from Gorgan Medicinal Plants agro-Industry Company and kept in a laboratory fridge at 3°C. Then, 20 g of leaves were divided into smaller pieces and used for drying. A Laboratory fluidized bed dryer manufactured in the Bio System Mechanics Engineering Department of Gorgan University of Agricultural Sciences and Natural Resources was used (Figure 1).

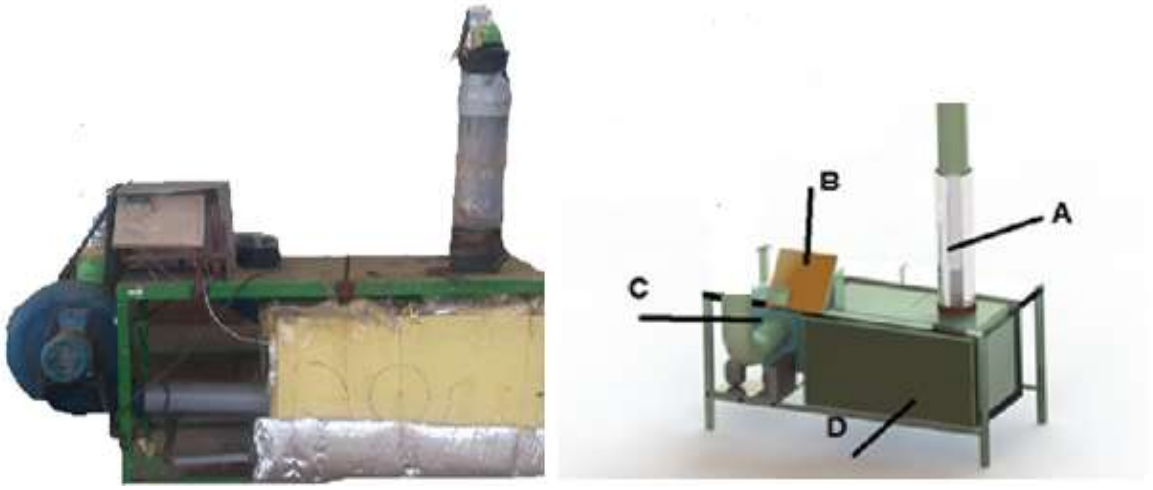

Fig. 1. Fluidized bed dryer and its components: A- Fluidizing chamber B- heater control C- fan D- heaters chamber

Method of testing

Drying of Artichoke leaves was performed in three states include: without pre-treatment, using microwave pre-treatment, and blanching pre-treatment. To apply blanching pre-treatment and given the sensitivity of the medicinal plant to contact with water; first, the water was heated to the boiling point so that the required steam for blanching operation was supplied. To determine the best level of applying blanching pre-treatment, samples were placed in the vicinity of steam at three different time of 30, 60, and 90 s and after completion of the pre-treatment process; the samples were placed on dry paper for two minutes to remove the moisture resulting from the blanching (steam) operation. The pre-treated samples were dried in a fluidized bed dryer and the percentage of free radical scavenging and phenol content of samples were determined after the drying process. The maximum level of effective compounds in blanching pre-treatment was obtained with a duration of 60 seconds.

Additionally, to apply microwave pre-treatment, the samples were placed in three different power levels of 90, 180, and 360 watts in the microwave chamber. To apply the same energy on the samples by microwave radiation, the microwave pre-treatment was applied on the samples at the power of 90 180, and 360 watts for 2.5, 5, and 1.25 minutes, respectively. The basis of the microwave condition in this experiment was the pre-tests that were performed. The pre-treated samples were transferred to the fluidized bed dryer environment and after the drying process, the phenol and antioxidant content were measured.

Considering the level of effective compounds, the pre-treatment of 90 Watts for 5 minutes was selected at the best experimental level. Thus, the samples were dried in three methods without using pre-treatment, blanching pre-treatment with steam for 60 seconds, and microwave pre-treatment with a power of 90 watts for 5 minutes at different temperatures of 40, 50 and 60°C and air flow velocity of 3, 5 and 7 m/s. A data logger (As Instrument Model 88598) with a precision of

0.1 m/s was used to measure the temperature. To measure the wind velocity of dryer, an anemometer (LUTRON, AM-2416) with a precision of 0.1 m/s was used. This dryer equipped with a temperature controller, operating automatically with a precision of 0.1°C. The weight of the samples was also measured using a Dj 2000A scale with a precision of 0.01 g.

Preparation of methanol extract

To measure total phenol and antioxidant activity, the samples were powdered using an electric mill, and one gram of each sample with 10 ml of 80% methanol (ratio of 1 to 10) was homogenized and samples were placed on a shaker device for 24 hours. The methanol extract of the sample was then filtered by filter paper and the extract was used to measure the considered biochemical characteristics.

Measuring the percentage of free radical scavenging of the extract

To calculate the percentage of free radical scavenging (ability to trap free radical), the Brand William method was used. The extraction method is the same as the methanol extraction method. The DPPH method was used to measure antioxidants. Accordingly, 1 ml methanol extract along with 1 ml of DPPH reagent (Dimethyl Sulfur Salicylic) of 1 mM (0.002 grams of DPPH reagent in 50 ml methanol) were added to the test tube. For the control sample, methanol of 80% was used instead of methanol extract and the remaining steps were similar to phenol measurement. The samples were stored in dark conditions for 30 minutes to inhibit the free radical to be applied by DPPH. After passing the required time, the absorption rate was measured by a spectrophotometer at 517 nm. For measurement, first, the device was calibrated with methanol of 80%. Then, the absorbance of control and other samples were recorded. Using the following formula the antioxidant activity was calculated (Brand-Williams *et al*., 1995).

$$\% \text{ DPPH} = \frac{Ac - As}{Ac} \times 100 \quad (1)$$

In which, A_C is the rate of control sample absorption and A_S is rate of absorption of each of the samples.

Measurement of total phenol content

To calculate the phenol content, the method used by Ragazi and Veronese (1973) was used. The amount of 20 µl of methanol extract was diluted with 100 µl of Folin Sioculteus reagent in 1.16 ml of deionized water. It was then placed in a dark place to rest and to apply the effect of the Folin-Sioculteus reagent for 6 min. After adding 300 µl of sodium carbonate, the solution was placed in a water bath at 40°C for 30 minutes. To prepare the control solution, only methanol of 80% was used and the rest of the steps were as described above. The control sample was used to calibrate the spectrophotometer. First, the spectrophotometer was calibrated using a control solution at 765 nm to measure the total phenol. Then, the methanol extract of the leaf was determined and the absorbance number was recorded at the given wavelength. Total phenol content was calculated in terms of gallic acid equivalent in 1g of dry plant leaf (Ragazzi *et al*., 1973).

Statistical Analysis

In this study, all experiments were performed in three replications and the results were analyzed using factorial experiments that can be analyzed using ANOVA analysis in a completely randomized design using SAS statistical software. The samples were tested at three temperatures (40, 50 and 60°C), air velocity (3, 5 and 7 m/s) for pre-treatments (without pre-treatment, blanching and microwave), an antioxidant and phenol content factors were measured.

Results and discussion

Table 1 presents analysis of variance results (ANOVA) for the effect of temperature, air velocity and their interaction.

The effect of different parameters on free radical scavenging

Figures 3, 4, and 5 show the level of free radical scavenging of artichoke leaf in different

states of experimental conditions (using blanching pre-treatment, using microwave pre-treatment, and without using pre-treatment).

The maximum level of this activity (72.08%) was obtained in the state of drying by using microwave pre-treatment at 60°C and air velocity of 7 m/s. The minimum anti-oxidant activity (19.36%) was obtained at the state of drying without using pre-treatment at 40°C and air velocity of 3 m/s. The results suggest that the antioxidant content increases with increasing temperature. With increasing temperature and faster absorption of moisture from the leaf surface, the intracellular vapor pressure increases and with the increasing pressure into the cellular cytoplasm, the cells are swollen and put pressure into the cell wall and increase the withdrawal of cellular contents from the cells (Wang *et al*., 2010; R. Wang *et al*., 2008).

Table1- Analysis of antioxidant and phenol content in different pre-treatment

		Antioxidant content		Phenol content	
		Microwave pre-treatment			
	DF	MS	F value	MS	F value
Temperature	2	1779.70	29.90**	0.215	4.36**
Air velocity	2	63.77	1.06ns	0.035	0.50ns
Temperature× Air velocity	4	598.03	9.93**	0.48	6.92**
Error			60.19		0.069
		Blanching pre-treatment			
Temperature	2	652.26	6.57**	5.76	88.65**
Air velocity	2	467.31	4.70*	0.084	1.31ns
Temperature× Air velocity	4	651.82	6.56**	1.12	17.22**
Error			99.33		0.065
		Without pre-treatment			
Temperature	2	299.41	1.86ns	1.072	14.01**
Air velocity	2	28.25	0.18ns	0.868	1.91ns
Temperature× Air velocity	4	871.95	5.41**	0.146	11.35**
Error			161.1		0.076

** Significant difference at the statistical level of 1%, * Significant difference at the statistical level of 5%, ns no significant difference

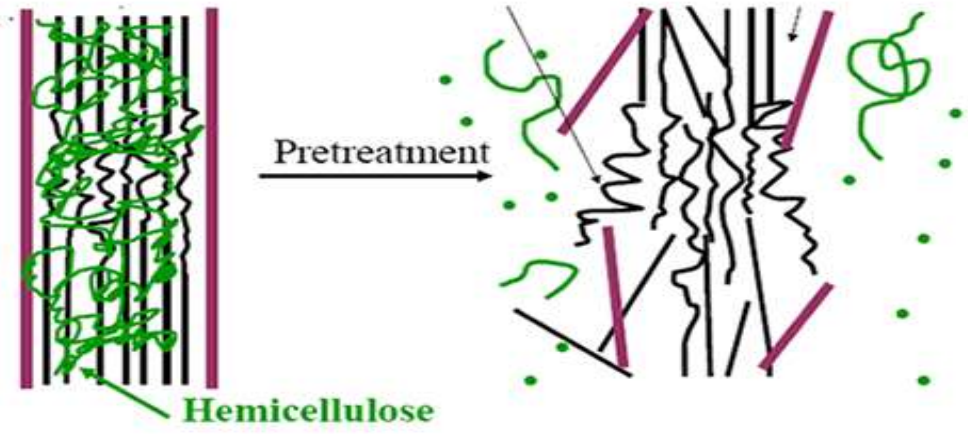

Fig. 2. The effect of using blanching pre-treatment

As shown in Figure 2, drying with blanching pre-treatment, destroys the cell wall and causes stress in the product texture. It also leads to porous products and a hard surface remains due to moisture withdrawal from the product. This increases the mass and moisture transfer from

the product. As the velocity of drying increases, the amount of extraction of free radical scavenging increases compared to the previous state.

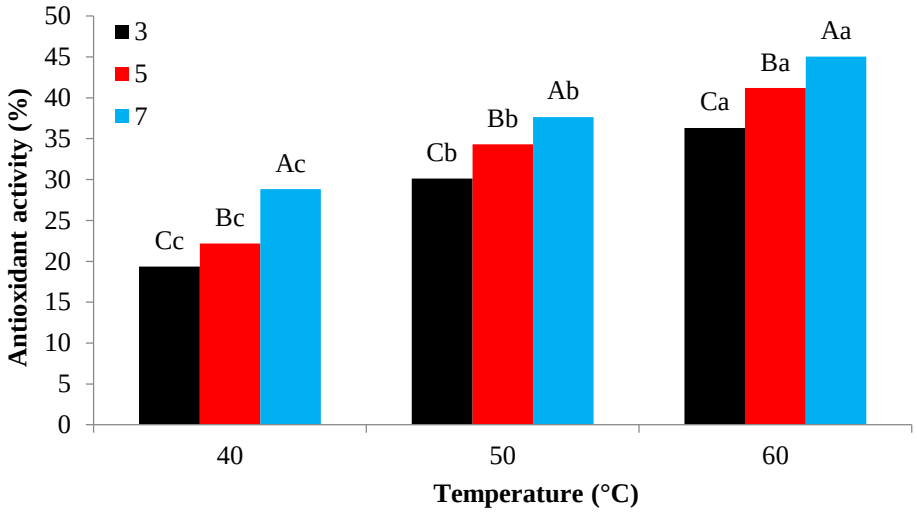

Fig. 3. The effect of temperature and air velocity on percentage of free radical scavengingin the state of drying without pre-treatment

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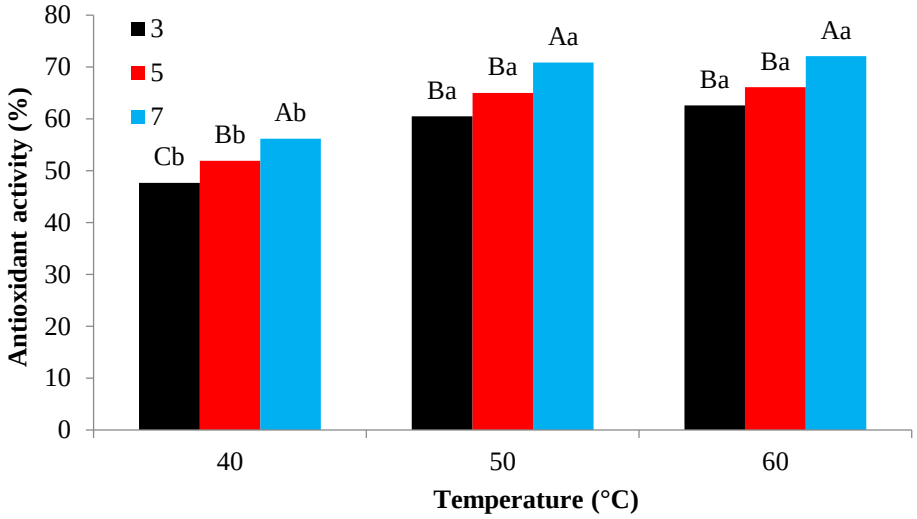

Fig. 4. The effect of temperature and air velocity on percentage of free radical scavengingin the state of drying with blanching pre-treatment

Big letters similar to no significant difference in a fixed Temperature and small letters similar to no significant difference in a fixed air velocity

These results are consistent with those of Madrau et al. (2009), who indicated that by increasing the temperature from 55 to 75°C, the apricot antioxidant capacity increases. The results also showed that blanching pre-treatment, compared to the control (drying without pre-treatment), decreased the drying time. These results are consistent with those of Heras-Ramírez et al. (2012), who examined the effect of blanching and drying temperature on phenol compounds and the free radical scavenging of apple in a cabinet dryer. Moreover, in the state of drying with microwave pre-treatment, the cell wall of the product is destroyed due to increase vapor pressure caused by applying microwave waves.

In such condition, the samples are swollen and try to return to their initial state. Their returning destructs the cell wall leading to the creation of more open pores. This creates a texture with lower resistance against moisture and mass transfer in the drying process, followed by reducing drying time and increasing the amount of extraction of free radical scavenging compared to the two previous pre-treatments. Our results are consistent with those of research conducted by Shamaei and EmamJome (2011) and Aslnejadi et al. (2015) in drying the mushroom layers

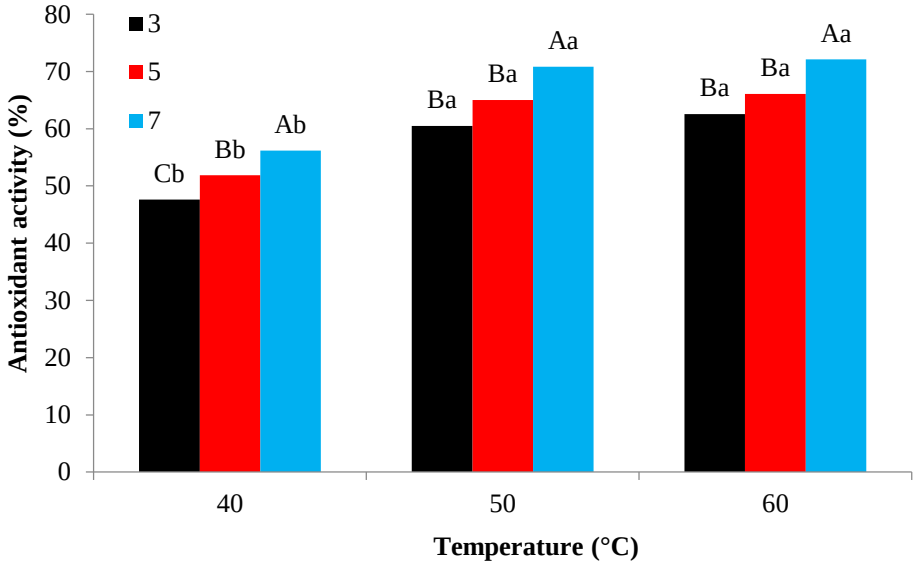

Fig. 5. The effect of temperature and air velocity on percentage of free radical scavengingin the state of drying with microwave pre-treatment

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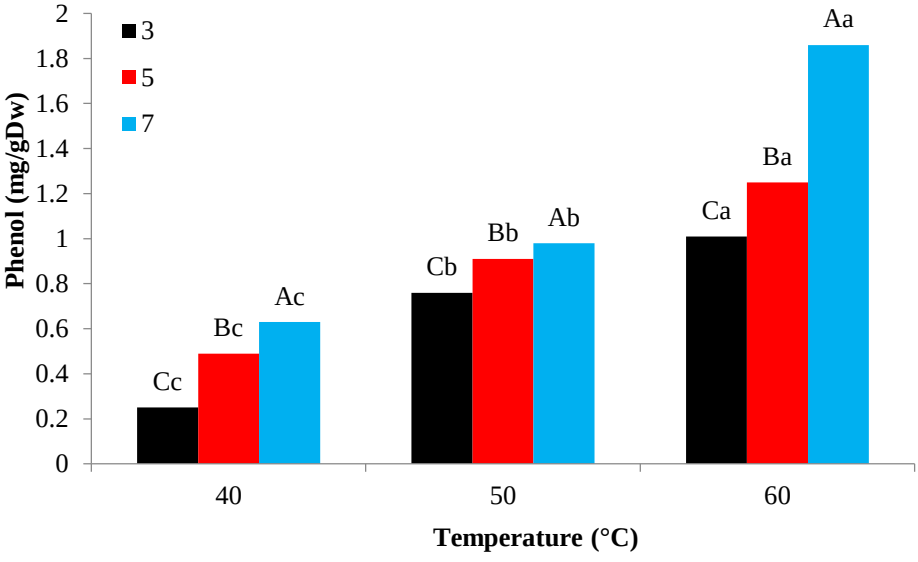

Fig. 6. The effect of temperature and air velocity on total phenol content in the state of drying without pre-treatment

Big letters similar to no significant difference in a fixed Temperature and small letters similar to no significant difference in a fixed air velocity

The effect of different parameters on total phenol content of leaf

the maximum and minimum phenol content were 3.55 and 0.25 mg/g dry matter at 60 and 40°C of air and 7 and 3 m/s of air velocity in the state of drying with microwave pre-treatment and without pre-treatment respectively. The results showed in Figures 6, 7 and 8. Increasing the temperature led to increase the phenol content of artichoke leaves. This finding was similar to GhasemNejad et al. (2014), on the effect of drying temperature on some of the qualitative characteristics of artichoke medicinal plant leaves. Their results revealed that the maximum phenol was obtained at a temperature of 60°C. It is also consistent with the research results conducted by Katsubeh et al. (2009), who investigated the effect of drying temperature on the free radical scavenging capacity and total phenol content in white berries.

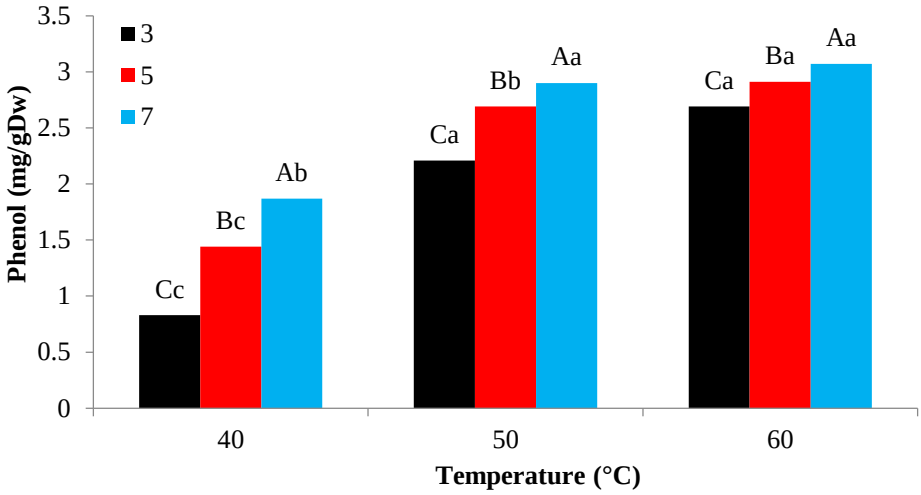

Fig. 7. The effect of temperature and air velocity on total phenol content in the state of drying with blanching pre-treatment

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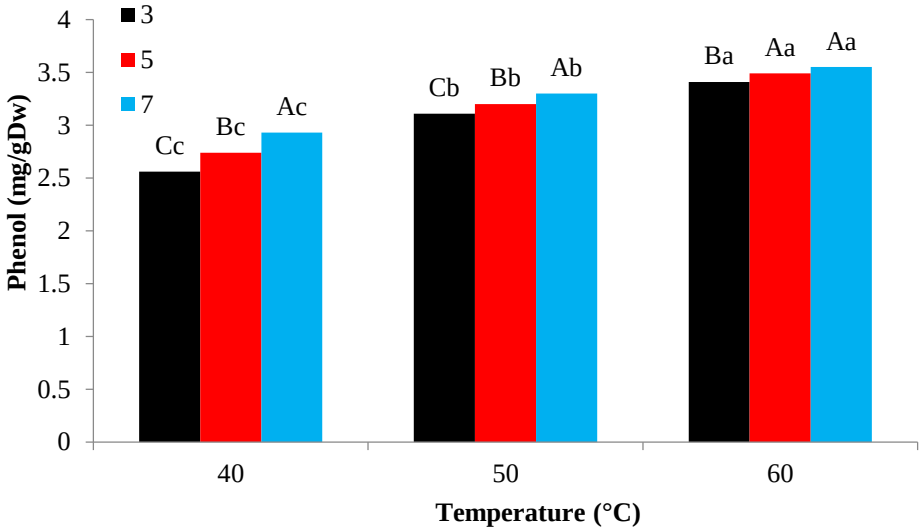

Fig. 8. The effect of temperature and air velocity on total phenol content in the state of drying with microwave pre-treatment

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Conclusion

The results revealed that the suitable method for drying artichoke medicinal plant leaves by using a fluidized bed dryer is the state of using microwave pre-treatment. Compared to the state of drying without pre-treatment, this method preserve the active ingredients significantly due to reduced drying time. According to the results, the best treatment for drying in terms of maximum quality of active components were 60°C and air velocity of 7 m/s and microwave pre-treatment. The lowest percentage of free radical scavenging and total phenol content were obtained at 40°C and an air velocity of 3 m/s in the drying state without using pre-treatment with19.36% and 0.25 mg/g of dry matter, respectively,

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بررسی اثرات پیش تیمارهای بلانچینگ و مایکروویو در تغییرات بعضی از عوامل فیزیولوژیکی برگ گیاه کنگر فرنگی در خشک کن بستر سیال

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چکیده

به‌منظور حفظ بیشترین میزان توانمندی آنتی‌اکسیدانی و ترکیبات موثر در این توانمندی از جمله فنل کل برگ کنگر فرنگی در یک خشک‌کن بستر سیال، اثرات دما، سرعت هوا و همچنین استفاده از پیش‌تیمارهای بلانچینگ و مایکروویو و مقایسه آن با حالت خشک شدن بدون پیش‌تیمار، در سه دمای ۴۰، ۵۰ و ۶۰ درجه سانتی‌گراد و سه سطح سرعت هوا ۳، ۵ و ۷ متر بر ثانیه مورد بررسی قرار گرفت. نتایج این تحقیق نشان داد که تغییرات دما و سرعت هوای محفظه خشک‌کن و روش‌های مختلف پیش تیمار تاثیر معنی‌داری بر میزان فعالیت آنتی‌اکسیدانی و فنل کل موجود در این گیاه داشت. بر اساس نتایج به‌دست آمده می‌توان نتیجه‌گیری کرد که با افزایش دما و سرعت هوا و استفاده از پیش‌تیمار بلانچینگ و مایکروویو مقدار درصد مهار رادیکال و میزان فنل کل افزایش می‌یابد. بیشترین درصد مهار رادیکال در دمای ۶۰ درجه سانتی‌گراد و سرعت هوای ۷ متر بر ثانیه در حالت خشک کردن و با استفاده از پیش‌تیمار مایکروویو مقدار (۷۲/۰۸٪) به‌دست آمد. همچنین بیشترین میزان فنل کل مقدار ۳/۵۵ میلی‌گرم بر گرم ماده خشک در دمای ۶۰ درجه سانتی‌گراد و سرعت هوای ۷ متر بر ثانیه در حالت خشک کردن استفاده از پیش‌تیمار مایکروویو به‌دست آمد.

واژه‌های کلیدی: کنگر فرنگی، خشک‌کن بستر سیال، مایکروویو، بلانچینگ.

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Full Research Paper

Carboxymethyl cellulose based bioactive edible films with *Lactobacillus casei* and fish protein hydrolysates

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Abstract

Nowadays, probiotics have been seriously considered for their potential application in healthy food formulations. The most highlighted concern about probiotics is that the number of probiotic bacteria at the time of consumption may be lower than the required value (10^7 CFU/g). A new approach is the immobilization of probiotic cells in the matrix of edible films. So in this study, edible carboxymethyl cellulose (CMC) probiotic films containing *Lactobacillus casei*, and the silver carp muscle protein hydrolysates (SCMH) prepared by using limited Alcalase hydrolysis, were analyzed and the viability of bacterial cells was determined at 25, 4, and -18°C for 30 days. An alkaline solubilization/acid precipitation method was used to isolate silver carp white muscle proteins. Protein isolate (3%, W/V) was hydrolyzed with 5% E/S ratio (w/w) Alcalase at 50°C and pH 8.0 for 3 min. Briefly, films were prepared by dissolving SCMH and CMC powder (1.5%, w/v) in a ratio of 1:2 in distilled water and *L. casei* was added to a final concentration of 10^8 CFU/mL. Probiotics were counted at intervals of 1, 10, 20, and 30 day. The physical, mechanical [Ultimate tensile strength (UTS) and elongation at break (EB)], thermal and structural properties were determined. XRD patterns of the film samples collected by X-ray diffractometer (XRD) and Fourier transform infrared (FT-IR) spectroscopy of the film samples were recorded. The results indicated that the addition of SCMH significantly ($p<0.05$) improved the *L. casei* viability at all three temperatures. Thickness, moisture absorption, and water vapor permeability (WVP) of the films were not influenced by addition of the probiotic. However, the addition of SCMH negatively affected the film's mechanical properties. The FT-IR analysis confirmed the formation of hydrogen bonds between *L. casei* and the CMC matrix, the XRD and differential scanning calorimetry (DSC) analyses confirmed the plasticizing effect of SCMH on the films. Thus, CMC films containing *L. casei* showed the highest UTS (3.7 MPa) and EB (29.9%). Generally, the results indicated that the SCMH incorporated CMC-based film can be a good carrier for probiotics as bioactive food packaging system with promising potential for shelf life extension of perishable foods.

Keywords: Protein hydrolysates, *L. casei*, Carboxymethyl cellulose, Edible films, Probiotic.

Introduction

Probiotics are increasingly being incorporated into the food products (Ma *et al*., 2019; Pavli *et al*., 2017) due to their ability to maintain the balance of intestinal microbiota, enhance the immune system and reducing the

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risk of gastrointestinal diseases such as inflammatory bowel diseases, diarrhea, and lactose intolerance (Fiocco *et al*., 2020; He *et al*., 2017). The stability and viability of probiotics are influenced by intrinsic food parameters, processing and storage conditions (Fiocco *et al*., 2020; Ebrahimi *et al*., 2018).

Bioactive edible films are thin-layer biopolymers constructed from natural polymeric ingredients that can be used on the surface of products as eco-friendly food packaging (Ma *et al*., 2019; Ebrahimi *et al*., 2018). Apart from their role in food protection (e.g., by reducing the rates of moisture and gas transfer) (da Cruz *et al*., 2007), they may contribute to human health (Espitia *et al*., 2016). Stabilization of probiotics in the matrix of edible films has been used to enhance the cells' viability (Altamirano-Fortoul *et al*., 2012). The production of probiotic edible films by incorporation of various probiotic bacteria into the different biopolymer-based films such as rice protein-shellac composite coating (Wang *et al.*, 2021), polyvinyl alcohol films (Hirsch *et al.*, 2021), bacterial cellulose film (Motalebi Moghanjougi *et al*., 2020), whey protein isolate (WPI) film (Karimi *et al.*, 2020), bacterial cellulose-cashew gum composite film (Oliveira-Alcântara *et al.*, 2020) and alginate films (Alvarez *et al.*, 2021) was the subject of some recent researches in food packaging field.

Hydrocolloids such as proteins and polysaccharides have been used for preparation of edible films. Carboxymethyl cellulose (CMC), a cellulose-derived polysaccharide, is one of those widely used industrial biopolymers in food packaging applications due to its low price, high compatibility, good biodegradability, high transparency, and acceptable mechanical properties (Azarifar *et al*., 2019; Tongdeesoontorn *et al*., 2011; Nie *et al*., 2004). Different bioactive compounds such as indigestible oligosaccharides have been used as prebiotics in the CMC-based probiotic films in order to increase the stability of probiotic bacteria (Zabihollahi *et al*., 2020; Yu *et al*., 2016). However, the potential of protein hydrolysates and peptides, as prebiotics, on growth or activity of beneficial microorganisms in edible film matrix is less studied (Yu *et al*., 2016).

Seafoods are a potential source of functional ingredients such as protein hydrolysates and bioactive peptides (BAP) that could potentially be used for food fortification (Nikoo *et al*., 2020; Sun *et al*., 2020; Xu *et al*., 2019).

Protein hydrolysates and BAP may beneficially modulate the physiological processes in the body and prevent oxidative stress associated with degenerative aging diseases (Wang *et al*., 2013; Kim & Wijesekara, 2013). Therefore, they may be used as promising ingredients for developing functional foods or active packaging due to their multiple health benefits (Sun *et al*., 2020). Silver carp (*Hypophthalmichthys molitrix*) is a major commercially aqua cultured carp species. The global production of silver carp has expanded steadily, rising from 3.8 million tonnes in 2006 to 5.3 million tonnes in 2016 (FAO FishStat). Protein hydrolysates and BAP from silver carp muscle showed various biological functions including antioxidant, anti-thrombotic, antihypertensive, and immunomodulatory activities (Wang *et al*., 2020; Jiang *et al*., 2014).

Lactobacillus casei is a well-known specie of mucosa, the production of antimicrobial Lactobacillus and has been recognized as safe by the US Food and Drug Administration (Arihara, 2006). The beneficial properties of *L. casei* such as resistance to stomach acid and bile salts, the ability to adhere to the cells of the intestinal substances and inhibition of the activity of pathogens have been documented (Rasdhari *et al*., 2008; Mishra & Prasad, 2005). Cold storage or high processing and storage temperatures might adversely affect the cell viability and proliferation in the food industry. Therefore, immobilization of *L. casei* in a matrix of edible films to improve its viability might be beneficial (Fiocco *et al*., 2020). Several studies have investigated the immobilization of *L. casei* in various biopolymer-based films. Mozaffarzogh et al. (2020) prepared probiotic CMC-sodium caseinate films containing *L. casei* for shelf life extension of fresh trout fillets. Khodaei et al.

(2020) developed *L. casei* loaded gelatin and low methoxyl pectin based probiotic films and observed the improved cell viability. Orozco-Parra et al. (2020) prepared and characterized bioactive synbiotic edible film based on cassava starch, inulin, and *L. casei*. Pruksarojanakul et al. (2019) developed synbiotic edible film from konjac glucomannan by incorporation of *L. casei* and OraftiGR, and used for coating of bread buns. Dianin et al. (2019) prepared edible films based on WPI and *L. casei* and evaluated the effect of resultant films for shelf life extension of tomato and grape fruits.

So far, immobilization of *L. casei* in the carboxymethyl cellulose edible films incorporated with fish protein hydrolysates has not been investigated. The aim of this study was to investigate the viability of *L. casei*, in CMC-based edible films containing silver carp muscle protein hydrolysates (SCMH) stored at different temperatures (25, 4, and -18°C). The effect of SCMH on the mechanical and thermal properties of the films containing *L. casei* was also evaluated.

Materials and Methods

The strain of *L. casei* PTCC 1608 (as freeze-dried culture) was purchased from Iranian research organization for science and technology (Tehran, Iran). Carboxymethyl cellulose (CMC) was provided by Caragum Parsian (Tehran, Iran, M_w = 250000 Da), and Silver carp (*Hypophthalmichthys molitrix*) was obtained from the local fish market (Urmia, Iran). Alcalase (Protease from *Bacillus licheniformis*, 2.4 L, 2.4 AU/g) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Other chemicals used in the research were purchased from Merck (Darmstadt, Germany).

Extraction of fish protein isolates

An alkaline solubilization/acid precipitation method was used to isolate silver carp white muscle proteins (Nikoo *et al*., 2019). Briefly, the pH of the homogenized mince was adjusted to 11 using 1 mol/L sodium hydroxide and continuously stirred using an overhead stirrer (FTDS-11, Sci Finetech Co., Seoul, South Korea) to solubilize proteins. After that, the mixtures were centrifuged at 4000 ×g and 4ºC for 10 min. Soluble proteins were then precipitated at pH 5.5 with the aid of 1 mol/L HCl and then centrifuged at 4000 ×g and 4ºC for 10 min to obtain protein isolate (PI). Finally, the PI paste was dried using a freeze-dryer (SBPE-SUT-02, Iran) and stored at -18°C until further use (<1 month).

Preparation of Silver carp muscle protein hydrolysates (SCMH)

Silver carp muscle PI (3%, w/v) was hydrolyzed with Alcalase at 5% E/S ratio (w/w) at 50°C and pH 8.0 for 3 min. The mixtures were heated in a boiling water bath (~95ºC) for 10 min to stop the reaction and then centrifuged at 4000 ×*g* and 4ºC for 10 min. The supernatants were dried using a freeze-dryer and the powders (SCMH) obtained were kept at -20ºC until used (<1 month) (Nikoo *et al*., 2019). Degree of hydrolysis was determined according to the pH-stat method described by Adler-Nissen (1986). SCMH prepared with Alcalase at 4.67% of degree of hydrolysis was extracted.

Preparation of probiotic cells

The vial of lyophilized culture of bacteria was broken down under sterile condition. About 1 g of lyophilized bacteria per 100 mL of sterilized MRS broth (Merck, Germany) was prepared and incubated at 37°C for 24 h. Cells were collected by centrifugation at 3500 rpm for 15 min at 4°C (Sigma Centrifuge, Osterode am Harz, Germany). The supernatant was drained and the pellet was centrifuged twice by sterile 0.9% NaCl (W/V) to be thoroughly washed. This bacterial suspension was used to prepare the desired inoculum (Ebrahimi *et al*., 2018; De Lacey *et al*., 2012).

In this study, McFarland Standards were used to standardize the approximate number of *L. casei* in a liquid suspension by comparing the turbidity of the test suspension with that of the McFarland Standard. Original McFarland Standards (0.5, 1, 2 to 10) were prepared by adding barium chloride and sulfuric acid; the

reaction between these two chemicals results in the production of a fine precipitate, barium sulfate. So, the turbidity of 8 McFarland Standard (approximate bacterial suspension/ml: 24× 10^8 CFU/mL) was used to obtain a *L. casei* suspension of known concentration. The accuracy of the density of bacterial suspension was checked by using a spectrophotometer (UV-Vis, CamSpec, M330, UK) at 650 nm (absorbance: 0.94-0.98). Finally, a bacterium suspension with a concentration of 24× 10^8 CFU/mL was acquired and used in the preparation of the examined films with a final concentration of 10^8 CFU/mL (McFarland, 1907).

Preparation of probiotic films

The method of Ebrahimi et al. (2018) was used to prepare the film solutions with some modifications. Briefly, 1.6 g SCMH was dissolved in 200 mL of distilled water. The resultant mixture was heated to the boiling point for 10 min to eliminate the possible pathogens. Then, 3 g of CMC powder was added into the mixture gradually. The solution was stirred using a magnetic stirrer at 1200 rpm for 2 h at room temperature. After that, Glycerol (50% of CMC weight) was added as a plasticizer and the solution was further stirred at 70°C for 30 min. The temperature of the solution was then allowed to decrease to 37°C and *L. casei* was added to a final concentration of 10^8 CFU/mL. The mixture was kept at 4ºC for 3 h. Afterward, 25 mL of the film-forming solution was poured and casted into sterile plastic plates with a diameter of 8 cm and the plates were dried at 37°C for ~40 h in an oven. The SCMH-loaded probiotic film was coded as a CMC-Pro-Pep sample. Two other CMC films containing probiotic bacteria (CMC-Pro) and SCMH (CMC-Pep) were prepared in the same way as stated. The pure CMC film was considered as a control sample (coded as CMC). The prepared films were stored in zipped bags until use for characterization at three different temperatures (25, 4 and -18°C) within 30 days. Probiotics were counted at intervals of 1, 10, 20, and 30 day.

Physical properties of the films

Thickness

The thickness of the films was randomly determined at 5 points using a digital micrometer (Flower, USA) with a resolution of 0.001 mm (Almasi *et al*., 2020).

Water vapor permeability (WVP)

The WVP was measured by a gravimetric approach using ASTM E96-05 (ASTM, 2005) standard method with minor modifications. Film samples were placed on a cap of glass tubes (diameter of 2 cm and 4.5 cm height) with pores in 7 mm diameter. The tubes were filled with 3 g of anhydrous $CaSO_4$ (RH= 0%), followed by placing them in a desiccator containing saturated K_2SO_4 solution (RH= 97%) at room temperature. The tubes were weighed every 3 h until reaching a fixed weight. The weight changes curves of tubes were plotted as a function of time. After calculating the slope of a line using linear regression, WVP was calculated as:

$$\text{WVP} = \frac{WVTR.X}{P(R_1 - R_2)} \quad (1)$$

where, P is the saturation vapor pressure of water (Pa) at the test temperature (25°C), R_1 is the RH in the desiccator (97%), R_2 is the RH inside the vial (0%), and X is the average thickness of the film samples (m). Under these conditions, the driving force [$P(R_1-R_2)$] was 3115.42 Pa. The water vapor transmission rate (WVTR) was the slope of the linear part of the curve (g/h) divided by the transfer area (7.85 × 10^{-5} m^2).

Moisture absorption

In order to uniformize the test conditions, the films specimens (2× 2 cm) were kept in a desiccator containing calcium sulfate (RH=0%) for 24 h. The samples were then weighed (W_0) and mounted in a desiccator containing calcium nitrate saturated solution (RH= 55%) and kept at 25°C. The weight of samples at a specified time was recorded until reaching a fixed weight (W_t). The numerical value of moisture absorption was obtained from the following

equation (Almasi *et al*., 2020; Ghadetaj *et al*., 2018; Angles & Dufresne, 2000).

$$\text{Moisture absorption (\%)} = \frac{Wt - Wo}{Wo} \times 100 \qquad (2)$$

where, W_0 (g) is the initial weight of the sample, and W_t (g) is the weight of the sample after t time.

Color properties

Instrumental color parameters of films were determined using a Hunter lab colorimeter (Minolta CR-400, Japan). Color characteristics were determined using L^* (lightness/brightness), a^* (red to green) and b^* (yellow to blue) parameters. The total color difference (ΔE) was calculated according to the following equation (Almasi *et al*., 2010).

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad (3)$$

where, ΔL^*, Δa^*, and Δb^* are the difference between the color of the standard white color plate (L^*= 93.49, a^*= -0.25 and b^*= -0.9) and the film samples.

Mechanical properties

Ultimate tensile strength (UTS) and elongation at break (EB) were determined using a Tensile Analyzer (QTS texture analyzer, CNS Farnell, Essex, UK) according to the ASTM D882-02 standard method (ASTM, 2002). The films were cut in dumbbell shape (8× 0.5 cm) and the distance between the two grips was 50 mm, while the seal velocity was chosen 10 mm/min. The relationship between stress and strain was recorded and UTS and EB were calculated as MPa and percentage, respectively.

X-ray diffraction (XRD) analyses

X-ray diffractometer (X Pert Pro, Panalytical, USA) was used to attain the XRD patterns of the film samples. The analysis was done at room temperature with a diffraction angle (2θ) from 5° to 40°. Cu Kα radiation source (k= 0.154 nm) operating at 40 kV and 40 mA was used for XRD analysis.

Field emission scanning electron microscopy (FE-SEM)

Surface and cross-section morphology of the films were investigated using FE-SEM (SIGMA VP, ZEISS, Germany) at accelerating voltage from 10 to 20 KV. Before observation, the surface of the samples was covered with a thin layer of gold using a direct current sputtering technique (DST1, Nanostructured Coating, Tehran, Iran).

Differential scanning calorimetry (DSC)

Thermal behavior of the films samples (~8 mg) at an approximate velocity of 10°C/min was measured using DSC (Netzsch 200 F3, Germany). The thermal range between -18 to 200°C was used under the constant flow of the nitrogen atmosphere to record the glass transition temperature (T_g) and melting temperature (T_m). An empty pan was used as reference.

Fourier transform infrared spectroscopy (FTIR)

FT-IR spectroscopy (Equinox 55LS 101, Bruker, Germany) of the film samples were recorded over the wavenumber range of 500-3500 cm^{-1} and for sample preparation, the KBr pellet method was used.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using SPSS 23 (IBM Corporation, Armonk, NY, USA) software. Differences between treatments were determined by Duncan′s mean comparison tests at a 5% significant level. All data were expressed as mean± standard deviation (SD).

Results and Discussion

Survival of *L. casei* in films

The survival of *L. casei* in the produced films (CMC and CMC-Pep) at different temperatures (25, 4 and -18°C) is shown in Table 1. During 30 days of storage at 25, 4 and -18°C, the viable cell numbers significantly ($p<0.05$) decreased in both types of the produced films. Ebrahimi et al. (2018) showed a negative relationship between probiotic survival and time storage. Regarding the effect

of temperature on bacterial viability, a lower viability was found at 25°C than 4°C. Gialamas et al. (2010) attributed the less viability at 4°C to the reduced bacterial metabolism at low temperatures.

There was no significant difference between the viable cell numbers of *L. casei* in two initial film-forming solutions (CMC and CMC-Pep films). However, after 10 days, regardless from the films' types, the highest *L. casei* viability (5.74 log CFU/g in CMC and 7.47 log CFU/g in CMC-Pep films) was related to the refrigerator temperature. The reduction rate of live bacteria at -18°C was higher than that at 25°C in the first 10 days of storage. After this period, the survival of frozen *L. casei* was higher than that at the room temperature. On the last day of storage at -18°C, the countable numbers of *L. casei* in CMC and CMC-Pep films were 4.1 and 6.23 log CFU/g, respectively.

Table 1- Survival of *L. casei* (log CFU/g) in CMC and CMC-Pep film during storage.

Type of films	Temperature (°C)	Time storage (day)			
		1	10	20	30
CMC	25	7.70 ± 0.05^{aA}	5.57 ± 0.2^{bA}	5.26 ± 0.21^{bA}	3.28 ± 0.2^{cA}
	4	7.70 ± 0.05^{aA}	5.74 ± 0.16^{bA}	5.52 ± 0.2^{bA}	4.85 ± 0.13^{cB}
	-18	7.70 ± 0.05^{aA}	5.5 ± 0.12^{bA}	5.38 ± 0.12^{bA}	4.1 ± 0.05^{cC}
CMC-Pep	25	7.79 ± 0.01^{aA}	7.39 ± 0.25^{bB}	6.89 ± 0.11^{cB}	4.91 ± 0.09^{dB}
	4	7.79 ± 0.01^{aA}	7.47 ± 0.13^{bB}	7.22 ± 0.13^{cB}	7.00 ± 0.01^{cD}
	-18	7.79 ± 0.01^{aA}	7.25 ± 0.07^{bB}	6.98 ± 0.02^{cB}	6.23 ± 0.19^{dE}

Data are represented as mean ± standard deviation [different capital and small letters in each column and row (respectively) indicate a significant difference ($p<0.05$) between data].

Călinoiu et al. (2016) stated that initial cold shock from freezing damaged the integrity of bacterial cell membrane. However, after adapting to temperature conditions, depending on the bacterium strain, the survival of the probiotics (especially lactic acid bacteria) would be improved. The viability of *L. casei* in both CMC and CMC-Pep films at the end of storage at 4°C was significantly ($p<0.05$) higher than other temperatures. The type of bacterial strains, water activity, water vapor, and oxygen permeability of film and storage temperature are the main factors that affect the survival of probiotics (Ebrahimi *et al*., 2018; Soukoulis *et al*, 2017; Cruz *et al*., 2009; Vasiljevic & Shah, 2008).

The results showed that the addition of SCMH to the films significantly caused less bacterial death in the films over the storage time. The remaining bacterial count in the CMC film at the end of 30 days was lower than 10^7 CFU/g at all temperatures. However, at 4 °C, it was significantly ($p<0.05$) higher compared to those stored at 25 and -18°C. Besides, *L. casei* at 4°C in CMC samples from the initial value of 7.70 log CFU/g reached to 4.85 log CFU/g at the end of storage time (showed decreasing approximately 3 log of CFU). In contrast, the countable number of *L. casei* cells in CMC-Pep film at refrigerated storage conditions remained above 10^7 CFU/g throughout the 30 days with ~1 log CFU /g decrease in viability.

The results of this study were similar to those of Yu *et al*. (2016), proving that whey peptide extract accelerated probiotics reproduction. Additionally, Settier-Ramírez et al. (2020) showed that the inclusion of casein hydrolysates in PVOH/Protein based films resulted in higher survival of *L. lactis subsp. Lactis*. Soukoulis et al. (2017) stated that the film composition significantly affected the viability of probiotics and biopolymer chemistry was a critical factor in bacterial stability. Furthermore, the enhanced *L. casei* viability with the addition of SCMH could be due to providing a micronutrient source (Soukoulis *et al*., 2017; Charalapopoulos *et al*., 2003), maintenance of suitable water activity (Zabihollahi *et al*., 2020; Soukoulis *et al*., 2017), scavenging of free radical and preventing lipid autoxidation (Soukoulis *et al*., 2017; Peng *et al*., 2010). The mentioned factors

were implicated in maintaining *L. casei* cell to keep its physical structure (Soukoulis *et al*., 2017). Similar results have been reported for the effect of other prebiotics such as inulin (Orozco-Parra *et al*., 2020; Zabihollahi *et al*., 2020), polydextrose (Karimi *et al*., 2020), oligofructose and inulin (Alvarez *et al*., 2021) on the viability improvement of probiotic bacteria within edible films matrix.

Physicochemical properties of the films

The results related to different physicochemical properties of the produced films are presented in Table 2. Thickness is an essential factor affecting the mechanical properties, transparency, and WVP of the films (Ebrahimi *et al*., 2018; Ghanbarzadeh & Almasi, 2011). The results showed that adding probiotics had little effect on the thickness, probably due to the good interaction of *L. casei* with the CMC polymer (Ye *et al*., 2018). However, the addition of SCMH significantly ($p<0.05$) increased this index. Zabihollahi *et al*. (2020) reported that the incorporation of inulin in the CMC-based probiotic films significantly elevated film thickness, because of changing the dry matter content. The lowest value (72 µm) was for the CMC film. This amount reached 72.95, 102, and 102.5 µm in the CMC-Pro, CMC-Pep, and CMC-Pro-Pep films, respectively. These results contradicted the data obtained by Ebrahimi et al. (2018), who observed that the addition of probiotics caused a significant increase in the thickness of the film and it reached from 40.3 µm in the control films to 50 µm in the films containing *L. casei*. Khodaei et al. (2020) reported that adding probiotics to the low methoxyl pectin films did not have meaningful effect on the film thickness.

Evaluation of the moisture absorption of the produced films showed that by adding SCMH, their moisture absorption increased significantly ($p<0.05$). The addition of *L. casei* also partially increased the moisture absorption, however, this increase was not significant and could be ignored. The highest observed value was 13.8% in the CMC-Pro-Pep film. The moisture absorption increased with the enhancement of available OH groups in the polymer. The hygroscopic nature of film composition was also involved in the value of moisture absorption index of the films (Ghanbari *et al*., 2018). The addition of SCMH apart from enhancing OH groups, due to their high hygroscopicity, could raise the moisture absorption. SCMH has polar carboxyl and amine groups, enabling it to attach to water molecules by hydrogen bonds (Shavandi *et al*., 2019; Nuanmano *et al*., 2015). Moreover, the interrupting effect of the additives and their prevention from interconnected network formation in CMC matrix, causes to increase the free reactive –OH and –COOH groups of CMC which leads to increase the moisture absorption capacity of probiotic films.

The water vapor barrier properties of films can be measured by the WVP index. To prevent moisture exchange between the environment and the food product, the packaging materials must have lowest permeability to water vapor as much as possible. Generally, various factors, such as the type of compounds and degree of interaction between them, the film thickness, solubility and permeability of water vapor molecules in film matrix affect the amount of WVP (Nuanmano *et al*, 2015; Kanmani, & Lim, 2013). Adding *L. casei* to the film did not make a significant change in WVP amounts. These results were similar to those of Gialamas *et al*. (2010), who showed that the addition of *L. sakei* to sodium caseinate film had no impact on the WVP. Unlike the *L. casei*, the effect of the SCMH was more noticeable. The numerical value of WVP increased from 1.12 in the CMC film to 1.68 ($\times 10^{-7}$ g/m.h.Pa) in the CMC-Pep film, attributable to the plasticizing effect of the low molecular weight SCMH. As mentioned, the presence of SCMH resulted in increased hydrophilic groups in the film structure and subsequently, the presence of more water molecules in thus leads to increase the WVP of films (Nuanmano *et al*, 2015; Kanmani, & Lim, 2013). Nuanmano et al. (2015) confirmed that gelatin hydrolysate could enhance the WVP values of fish myofibrillar protein film and the higher degree of hydrolyses led to a more upward trend because of increasing content of

hydrophilic groups. Dianin et al. (2019) reported that the incorporation of *L. casei* increased the WVP and water solubility of WPI film. Mozaffarzogh et al. (2020) observed profound effect of different probiotic bacteria on the weakening of water barrier properties of CMC-sodium caseinate films. They stated that the probiotic microbial cells probably exist in the polymeric matrix as discontinuous particles and therefore increase the chain mobility of the polymers that leads to increase WVP and moisture absorption of films.

Table 2- Physicochemical properties of produced films

Film samples	Thickness (µm)	Moisture absorption (%)	WVP ($\times 10^{-7}$ g/m.h.Pa)
CMC	72± 2.64^{a}	10.60± 0.6^{a}	1.12± 0.05^{a}
CMC-Pro	72.95± 3.69^{a}	11.24± 1.35^{a}	1.26± 0.09^{a}
CMC-Pep	102.00± 2.6^{b}	13.00± 0.04^{b}	1.68± 0.07^{b}
CMC-Pro-Pep	102.5± 3.5^{b}	13.80± 1.12^{b}	1.98± 0.10^{c}

Different letters in each column indicate a significant difference ($p<0.05$) between data

Mechanical and optical properties

The color of the film can be affected by its constituents (Shahrampour *et al*, 2020). The color parameters of the produced films are shown in Table 3. There was no visual difference between the probiotic and non-probiotic films. The results of the colorimetric analysis also revealed that probiotics had no significant effect on the lightness (L^*) of the films, but significant changes were made by adding SCMH i.e. lightness decreased. Apart from films' components and the nature of polymer, the thickness of the films can change the color parameters (Liu and Han, 2005). Addition of SCMH resulted in higher thickness of the films, which in turn causes lower L^* values (Shahrampour *et al*, 2020). The L^* values ranged from 84.21 (CMC-Pro-Pep film) to 89.38 (CMC film). The SCMH was green in color and its addition to the film compositions resulted in the film turning from colorless to green. The highest tendency for green (a^*= -9) was in CMC-Pep and CMC-Pro-Pep films. Although the produced films were somewhat yellowish, the trend was higher in the SCMH-loaded films. Piermaria et al. (2015) stated that when the difference of ΔE was over 3, the color difference between the films was detectable by the naked eyes. The ΔE in the SCMH-containing films was significantly ($p<0.05$) higher than that of the CMC films and the color difference between these two categories of films was clearly detectable. Similarly, previous studies showed that the immobilization of probiotics in the edible films did not cause significant changes in color parameters including L^*, a^*, b^*, and ΔE values (Shahrampour *et al*, 2020). Moreover, in agreement with our results on the effect of SCMH prebiotics on color parameters, Orozco-Parra et al. (2020) and Zabihollahi et al. (2020) reported that the addition of inulin even at lower concentrations, caused an increase in the ΔE values of CMC and cassava starch-based probiotic films respectively.

Table 3- Mechanical and color properties of produced films

Film samples	UTS (MPa)	EB (%)	L^*	a^*	b^*	ΔE
CMC	2.548± 0.5^{a}	17.54± 0.48^{a}	89.38± 0.18^{a}	-1.46± 0.02^{a}	4.22± 0.02^{a}	6.078± 0.07^{a}
CMC-Pro	3.679± 0.9^{a}	29.91± 0.81^{b}	89.25± 0.29^{a}	-1.70± 0.01^{b}	3.42± 0.001^{b}	5.69± 0.19^{b}
CMC-Pep	2.186± 0.6^{a}	17.05± 0.02^{a}	84.42± 0.31^{b}	-9± 0.02^{c}	7.46± 0.05^{c}	14.69± 0.20^{c}
CMC-Pro-Pep	3.138± 0.2^{a}	21.58± 1.13^{c}	84.21± 0.001^{b}	-9± 0.001^{c}	6.91± 0.08^{d}	14.55± 0.009^{c}

Different letters in each column indicate a significant difference ($p<0.05$) between data

Mechanical properties show the film's durability and ability to preserve food integrity. The effect of adding SCMH was different from that of the probiotic microorganism. The

addition of SCMH reduced both the UTS and EB indices; however, in the presence of *L.casei*, both factors increased, possibly because of plasticizing effect of SCMH in the films which reduces the intra- and intermolecular interactions between CMC polymer chains. As a result, the mobility of the polymer chains increased, which could reduce both UTS and EB values (Mandal and Chakrabarty, 2019; Nuanmano *et al*, 2015). Previous studies reported that mechanical properties were reduced with the incorporation of fish protein hydrolysates into the edible films (Hasanzati Rostami *et al*., 2017; Hoque *et al*., 2011; Giménez *et al*., 2009). The reducing of mechanical stiffness by incorporation of other prebiotics such as inulin (Zabihollahi *et al*., 2020) and polydextrose (Karimi *et al*., 2020) to the biopolymer-based films formulations, has also been approved.

As mentioned, probiotic bacteria improved both UTS and EB, probably due to good interaction between the bacteria and the film components (Ye *et al*, 2018). The UTS value increased from 2.548 MPa in the CMC film to 3.679 MPa in the CMC-Pro film. The amount of EB in these two films also increased after the addition of *L. casei* and reached from 17.54% to 29.91%. The results of this study contradicted those of Ebrahimi et al. (2018), showing that probiotics interfered with the film cohesiveness and had a negative effect on the mechanical properties. However, Piermaria et al. (2015) reported similar results. They revealed that the subjoining of microorganisms (yeast and lactic acid bacteria) in glycerol-plasticized kefiran films enhanced the UTS, attributable to the positive effect of microorganisms on reducing the plasticizing properties of glycerol. Khodaei et al. (2020) incorporated three different probiotics (*Lactobacillus plantarum*, *L. casei*, and *Saccharomyces boulardii*) into the two different biopolymer films (gelatin and low methoxyl pectin), separately. They achieved more interesting results indicating that mechanical properties of probiotic films strongly depends on the type of both biopolymer and bacteria. For example, *L. plantarum* improved the UTS of low methoxyl film but decreased the UTS of gelatin film. According to their results, the type of microorganism and biopolymer determine the effect on mechanical properties of probiotic films and this should be investigated case by case.

Fourier transform infrared spectroscopy (FTIR)

Fig. 1 shows the FTIR spectra of the CMC-based probiotic films. The pure CMC films exhibited specified peaks at 3436, 2926, 2151, 1614, 1418, 1054, 922, 855, and 719 cm^{-1}. The peaks at 3436 and 2926 cm^{-1} were related to the stretching vibration of –OH and C-H groups, respectively. Besides, the asymmetric and symmetric vibrations of –COOH groups of CMC were assigned by the peaks appeared in 1614 and 1418 cm^{-1}, respectively. The peak at 1054 cm^{-1} could be attributed to the C-O-C stretching vibrations of the polysaccharide skeleton. Furthermore, the peaks observed in the region of 700-950 cm^{-1} indicated the bending vibrations of C-H groups (Akhtar *et al*., 2018).

The incorporation of probiotic bacterium caused some changes in the spectra of the CMC film. The most important changes were the shifting of the peaks at 2926 and 1054 cm^{-1} to lower wavenumbers (2915 and 1040 cm^{-1}, respectively) and disappearing of –COOH symmetric vibration peaks at 1418 cm^{-1}. Moreover, the addition of SCMH caused a change in the FTIR spectrum of the CMC film. The decrease in the intensity of 3436 cm^{-1} peak, assigned to –OH groups, was the main observable change in the spectrum of the SCMH-loaded CMC film. In the film containing both of probiotics and SCMH, the effect of bacteria on FTIR spectrum was more than SCMH. The changes observed in the bands corresponding to –COOH and –OH groups by addition of *L.casei* and SCMH, respectively, confirmed the possible interactions (hydrogen bonds) between CMC, SCMH and probiotic bacteria, which was in agreement with the results of Zabihollahi et al. (2020), who reported the formation of hydrogen bonds

between inulin, *L. plantarum* probiotic and CMC matrix.

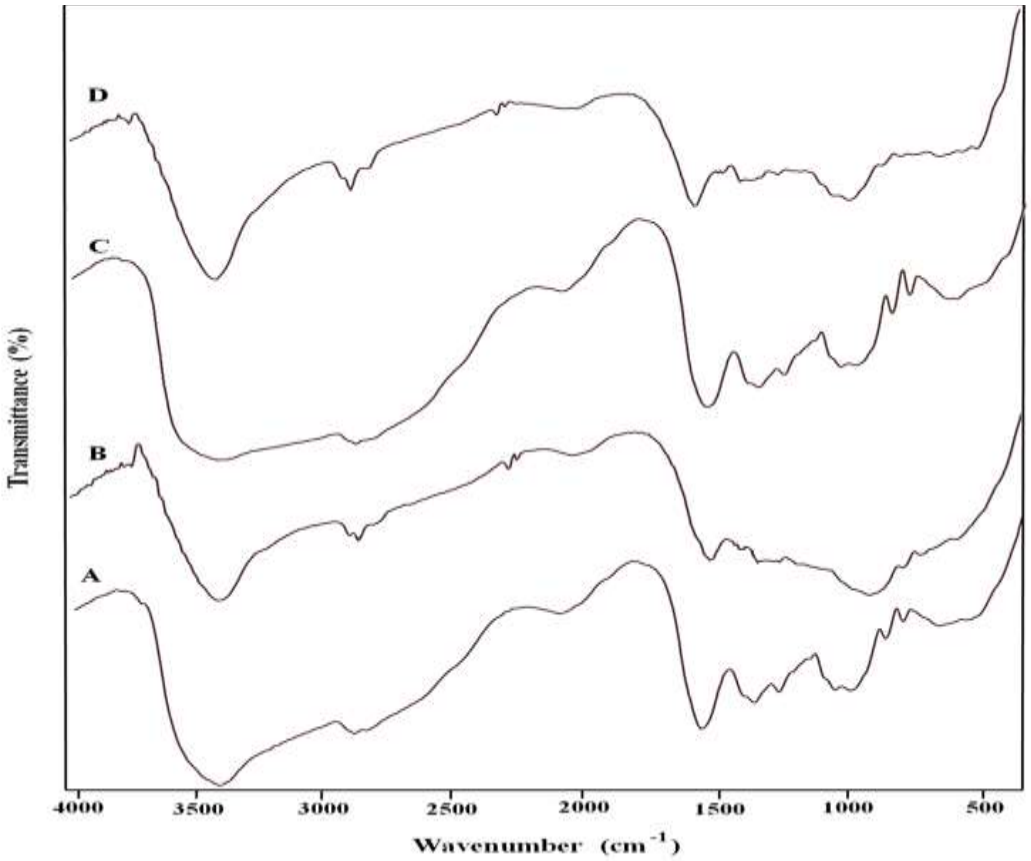

Fig. 1. FT-IR spectra of probiotic active films; (A: pure CMC film, B: *L. casei* loaded CMC film, C: SCMH loaded CMC film and D: *L. casei* and SCMH loaded CMC film).

X-ray diffraction (XRD) analysis

The XRD analysis was carried out in order to study the crystalline structure of the films. Fig. 2 shows the XRD patterns of pure and probiotic CMC films. As shown in this figure, the pure CMC film exhibited two specific peaks at 2θ of 7.8° and 20.5°, indicating the semi-crystalline structure of the CMC film. These results were in line with the previously reported studies on the CMC films (Zabihollahi *et al*., 2020; Dai *et al*., 2018). According to Fig. 2, the incorporation of probiotic bacteria had no significant effect on crystalline structure of the CMC film. However, with the addition of SCMH, the intensity of peaks, particularly the peak at 7.8°, decreased. This observation approved the plasticizing effect of low-molecular weight SCMH chains in the CMC matrix, leading to the decrease in the compactness of CMC matrix and thus the decrease of the crystalline domains in its structure. Arfat et al. (2014) reported similar results for fish protein isolate incorporated to the fish skin's gelatin films. As shown in Fig. 2, by simultaneous incorporation of *L. casei* and SCMH, the semi-crystalline structure of the CMC was preserved and probiotic bacteria decreased the negative effect of SCMH on structure of the CMC film. Karimi et al. (2020) reported similar results for the effect of *L. plantarum* and polydextrose as probiotic and prebiotic agents, respectively, on the semi-crystalline structure of the WPI films. Mozaffarzogh et al. (2020) prepared CMC-sodium caseinate probiotic films containing various probiotic bacteria including *Lactobacillus acidophilus*, *L. reuteri*, *L. casei*, *L. rhamnosus*, and *Bifidobacterium bifidum*. They observed that none of them had any effect on the crystallinity of composite film.

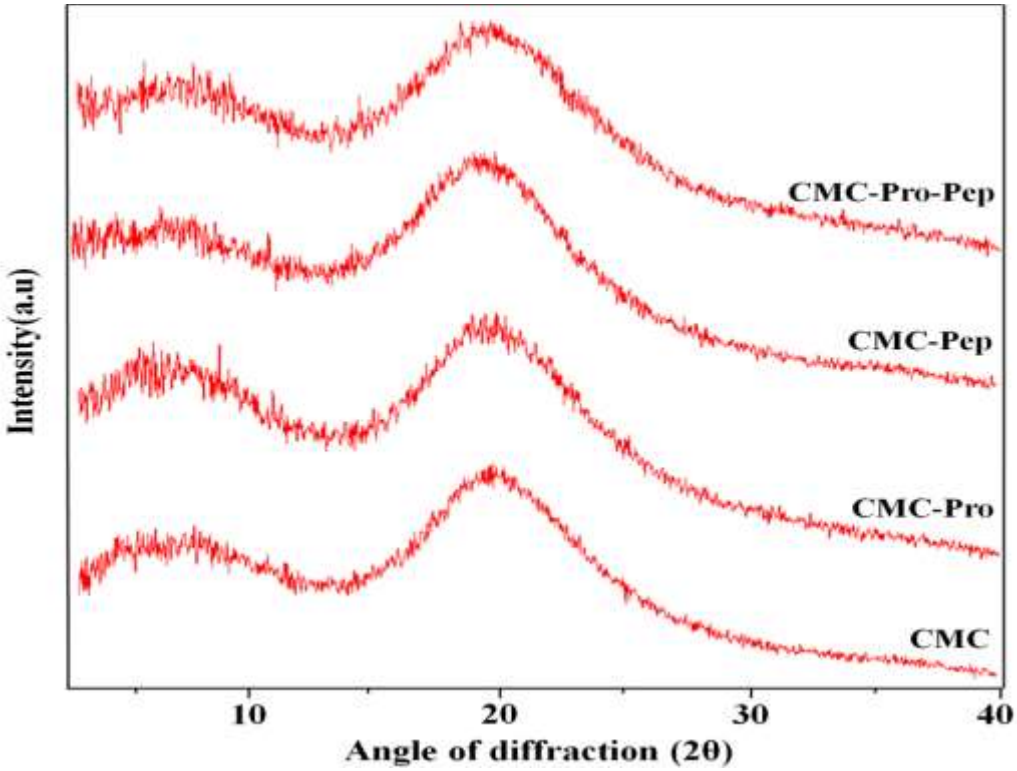

Fig. 2. XRD patterns of probiotic active films; (CMC: pure CMC film, CMC-Pro: *L. casei* loaded film, CMC-Pep: SCMH loaded film and CMC-Pro-Pep: *L. casei* and SCMH loaded film).

Field emission scanning electron microscopy (FE-SEM)

Fig. 3 depicts the FE-SEM images of the cross-section of the CMC films. All films exhibited a dense and pores-free cross section, indicating the compact microstructure of the CMC films. The only change was the increase of the projections after incorporation of bacterial cells and SCMH which is common for the heterogeneous films. Ebrahimi et al. (2018) reported similar results for the probiotics-incorporated CMC films. The surface images of films are shown in Fig. 4. The pure CMC film exhibited a smooth and homogenous surface without any cracks and projections, in agreement with results of Akhtar et al. (2018) reported for the pure CMC film having glycerol plasticizer. However, after incorporation of *L. casei*, some cracks were observed on the surface of the CMC-Pro film. The addition of SCMH caused an increase in discontinuity in the film surface. Moreover, the phase separation and aggregation phenomena were observed in the CMC-Pep film, confirming that the SCMH could be accumulated without uniform distribution in the CMC matrix. This observation is in accordance with XRD results. Moreover, the decrease of tensile strength and increase of WVP of CMC films after the incorporation of SCMH could be explained by this observation in FE-SEM analysis. Orozco-Parra et al. (2020) reported that inulin incorporation increases the discontinuity of cassava starch film microstructure. However, Karimi et al. (2020) observed more compact and uniform structure of WPI film after polydextrose incorporation indicating that polydextrose acted as a filler of the interspaces in the WPI network. Therefore, the effect of prebiotics on the microstructure of edible films depends on the nature of compounds and their miscibility with biopolymer matrix. The simultaneous incorporation of probiotic bacteria and SCMH prevented the aggregation of SCMH, but the cracks were observable in the CMC-Pro-Pep sample. In general, the presence of bacterial cells diminished the adverse effect of SCMH on the structural properties of the CMC films.

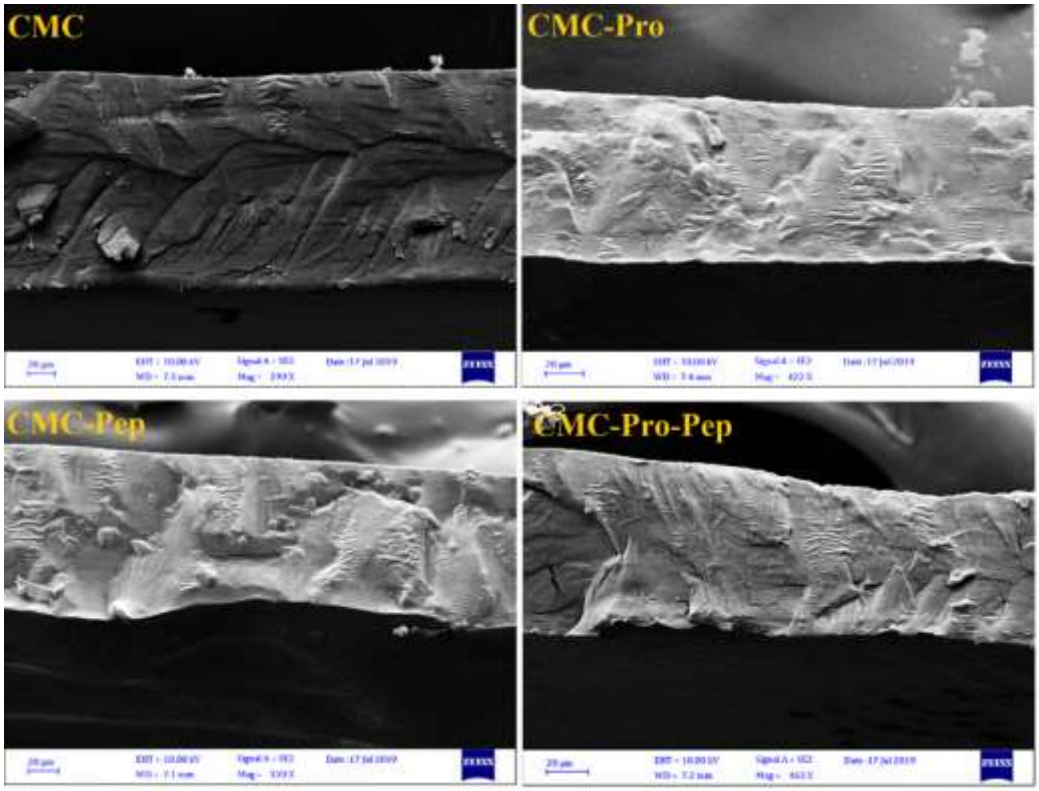

Fig. 3. FE-SEM images of cross-section of probiotic active films; (CMC: pure CMC film, CMC-Pro: *L. casei* loaded film, CMC-Pep: SCMH loaded film and CMC-Pro-Pep: *L. casei* and SCMH loaded film).

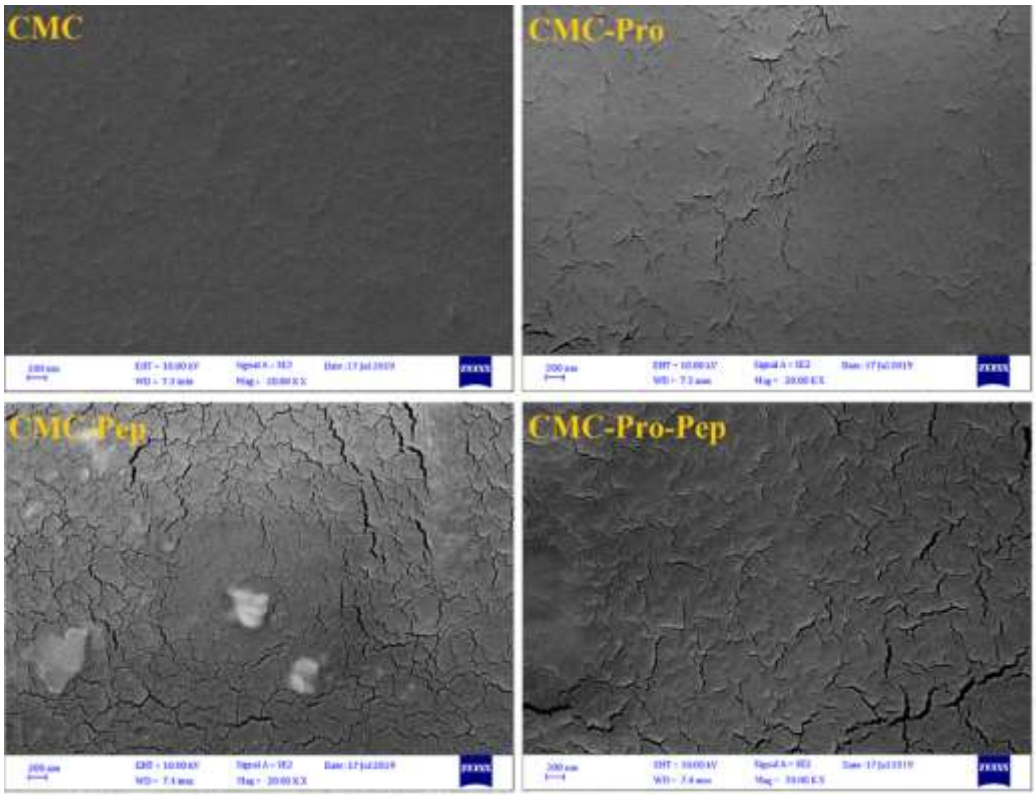

Fig. 4. FE-SEM images of surface of probiotic active films; (CMC: pure CMC film, CMC-Pro: *L. casei* loaded film, CMC-Pep: SCMH loaded film and CMC-Pro-Pep: *L. casei* and SCMH loaded film).

Differential scanning calorimetry (DSC)

Table 4 shows the results of DSC analysis of the CMC films. The T_g value of the CMC film increased from 41.2 to 42°C after the incorporation of *L. casei*. However, SCMH addition decreased T_g value. Karimi et al. (2020) reported similar results for the effect of polydextrose on thermal properties of WPI films. The SCMH-loaded film had the lowest melting point and increased by incorporation of probiotic cells. Similar to other results, the presence of bacteria decreased the adverse effect of SCMH on the thermal properties of the CMC film. As shown in table 4, the CMC-Pro-Pep sample had the highest T_m (112.4°C). The reduction of plasticizing effect of SCMH, due to the tight entrapment of bacterial cells in the CMC matrix, and the formation of new interactions after incorporation of probiotics were the reasons of this observation. In spite of forming new bands (approved by FT-IR test), the plasticizing effect of SCMH was higher, resulting in weakened thermal properties of the CMC films. The decrease of T_g and T_m of the CMC films after the incorporation of inulin as prebiotic agent was reported by Zabihollahi et al. (2020).

Table 4- Thermal properties (glass transition temperature (T_g) and melting temperature (T_m)) of CMC-based probiotic film.

Sample	T_g (°C)	T_m (°C)
CMC	41.2	109.0
CMC-Pro	42.0	109.6
CMC-Pep	38.4	108.0
CMC-Pro-Pep	41.5	112.4

Conclusions

The CMC-based probiotic films were successfully developed and characterized. Based on the results, the incorporation of probiotic cells showed no significant effects on the water barrier properties of CMC-based films. The FT-IR, FE-SEM, DSC and XRD results revealed the formation of interactions between CMC matrix and SCMH, indicating their good compatibility. In addition, the thermal properties, and crystallinity of *L. casei* and SCMH incorporated CMC-based probiotic films were acceptable. The viability of probiotic bacteria in the CMC-based films was improved through the addition of SCMH. In conclusion, the SCMH incorporated CMC-based film can be a good carrier for probiotics as a bioactive food packaging system. The as prepared films may be applied as coatings or wrappings to a variety of foods including meat products, fruits and vegetables, cheese and butter, and bakery products, providing them with potential health benefits to the consumers, besides being potentially able to inhibit the growth of spoilage microorganisms on food surface, thus increasing food shelf life. Further studies are required to investigate the effects of developing probiotic films on the shelf-life extension of real food systems.

Conflicts of interest

The authors certify that they have no conflict of interests with respect to this manuscript.

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فیلم خوراکی زیست فعال برپایه کربوکسی متیل سلولز حاوی لاکتوباسیلوس کازئی و پروتئین هیدرولیز شده ماهی

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چکیده

به‌دلیل تمایل به غذاهای طبیعی و سالم، بازار غذاهای فراسودمند به سرعت در حال رشد است. در این میان، پروبیوتیک‌ها به دلیل توانایی بالقوه آن‌ها، در فرمولاسیون‌های غذایی سالم، به‌طور جدی مورد توجه قرار گرفته‌اند. بیشترین نگرانی در مورد پروبیوتیک‌ها این است که ممکن است تعداد باکتری‌های پروبیوتیک در زمان مصرف کمتر از مقدار مورد نیاز (10^7 CFU/g) باشد. بنابراین در این مطالعه، فیلم‌های خوراکی پروبیوتیک کربوکسی متیل‌سلولز (CMC) حاوی *لاکتوباسیلوس کازئی* و پروتئین هیدرولیز شده عضله ماهی کپور نقره‌ای (SCMH)، تهیه شد و زنده‌مانی سلول‌های باکتری در طول ۳۰ روز نگهداری (در فواصل زمانی ۱، ۱۰، ۲۰ و ۳۰ روز) در دماهای ۲۵، ۴ و ۱۸- درجه سانتی‌گراد بررسی گردید. جهت استخراج پروتئین از روش انحلال قلیایی/ ترسیب اسیدی استفاده شد. ایزوله پروتئین استخراجی به‌وسیله آنزیم آلکالاز (۵٪ وزنی/ وزنی) در دمای ۵۰°C و pH= ۸ به مدت ۳ دقیقه هیدرولیز گردید. فیلم‌ها با انحلال SCMH و CMC با نسبت ۲:۱ در آب مقطر، تهیه شدند و *لاکتوباسیلوس کازئی* با غلظت 10^8 CFU/mL به فیلم‌ها اضافه شد. خصوصیات رنگی، فیزیکی، استحکام کششی نهایی (UTS) و ازدیاد طول در نقطه شکست (EB) فیلم‌ها بررسی شد. الگوهای ساختاری نمونه‌های فیلم با پراش‌سنج اشعه X در دمای اتاق با زاویه پراش (۲θ) از ۵ تا ۴۰ درجه به‌دست آمد. طیف‌سنجی FT-IR فیلم‌ها در طول موج $3500-500\ cm^{-1}$ ثبت شد. نتایج آنالیز FT-IR، XRD و DSC، حاکی از شکل‌گیری پیوند هیدروژنی بین *لاکتوباسیلوس کازئی* و ماتریس فیلم و همچنین اثر پلاستی‌سایزری SCMH بودند. به‌طوری که فیلم CMC خالص حاوی باکتری، بالاترین خصوصیات مکانیکی (EB=۲۹/۹٪، UTS=۳/۷ MPa) را داشت. افزودن SCMH به فیلم‌ها، به‌طور قابل توجهی ($p<0.05$) زنده‌مانی *لاکتوباسیلوس کازئی* را در همه دماها افزایش داد و توانست در پایان دوره نگهداری در دمای ۴°C مقدار آن را در حد ۰/۰۱ ±۷ log CFU/g نگه دارد.

واژه‌های کلیدی: پروتئین هیدرولیز شده، *لاکتوباسیلوس کازئی*، کربوکسی متیل‌سلولز، فیلم خوراکی، پروبیوتیک.

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Full Research Paper

Mass transfer simulation of pistachio nuts using computational fluid dynamic (CFD) during fluid bed drying

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Abstract

The drying of food can extend the shelf life of food, reduce transportation and storage costs. Fick's second law is commonly used to evaluate the mass data in the drying process in a standard way and is based on many assumptions. Understanding the meaning of mass transfer in products can improve the drying process and product quality. Computational fluid dynamics (CFD) models fluid flow situations utilizing powerful computer and applied mathematics in order to predict mass transfer in industrial processes. The aim of this research was numerical study of the drying behavior of pistachio nut using CFD method and evaluating the numerical results in the bed condition of fluid, semi fluid and fix bed as well as air temperatures of 90, 75, 60 and 45°C. During drying using computational fluid dynamic and the Fluent CFD code, the external flow and temperature fields around the cylindrical object (7.5× 10 millimeter) will be predicted in the numerical analysis. A laboratory fluid bed dryer was used for drying experiments. The main parts of the dryer are forward radial fan, drying chamber, electrical heater, inverter, temperature controller. The dryer attachment tools are input and output temperature sensors, anemometer and computer. The numerical part was verified and juxtaposed with the experimental data. The numerical solution result at 60, 75 and 90°C were so close to experimental results except for air temperature of 45°C. Mean absolute error in fix bed, at 60, 75 and 90°C were 0.2123, 0.1257 and 0.0337 which were lower than 45°C temperature and R^2 values for these temperatures were 0.9903, 0.9705 and 0.9807, respectively. As the temperature decreased, the values of E_{abs} and X^2 increased in all bed conditions. The average value of R^2 for all applied bed conditions was 0.9850. This value showed high correlation between experimental and numerical results.

Keywords: Semi fluid bed drying, Fluid bed drying, Fix bed drying, Experimental results, Modeling, Numerical solution.

Introduction

Nuts are rich in nutrients, fiber, protein, phytosterols and antioxidants (Fantino *et al.*, 2020). The edible seeds harvested from the pistachio tree are pistachios. Among the different species of the genus Pistachio, pistachio (Pistacia Vera) is the only known commercially edible nut. Its main cultivation areas include the Mediterranean region. It is not affected by drought and salinity (Noguera Artiaga *et al*., 2020). For consumers to consider it an edible nut, the fruit of the Pistacia Vera variety must be large enough (Shokraii and Esen, 1998). Pistachios are used as ingredients

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in the confectionery, snacks, ice cream and pastry industries. Pistachios were originally grown in the United States, the Middle East, and especially in Iran (Kouchakzadeh and Shafeei, 2010).The quality of pistachios depends on proper harvesting and post-harvest processing. Drying is very important in the marketing of the final product. The optimal moisture content for storing pistachios is 7%-9% (w.b.) (USDA Std., 1990).

Studies have shown that drying methods and different changes are key factors in determining the quality of dried food products (Naidu *et al*., 2016). In other words, the deterioration and damage of certain characteristics, including their color, structure, aromatic compounds and nutrients, may lead to a decline in product quality (Izli and Isik, 2015). The most common method used to dry pistachios is the hot air drying method. Fluidized bed drying is a profitable drying method for pistachios. When passing through the pistachio bed, a fixed bed is formed when a low-velocity airflow flows upward. When the highest air velocity is applied, the whole pistachio starts to float, which is called minimum fluidization (AmiriChayjan *et al*., 2012). The semi-fluidized bed condition occurs at the maximum static pressure drop. When the inflow airflow increases, a bubbling fluidized bed is created, causing the mixing of pistachios (Kunii and Levenspiel, 1991; Kaveh and AmiriChayjan, 2015).

In the drying mechanism of a system, the phenomenon of simultaneous transmission (energy, momentum and mass) will occur. Drying can extend the shelf life of food, reduce transportation and storage costs, and develop new consumption methods (Lindsay Rojas and Augusto, 2018). Fick's second law (Fick, 1855) is commonly used to evaluate the mass data process in the drying process in a standard way and is based on many assumptions (Lindsay Rojas and Augusto, 2018). Understanding the meaning of mass transfer in products can improve the drying process and product quality. External factors include temperature, relative air humidity, and air velocity. However, internal factors include factors such as the density and permeability of the material, as well as thermophysical properties (Kaya *et al*., 2006).

Compared with experimental research, numerical simulation is a common method applied for drying process analysis due to lower cost and time (Kaya *et al.*, 2006). Numerical methods for describing food drying mechanisms can provide useful information to help understand temperature and humidity requirements more clearly (Haghighi *et al.*, 1990; Rafiee and Kashaninejad, 2005; Rafiee *et al.*, 2005). In computational fluid dynamics (CFD), numerical methods are often used to approximate the equation that governs fluid dynamics in the target fluid region. It uses numerical methods to predict chemical reactions, mass transfer, heat transfer, fluid flow, and related phenomena by solving mathematical equations that control these processes (Puma Chandra, 2017). CFD technology can also help solve complex transmission phenomena and make drying process more cost and time-effective (Kaya *et al*., 2006; Kaya *et al*., 2008a).

Many researchers studied numerical modeling of mass transfer for a wide variety of agricultural and food products. These include hazelnut (Topuz *et al*., 2004), garlic (AbbasiSouraki and Mowla, 2008), kiwi fruit (kaya *et al*., 2008b), apple slices (Mabrouk *et al.*, 2012), soybean meal (Silva et al., 2012), shrinkable products such as lentils (Carmo and Lima, 2004), fruits and vegetables (Kowalski and Mierzwa, 2013). However, no studies have specifically examined numerical modeling for pistachio during convective drying of fluid, semi fluid and fix bed.

Researchers around the world have studied the drying of various products using CFD simulation (Malekjani and Jafari, 2018; Demissie *et al.*, 2019).Studies have also been performed on the simulation of pistachio nuts drying by finite element (Rafiee *et al*., 2007; Rafiee *et al*., 2009). No study has been done on simulating the drying of pistachio nuts using CDF simulation. The aim of this research was numerical study of the drying behavior of pistachio nut using CFD method and e

evaluating the numerical results in the bed condition of fluid, semi fluid and fix bed as well as air temperatures of 90, 75, 60 and 45°C. During drying process using computational fluid dynamic and the Fluent CFD code, the external flow and temperature fields around the cylindrical object (7.5× 10 millimeter) were predicted in the numerical analysis. The numerical part was verified and the experimental data relevant to the method of thin-layer drying were juxtaposed.

Materials and method

Experimental setup and procedure

Ohadi cultivar of pistachio was selected for conducting the study. The initial moisture content of the samples collected was 50.3±0.2% (d.b.), at 130°C and 24 h using the oven process (AOAC, 1995).

To indicate bed conditions (air velocities) in tests, the increasing air velocity against pressure drop were recorded and then plotted as Fig. 1. An estimating and recording unit for differential digital manometer and a vane type advanced anemometer (StandardST-8897) was utilized to get the fluidization curve. To accomplish the net air pressure drop across the pistachio bed, from the start, total static pressure drop because of pistachio column and bed plate was estimated, and afterward was deducted from air pressure drop because of empty chamber.

The greatest value of static pressure drop against a specific air velocity in fluidization curve is characterized as least fluidization point or semi fluidized bed (Kunii and Levenspiel, 1991). Fluidization tests were acted in three recreates for thin layer drying of pistachio tests with around 100 g load. In the wake of getting the semi fluid bed point (air velocity about 2.6 ms^{-1}), two focuses with air velocities of 1.6 ms^{-1} and 4.1 ms^{-1} were chosen as fix and fluid bed conditions, respectively.

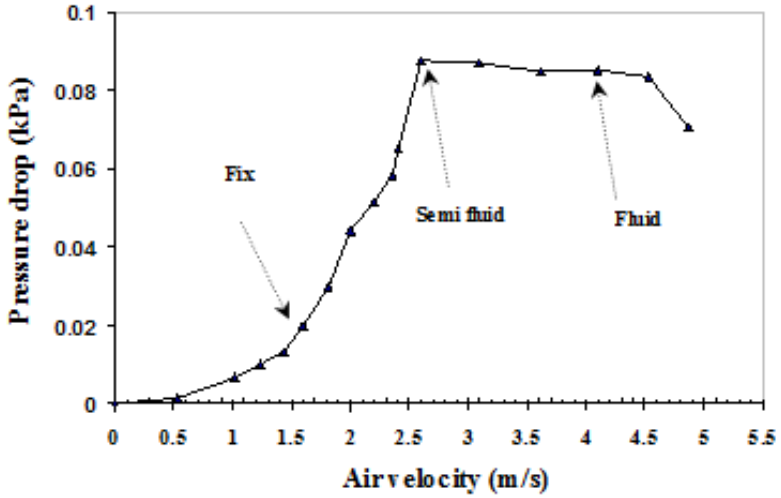


Fig. 1. Pistachio nuts' fluidization curve

The drying process was ended, when the moisture content of the samples reached an average of about 9 percent (d.b.) (Amirichayjan *et al.*, 2012). The air relative humidity and air temperature were 21%-33% and 28-32°C, respectively. A laboratory fluid bed dryer was used for drying experiments. This apparatus was fabricated in agricultural machinery engineering of Bu-Ali Sina University (Figure 2). The main parts of the dryer are forward radial fan, drying chamber, electrical heater, inverter, and temperature controller. The dryer attachments tools are input and output temperature sensors, anemometer and computer. 45, 60, 75 and 90°C air temperature levels and fix, semi fluid and fluid bed conditions were used as input variables in the experiments. Each experiment was replicated three times (Amirichayjan *et al.*, 2012).

In each drying test, around 100 g pistachio sample was stacked in drying chamber. Pistachio nuts were extended in a thin layer form in the drying chamber and the test was begun. The ambient air temperature, input and output air temperature, air velocity, air relative humidity and sample weight were consistently checked and recorded during drying process. A digital balance (AND GF-6000) with 0.01 g accuracy was used to online weighing the nuts during the drying experiments. Moisture content of the pistachio samples in each drying run was calculated based on the initial and final moisture content of the samples and initial samples mass.

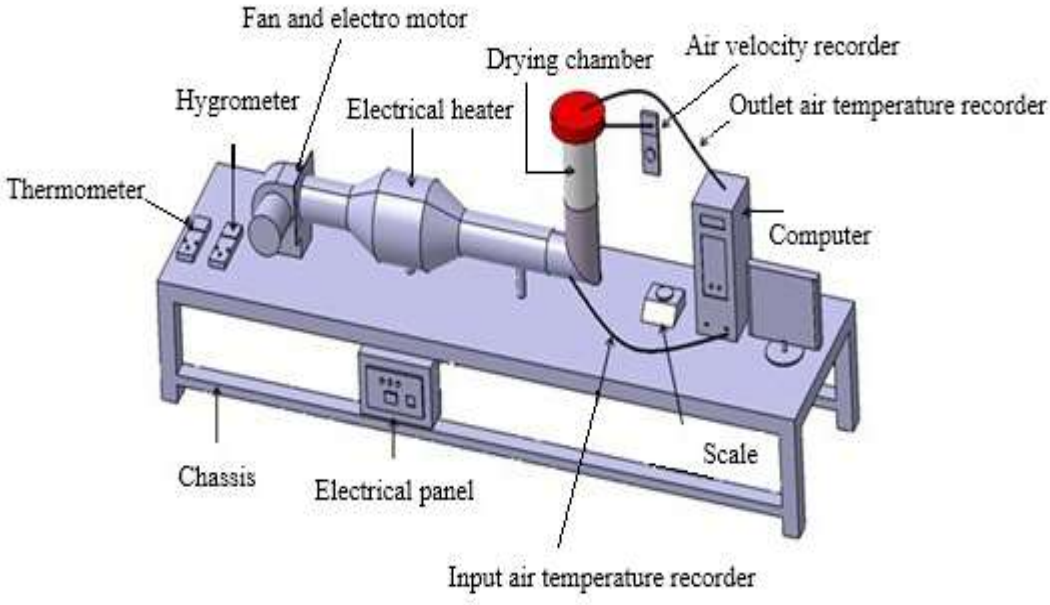


Fig. 2. Laboratory Scale Schematic Diagram of the Fluidized Bed Dryer (Golpour et al, 2021)

Modeling

Partial differential equations that govern the forced convection motion of a fluid bed drying in a two-dimensional geometry are included in the energy, mass and momentum conservation equations. In some cases that are not complex, physical and thermal properties are considered constant. For a two-dimensional cylindrical problem, considering the flow incompressible, the Navier-Stokes equations are described in their most general form:

The equation of mass conservation (i.e. continuity) is (Norton and Sun, 2006):

$$\frac{1}{r}\frac{\partial}{\partial r}(ru_r)+\frac{\partial u_z}{\partial z}=0 \quad (1)$$

where the radial coordinate is r and the axial coordinate is z .

The momentum equations are (Norton and Sun, 2007):

$$\rho(u_r\frac{\partial u_r}{\partial r}+u_z\frac{\partial u_r}{\partial r})=-\frac{\partial p}{\partial r}+\mu\left[\frac{1}{r}(\frac{\partial}{\partial r}(r\frac{\partial u_r}{\partial r})+\frac{\partial^2 u_r}{\partial z^2}-\frac{u_r}{r^2}\right] \quad (2)$$

$$\rho(u_r\frac{\partial u_r}{\partial r}+u_z\frac{\partial u_z}{\partial r})=-\frac{\partial p}{\partial r}+\mu\left[\frac{1}{r}(\frac{\partial}{\partial r}(r\frac{\partial u_z}{\partial r})+\frac{\partial^2 u_z}{\partial z^2}\right] \quad (3)$$

where ρ represents density (kg/m^3), μ denotes the dynamic viscosity (Pa.s), p is the pressure (Pa) and u shows the velocity in x-direction (m/s).

The energy equation is (Ferziger and Peric, 20002):

$$\frac{\partial T}{\partial t}+u\frac{\partial T}{\partial r}+v\frac{\partial T}{\partial z}=\alpha\left[\frac{1}{r}\frac{\partial}{\partial r}(r\frac{\partial T}{\partial r})+\frac{\partial}{\partial z}(\frac{\partial T}{\partial z})\right]$$

which T shows the air temperature (K), t is the drying time (h), v represents the velocity in y-direction (m/s) and α denotes the thermal diffusivity (m^2/s).

The Fluent V6.3.26 CFD package based on the finite volume method has been used for transforming and solving these equations. The boundary conditions assumed are as follows:

1) For velocity, no-slip conditions

2) The constant surface temperature of the drying material.

The following governing equations related to two-dimensional heat and moisture transfer can be written as follows, taking into account the above assumptions (Anderson, 1992):

$$\rho C_p\frac{\partial T}{\partial t}=\frac{1}{r}\frac{\partial}{\partial r}(kr\frac{\partial T}{\partial r})+\frac{\partial}{\partial z}(k\frac{\partial T}{\partial z}) \quad (6)$$

$$\frac{\partial W}{\partial t}=\frac{1}{r}\frac{\partial}{\partial r}(Dr\frac{\partial W}{\partial r})+\frac{\partial}{\partial z}(D\frac{\partial W}{\partial z}) \quad (7)$$

where C_p represents the constant pressure specific heat (J/kg K), k stands for the thermal conductivity (W/m K), D is the moisture diffusivity (m^2/s) and W denotes the moisture content (kg/kg, d.b.).

In both instances, the following initial and boundary conditions were used (Anderson, 1992):

$$T(r,z,0)=T_i \text{ and } M(r,z,0)=M_i$$

Where M_i is the initial moisture content (kg/kg, d.b.), n is normal to surface, s represents the surface coordinate, h denotes the heat transfer coefficient (W/m^2 K) and h_m shows the mass transfer coefficient (m/s).

The spectrum of coefficients associated with convective heat and mass transfer (h and h_m) on the surface of the material was assumed. For

$$\frac{\Delta T}{\Delta t}=\alpha\nabla^2 T \quad (4)$$

Or:

(5)

solving the mass transfer equations under the initial and boundary conditions of interest, the finite difference method was applied.

$$\begin{aligned}
&k\frac{\partial T}{\partial r}\Big|_{r=R}=h(T_s-T_\infty)\\
&k\frac{\partial T}{\partial z}\Big|_{z=0}=h(T_s-T_\infty)\\
&k\frac{\partial T}{\partial z}\Big|_{z=1}=h(T_s-T_\infty)\\
&k\frac{\partial T}{\partial r}\Big|_{r=0}=0\\
&D\frac{\partial M}{\partial r}\Big|_{r=R}=h_m(M_s-M_\infty)\\
&D\frac{\partial M}{\partial r}\Big|_{r=0}=0\\
&D\frac{\partial M}{\partial z}\Big|_{z=0}=h_m(M_s-M_\infty)\\
&D\frac{\partial M}{\partial z}\Big|_{z=1}=h_m(M_s-M_\infty)
\end{aligned} \quad (8)$$

Various researchers have used the following correlations for calculating parameters for the pistachio, as follows:

For bulk density (Hsu *et al*., 1991); $\rho=439+5.003M$ $(R^2=0.959)$

For thermal diffusivity (Hsu *et al*., 1991); $\alpha=51.1\times10^{-9}-0.568\times10^{-9}M$ $(R^2=0.983)$

For thermal conductivity (Hsu *et al.*, 1991); $k=0.0866+0.2817\times10^{-3}M$ $(R^2=0.963)$

For constant-pressure specific heat (Hsu *et al*., 1991); $C_p=1074+27.79M$ $(R^2=0.920)$

For moisture diffusivity (AmiriChayjan *et al*., 2012); $D_{eff}=4\times10^{-9}m^2/s$

Finally, Four indices such as correlation coefficient (R^2), absolute error (E_{abs}), mean squared error (*MSE*) and chi-square (χ^2) were used as the goodness of fit and agreement between experimental results and numerical solutions. These indices are as follow (AmiriChayjan *et al*., 2012):

$$R^2 = 1 - \frac{\sum_{i=1}^{N}\left[M_{\exp,i} - M_{num,i}\right]}{\sum_{k=1}^{N}\left[M_{num,i} - \frac{\sum_{k=1}^{n} M_{num,i}}{N}\right]} \quad (9)$$

$$E_{abs} = \left[\frac{1}{N}\sum_{i=1}^{N}\left|\frac{M_{\exp.i} - M_{num,i}}{M_{\exp,i}}\right|\right] \quad (10)$$

which $M_{\exp.i}$ represents the experimental moisture ratio of i^{th} data, $M_{num,i}$ denotes the numerical moisture ratio of i^{th} data and N shows the number of observations.

$$MSE = \frac{1}{N}\sum_{i=1}^{N}(f_i - y_i)^2$$

where N stands for the number of observations, f_i represents the numerical value of i^{th} data and y_i shows the experimental value of i^{th} data.

$$\chi 2 = \sum_{i=1}^{N}\frac{(O_i - E_i)^2}{E_i}$$

In which N denotes the number of observations, O_i is the experimental value of i^{th} data and E_i is the numerical value of i^{th} data.

Results and discussion

Comparison of experimental and numerical results

Drying curves of pistachio nuts in all the three bed conditions and temperature conditions using both experimental and numerical methods are presented in Figs. 3-5.

After drying the pistachio nuts to nearly 9% (d.b.), the simulated moisture contents at 45°C were found to be higher compared to the measured values (Fig. 3A). Results related to the simulated moisture curve and to the experimental variation of moisture at an air temperature of 60°C are shown (Figure 3B). Based on this result, the simulated values within the range of 6000 and 10000 seconds were slightly higher than the measured values. Because the properties of the product are expressed as averages, so for half of the conditions, the forecast is done properly (Makarichian *et al.*, 2021; Rashidi *et al*., 2021).

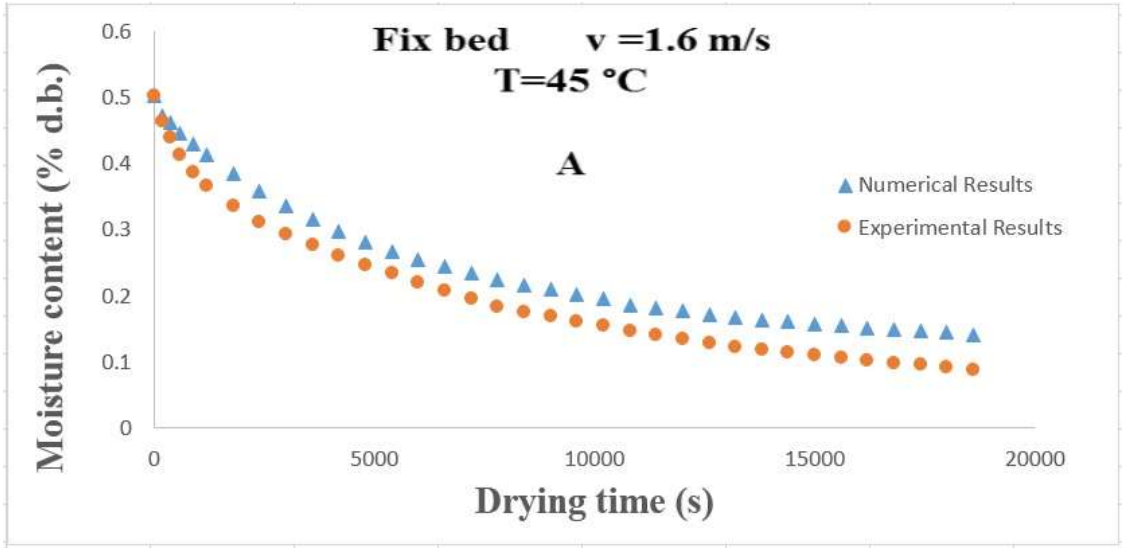
At 75°C the simulated values between 8500 and 30000 seconds were slightly lower and those between 47000 and 66000 seconds were higher compared to the measured values (Fig. 3C) and at 90°C the values simulated were lower than the values measured (Fig. 3D).

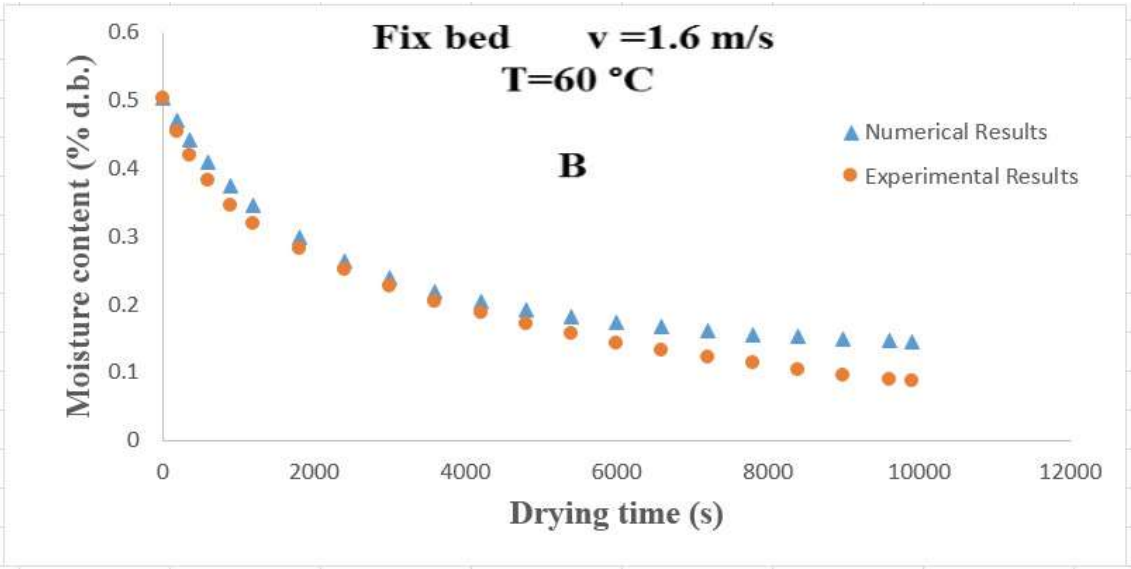
Fig. 4A till Fig. 4D show the simulated moisture contents for semi fluid bed at 45°C and 90°C, respectively. They had the same trend in contrast with fix bed. The reason for this condition can be the average amount of physical and thermal properties

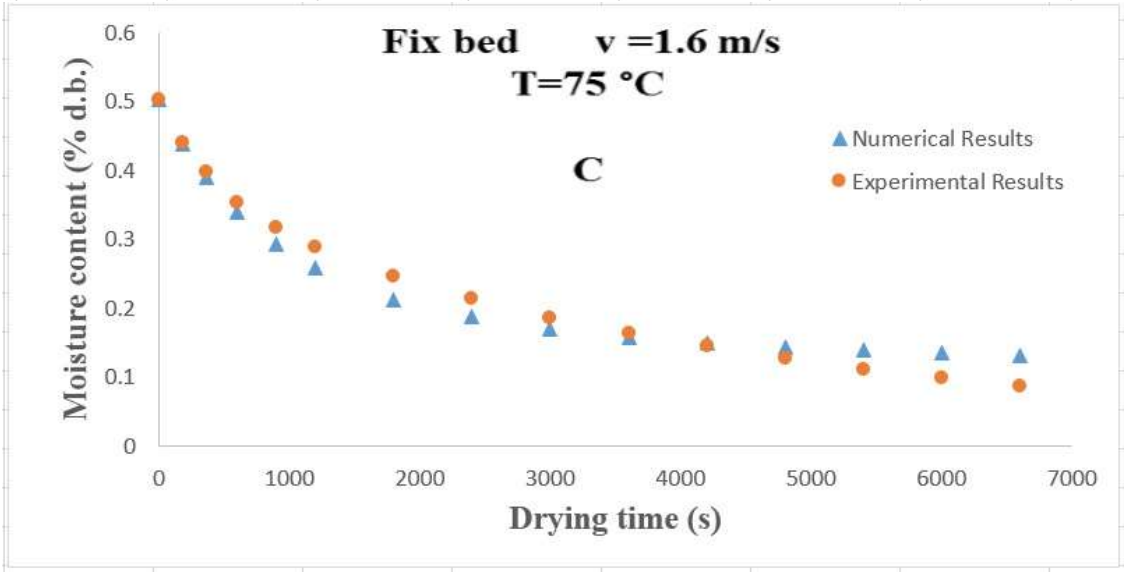
At 60°C the simulated values between 60000 and 10800 seconds were slightly higher than the measured values (Fig. 4B). Fig. 4C showed that the simulated values from 48000 to 66000 seconds were slightly higher than the measured values (at 75°C).

The same trends were found for fluid, fix and semi fluid bed at 45°C and 90°C (Fig. 5A and Fig. 5D), also the curve trends for fluid bed was the same against semi fluid bed at 60°C and 75°C (Fig. 5B and Fig. 5C).

According to Figs. 3- 5, following an increase in air temperature, drying time decreased.


Fix bed v =1.6 m/s
T=45 °C
A
Numerical Results
Experimental Results
Moisture content (% d.b.)
Drying time (s)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5000
10000
15000
20000


Fix bed v =1.6 m/s
T=60 °C
B
Numerical Results
Experimental Results
Moisture content (% d.b.)
Drying time (s)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2000
4000
6000
8000
10000
12000


Fix bed v =1.6 m/s
T=75 °C
C
Numerical Results
Experimental Results
Moisture content (% d.b.)
Drying time (s)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1000
2000
3000
4000
5000
6000
7000

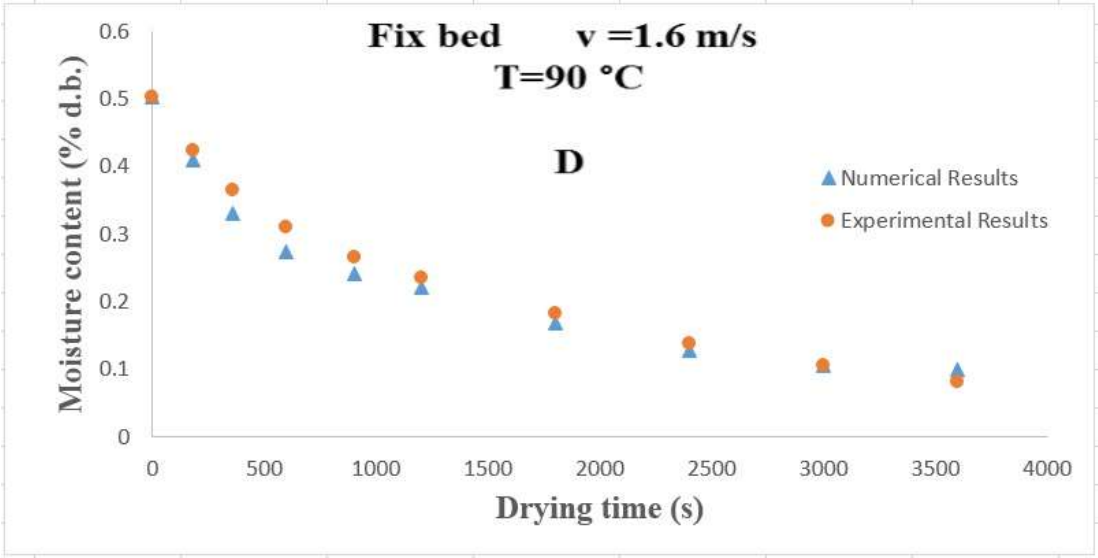
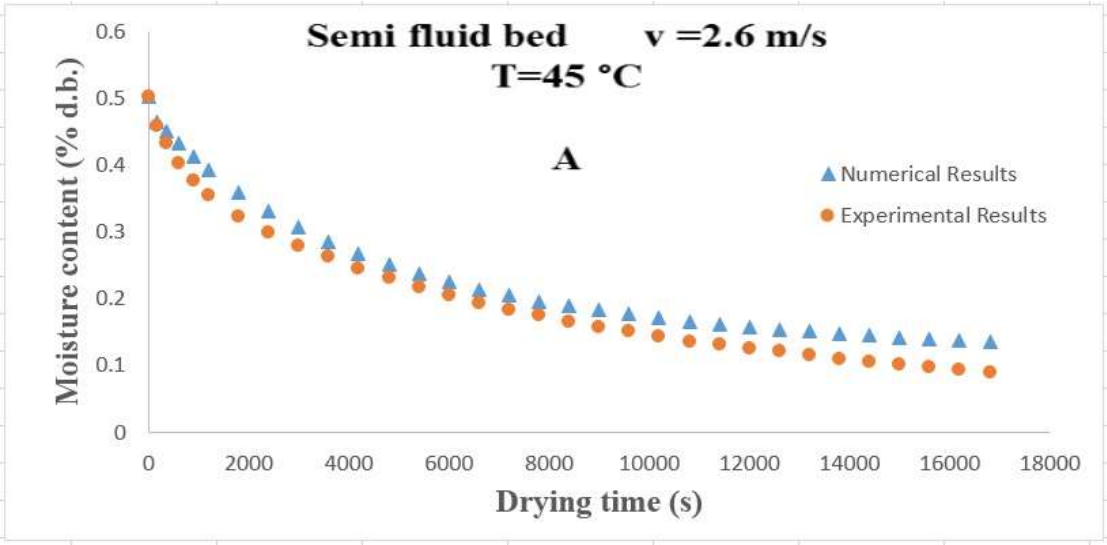
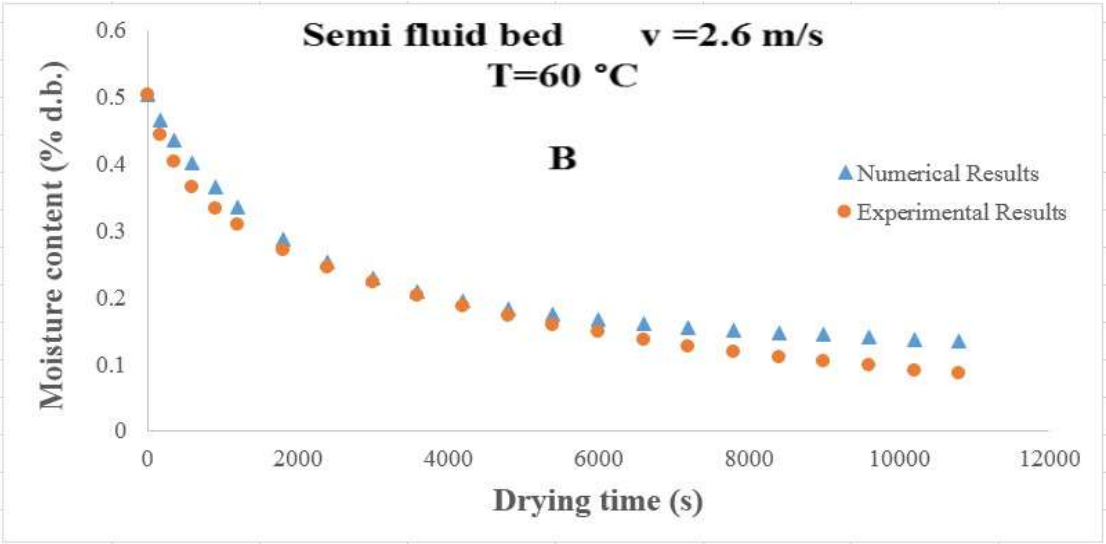


Fig. 3: Measured and simulated moisture contents at fix bed (velocity=1.6 m/s).





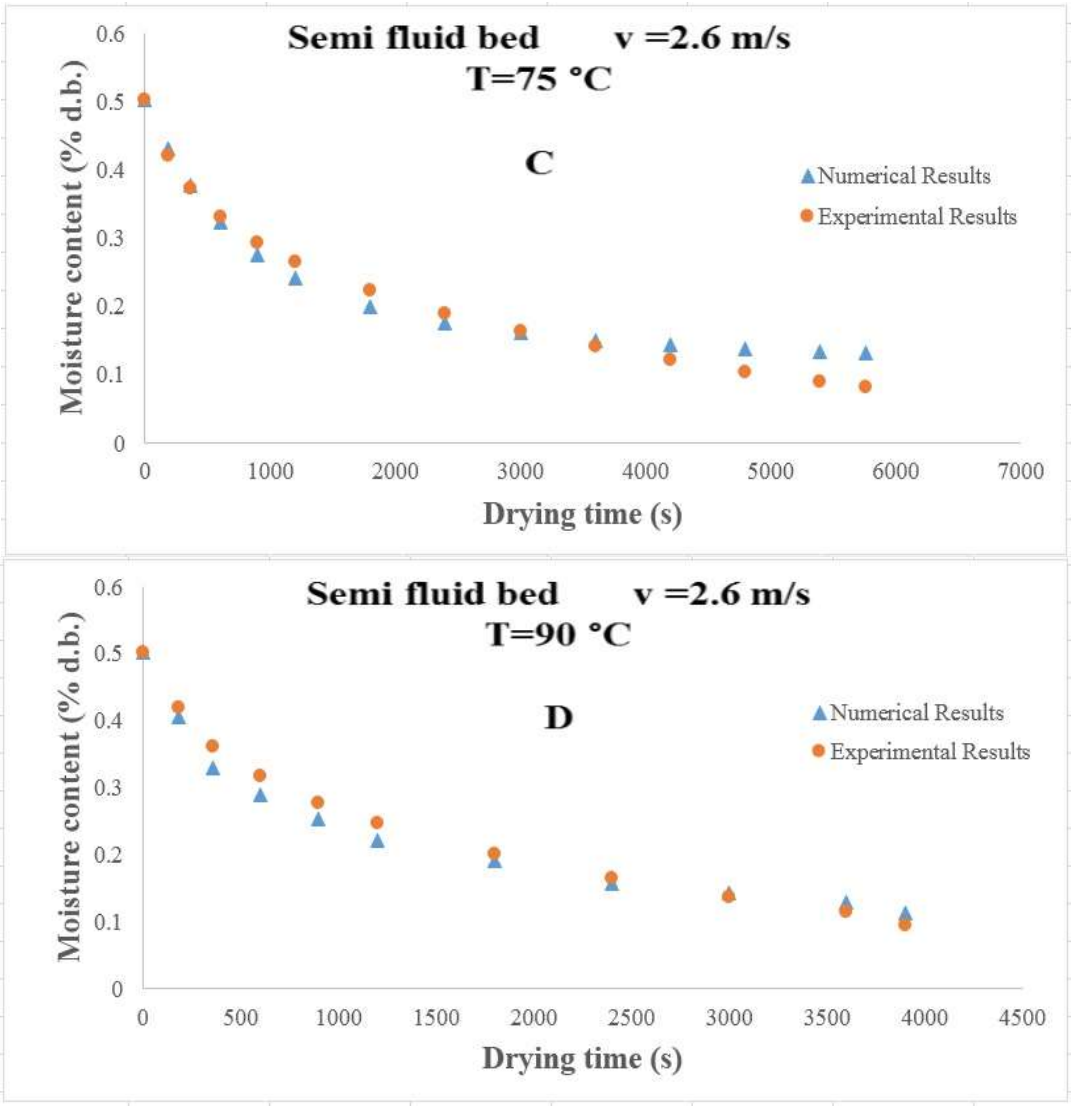
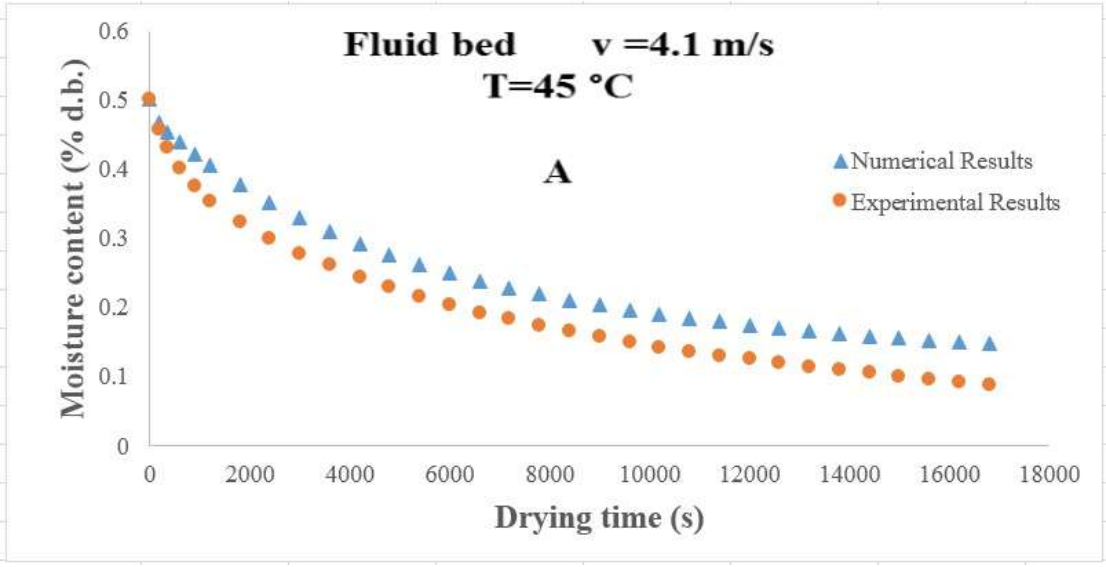


Fig. 4. Measured and simulated moisture contents at semi fluid bed (velocity=2.6 m/s).



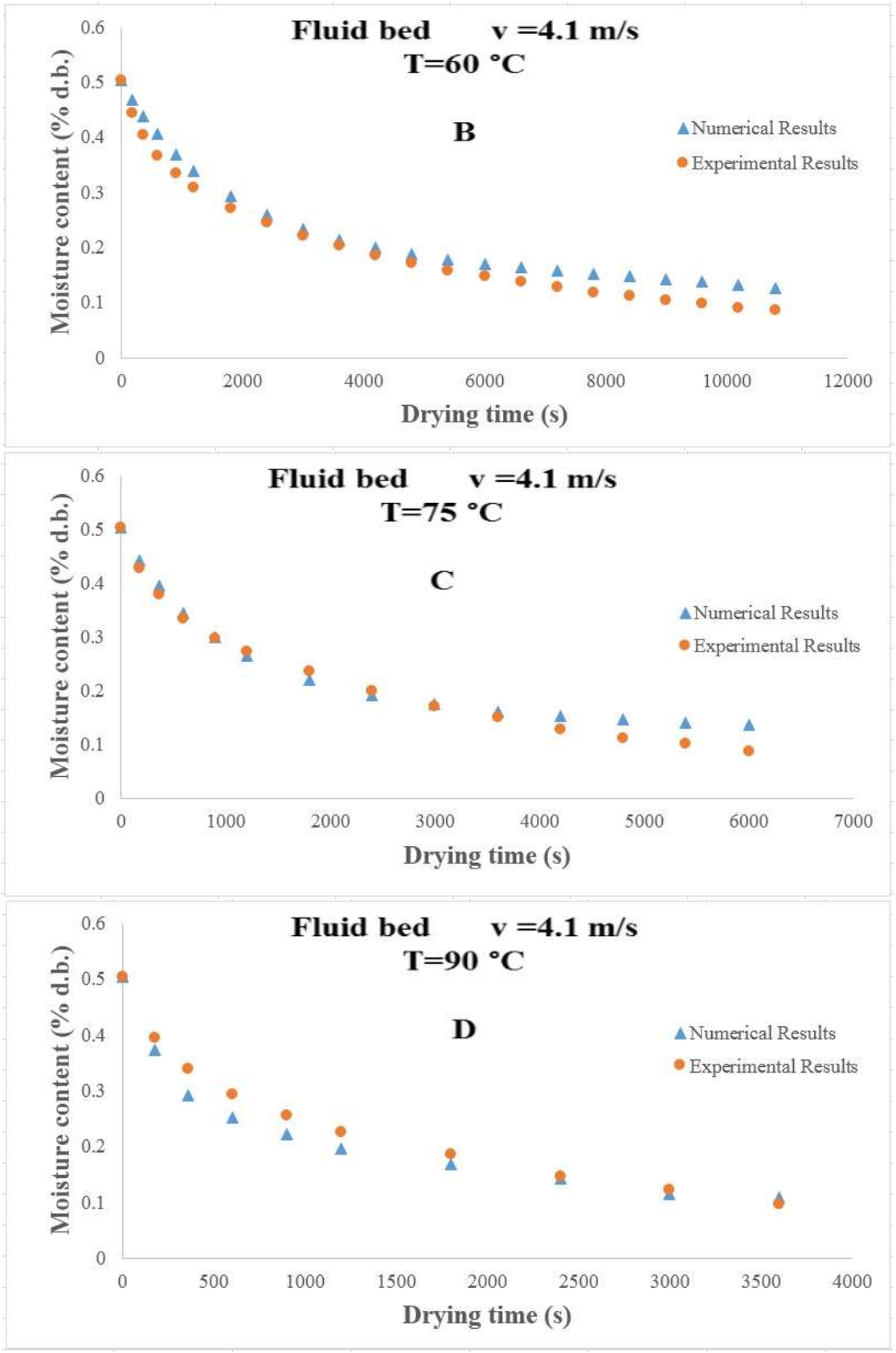


Fig. 5. Measured and simulated moisture contents at fluid bed (velocity=4.1 m/s).

The rate of energy exerted to the pistachio sample bed and then the drying rate increased following increased the temperature being applied to the pistachio bed. Increased temperature allows the rate of transfer of heat to the product to be increased. Air velocity also has no major effect on the rate of drying. An increase in the coefficients of convective heat and mass transfer between the drying air and the pistachio nuts caused an increase in the rate of drying and decreased the drying time.

Similar results were reported by other studies on drying other products including hazelnuts (Topuz *et al.*, 2004), eggplant (Akpinar and Bicer, 2005), peach (Kingsly *et al*., 2007), plum (Goyal *et al*., 2007), berberis fruit (Aghbashlo *et al*., 2007), corn (Vukić *et al*., 2015), ginger (Parlak, 2015), chilean berry (Quispe-Fuentes *et al*., 2016) and turnip (Kaveh and AmiriChayjan, 2016).

Table 1 shows the values related to R^2, E_{abs}, MSE and $\chi 2$, which were calculated for all examined bed conditions. The highest and the lowest values of R^2 and E_{abs}, confirm the highest agreement between experimental results and numerical solutions.

Figure 6 A-C shows the comparison between the numerical solution and experimental results on each bed condition.

According to Fig 6, it seems that the numerical solution result at 60, 75 and 90°C were so close to experimental results except for air temperature of 45°C. The values of each parameter in Table 1 proved this result. In fix bed, the value of E_{abs} at 60, 75 and 90°C were 0.2123, 0.1257 and 0.0337 that were lower than the air temperature of 45°C (0.2595) and R^2 values for these temperatures were 0.9903, 0.9705 and 0.9807. These trends were repeated for the bed conditions of semi fluid and fluid bed with different values.

The average values of R^2 for all applied bed conditions was calculated 0.9850. This value showed the high agreement between experimental results and numerical solutions.

According to the values of statistical indices for different conditions, the errors created by using the model type and fixed numbers of physical and thermal properties are not significant and the results of this study can be used to predict the kinetics of dried pistachios at any temperature and used air velocity in the study area. The advantage of this numerical method is its accuracy and comprehensiveness. However, to predict the drying kinetics of pistachios with the help of experimental and semi experimental models, it will be necessary to adjust the model coefficients (Azharul Karim and Hawlader, 2005; Shahbazi and Rahmati, 2013; Castro *et al*., 2018; Nguyen *et al*., 2021).

Table 1- Values of R^2, E_{abs}, MSE and X^2 for all applied bed conditions

Bed condition	Temperature (°C)	R^2	E_{abs}	MSE	X^2
Fix bed (1.6 m/s)	45	0.9955	0.2595	0.0017	0.3
	60	0.9903	0.2123	0.0010	0.13
	75	0.9705	0.1257	0.0005	0.059
	90	0.9807	0.0337	0.0004	0.018
Semi fluid bed (2.6 m/s)	45	0.9944	0.1850	0.0008	0.15
	60	0.9879	0.1706	0.0007	0.098
	75	0.9715	0.1475	0.0005	0.053
	90	0.9856	0.0120	0.0003	0.017
Fluid bed (4.1 m/s)	45	0.9942	0.2908	0.0022	0.34
	60	0.9911	0.1746	0.0008	0.098
	75	0.9808	0.1278	0.0004	0.045
	90	0.9782	0.0662	0.0007	0.029

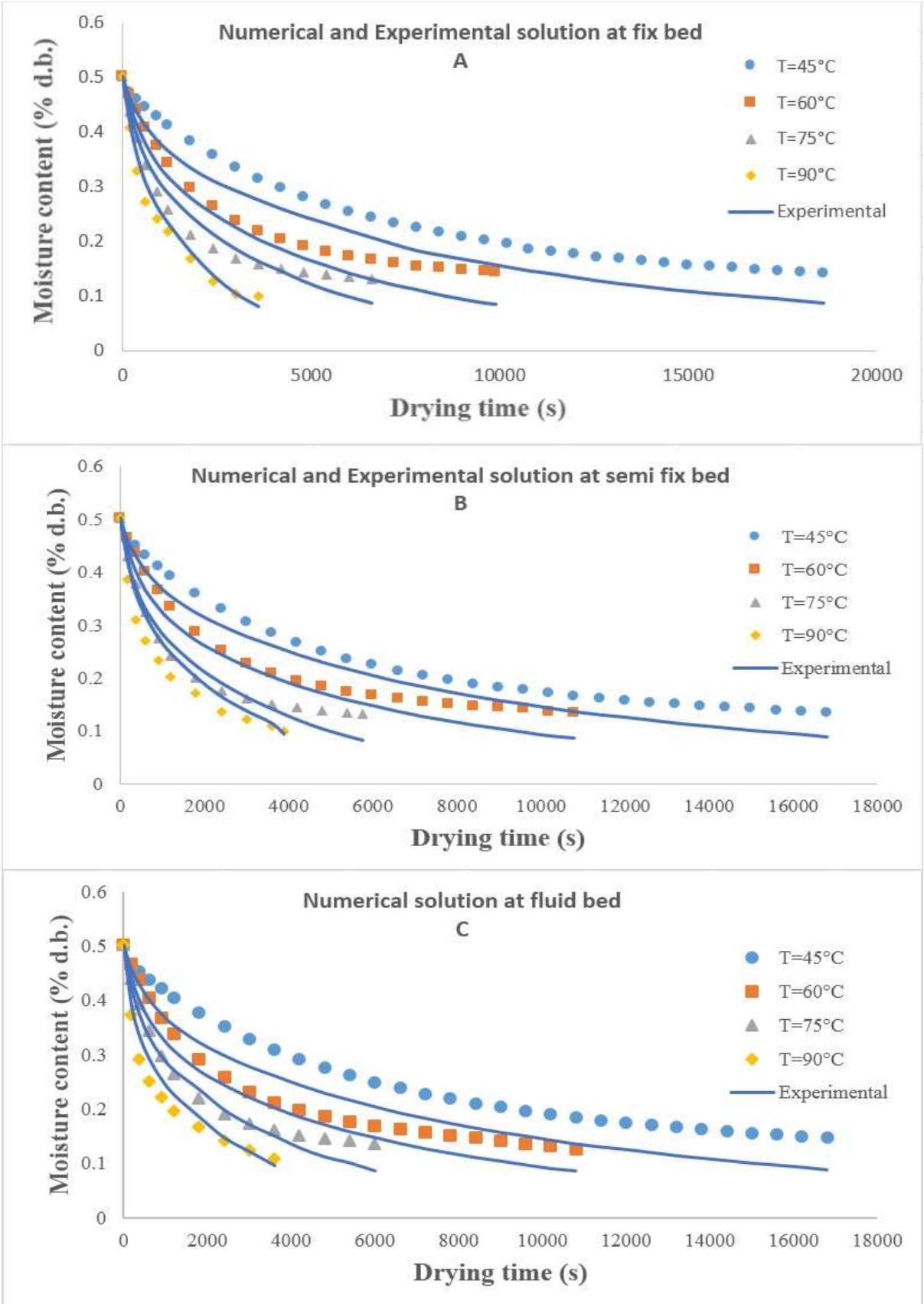


Fig. 6. Experimental results and the numerical solution of drying kinetic of pistachio nuts in bed conditions

Conclusions

The results of this research work are as follows:

1) Comparison between the experimental data with the results obtained in the numerical solution with Fluent CFD code showed that there is a high agreement between them.

2) In fix bed (1.6 ms^{-1}), the lowest values of E_{abs} (0.0337) and $\chi 2$ (0.0004) were related to the temperature of 90°C. The R^2 value of this temperature was 0.9807, which shows the highest agreement between experimental data and numerical solution compared to other examined temperatures.

3) The lowest values of $\chi 2$ and E_{abs} in the semi fluid bed (2.6 ms^{-1}) were 0.017 and 0.0120 and belong to the temperature of 90°C with R^2= 0.9856. In semi fluid bed, the highest agreement was obtained between experimental data and numerical solution for 90°C. After 90°C, temperatures of 75, 60 and 45°C showed the greatest agreement between experimental and numerical results, respectively.

4) The temperature of 90°C showed the highest agreement in the fluid bed (4.1 m s-1). The values of R^2, $\chi 2$ and E_{abs} for this temperature were 0.9782, 0.029 and 0.0662, respectively.

5) The average value of R^2 for all applied bed conditions was calculated 0.9850. This value showed the high agreement between experimental results and numerical solutions.

6) The highest agreement in all bed conditions was related to temperature of 90°C.

7) As the temperature decreased, the values of E_{abs} and $\chi 2$ increased in all bed conditions.

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Nomenclatures

a	thermal diffusivity (m^2/s)	p	pressure (Pa)
C_p	constant pressure specific heat (J/kg K)	r	radial coordinate
D	moisture diffusivity (m^2/s)	s	surface coordinate
E_{abs}	absolute error	T	air temperature (K)
h	heat transfer coefficient (W/m^2 K)	t	Drying time
h_m	mass transfer coefficient (m/s)	u	velocity in x direction (m/s)
k	thermal conductivity (W/m K)	v	velocity in y direction (m/s)
$M_{\exp,i}$	experimental moisture ratio	W	moisture content (kg/kg, db)
$M_{num,i}$	numerical moisture ratio	z	axial coordinate
M_i	initial moisture content (kg/kg, db)	ρ	density (kg/m^3)
N	number of observations	μ	dynamic viscosity (Pa.s)
n	normal to surface		
R^2	correlation coefficient		

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چکیده

خشک کردن مواد غذایی می‌تواند ماندگاری آن را افزایش داده و موجب کاهش هزینه حمل و نقل و نگهداری آن شود. قانون دوم فیک معمولاً برای ارزیابی فرآیندهای انتقال جرم در فرآیند خشک کردن به روش استاندارد مورد استفاده قرار می‌گیرد و بر اساس بسیاری از فرضیات است. درک معنای انتقال جرم در محصولات می‌تواند روند خشک شدن و کیفیت محصول را بهبود بخشد. دینامیک سیالات محاسباتی (CFD) با استفاده از ریاضیات قدرتمند رایانه‌ای و کاربردی به‌منظور پیش‌بینی انتقال جرم در فرآیندهای صنعتی، شرایط جریان سیال را مدل می‌کند. هدف از این تحقیق بررسی عددی رفتار خشک شدن مغز پسته با استفاده از روش CFD و ارزیابی نتایج عددی در شرایط بستر سیال، نیمه سیال و بستر ثابت و همچنین دمای هوا ۴۵ ، ۶۰ ، ۷۵ و ۹۰ درجه بود. در حین خشک کردن با استفاده از دینامیک سیال محاسباتی و کد CFD Fluent، جریان‌های خارجی و زمینه‌های دما در اطراف جسم استوانه‌ای (۱۰ ×۷/۵ میلی‌متر) در تجزیه و تحلیل عددی پیش بینی می‌شود. برای آزمایشات خشک کردن از خشک‌کن بستر سیال آزمایشگاهی استفاده شد. قسمت‌های اصلی خشک‌کن فن شعاعی جلو، محفظه خشک‌کن، بخاری برقی، اینورتر و کنترل‌کننده دما است. اتصالات خشک‌کن سنسورهای دمای ورودی و خروجی، بادسنج و کامپیوتر هستند. بخش عددی انجام و با داده‌های تجربی کنار هم قرار گرفت. نتیجه حل عددی در دمای ۶۰ درجه سانتی‌گراد، ۷۵ درجه سانتی‌گراد و ۹۰ درجه سانتی‌گراد به جز در دمای هوا ۴۵ درجه سانتی‌گراد ، بسیار نزدیک به نتایج تجربی بود. میانگین خطای مطلق در بستر ثابت، در دمای ۶۰ ، ۷۵ و ۹۰ درجه سانتیگراد به‌ترتیب ۰/۲۱۲۳، ۰/۱۲۵۷ و ۰/۰۳۳۷ بود که کمتر از دمای ۴۵ درجه سانتی‌گراد و مقادیر R^2 برای این دماها به‌ترتیب ۰/۹۹۰۳، ۰/۹۷۰۵ و ۰/۹۸۰۷ بود. با کاهش دما، مقادیر E_{abs} و $\chi 2$ در تمام شرایط بستر افزایش می‌یابد. مقادیر متوسط R^2 برای تمام شرایط بستر اعمال شده ۰/۹۸۵۰ محاسبه شد. این مقدار نشان داد که توافق زیاد بین نتایج تجربی و راه حل‌های عددی وجود دارد.

واژه‌های کلیدی: خشک‌کن بستر سیال، خشک‌کن بستر نیمه سیال، خشک‌کن بستر ثابت، نتایج تجربی، مدل‌سازی، حل عددی.

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Investigation of antibacterial activity of heated Kombucha beverages prepared with several herbal teas using response surface methodology

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Abstract

The composition and biological activities of kombucha beverage depends on type of herbal tea, concentration of sucrose and the fermentation time. This study aimed to investigate the effect of different preparation conditions on antibacterial activity of heated kombucha beverages by Response Surface Methodology (RSM). Four types of herbal teas, including black tea, green tea, lemon verbena and peppermint were prepared with three concentrations of sucrose (2, 5 and 8%) and inoculated with active kombucha culture. After 7, 14 and 21 days, beverages were heated by autoclaving and their antibacterial activity against four bacteria including *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus cereus* was evaluated by well method. The RSM was used to investigate the effect of sucrose concentration, fermentation time and type of herbal tea on antibacterial activity of heated beverages. Increasing sucrose concentration had significant effect ($P<0.0001$) on antibacterial activity of heated beverages against all tested bacteria. Increasing of fermentation time had significant effect ($P< 0.0001$) on antibacterial activity of heated beverages against *E. coli* and *S. dysenteriae*. Type of herbal tea had significant effect on antibacterial activity against *S. aureus* and *S. dysenteriae*. The highest antibacterial activity against *E. coli* and *S. dysenteriae* was observed in beverages prepared with lemon verbena. Beverages prepared with green tea showed highest antibacterial activity against *S. aureus*. The highest antibacterial activity against *B. cereus* was observed in heated beverages prepared with black tea and peppermint. In general, the results showed significant antibacterial activity of heated kombucha beverages against the tested bacteria.

Keywords: Antimicrobial effect; Fermented beverage; Fermentation conditions; Sucrose concentration; Fermentation time

Introduction

Considering the disadvantages of consuming carbonated beverages with high sugar content, kombucha with its probiotic properties can be a good alternative. Kombucha is a sweet and sour drink prepared by fermenting tea and sugar by a symbiotic microbial consortium, which is mainly composed of acetic acid bacteria and yeasts, contains nutritious compounds that enhance the immune system and eliminate toxins. It also offers a new taste to the consumers (Jayabalan *et al.*, 2014; Velicanski *et al.*, 2007). Kombucha beverage consists the

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cellulose layer floating on the surface as well as fermented liquid. The cellulosic layer consists of a combination of yeasts and bacteria (Atiyeh and Duvnjak, 2003). The most abundant bacteria in the cellulose layer are acetic acid bacteria, especially Komagataeibacter, Gluconobacter, and Acetobacter species (Roos and Vuyst, 2018). *Schizosaccharomyces pombe*, *Saccharomycodes ludwigii*, *Kloeckera apiculata*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Torulaspora delbrueckii*, *Brettanomyces bruxellensis* are the most abundant yeasts (Coton *et al.*, 2017). In addition, lactic acid bacteria (Lactobacillus and Lactococcus) comprise less than 1% of the microorganisms of this fermented beverage (Marsh *et al.*, 2014).

The microorganisms are embedded in a cellulose floating matrix that is produced by acetic acid bacteria and form a biofilm (Watawana *et al.*, 2016). *Acetobacter xylinum* is the most important species in the production of this cellulose matrix (Yamada *et al.*, 2012).

Besides, the Kombucha beverage contains various organic acids, enzymes and amino acids. Glucuronic acid, Gluconic acid, Lactic acid, Acetic acid, Malic acid, Tartaric acid, Carbonic acid, Butyric acid, Malonic acid, Oxalic acid and ethanol, 14 types of amino acids, water soluble vitamins, especially the family of vitamins B and C and some of the hydrolytic enzymes considered as the most important compounds in this beverage (Malbasa *et al.*, 2011).

Glucuronic acid, which is a vigorous compound of Kombucha tea, plays vital roles in body such as: resistance to diseases, detoxification of the body, transfer of hormones and other important substances, and as an intermediate agent in producing vitamin C. The reusability of this Kombucha culture is considered as one of its advantages because this Kombucha culture is frequently propagated in a favorable condition) (Teoh *et al.*, 2004; Zarowska *et al.*, 2001).

One of the biological activities attributed to this fermented beverage is its antibacterial effects. Various mechanisms have been proposed for this biological activity. Including the production of antimicrobial proteins and enzymes by microorganisms in kombucha, the acidic pH of this beverage, the high content of organic acids such as acetic acid (Sreeramulu *et al.*, 2000; Greenwalt *et al.*, 1998).

The metabolic concentration and composition of this beverage depends on several factors, including type of herbal tea, sucrose concentration and fermentation time and any change in the fermentation conditions might affect the final product and therefore the biological activities including antibacterial effects (Villarreal soto *et al.*, 2018; Wolfe and Dutton, 2015)

Usually black and green tea is used to prepare this beverage. In the present study, kombucha beverages were prepared with black tea, green tea, lemon verbena and peppermint, different concentrations of sucrose and at different fermentation times. The beverages were then heated by autoclaving and the effects of independent variables on the antibacterial activity of the heated fermented beverages were analyzed using by response surface methodology.

Materials and methods

Preparation of kombucha beverages

Leaves of lemon verbena (*Lippia citriodora*) prepared from research farm of Azadshahr Islamic Azad University (Iran), black tea (*Camellia sinensis*) and green tea (*Camellia sinensis*) were prepared from tea gardens of the Lahijan City in northern Iran. Peppermint (*Mentha piperita*) was purchased from medicinal herbs store. Plants were scientifically approved in the Botany Laboratory of Azadshahr Islamic Azad University. One g of the dried leaf of each plant was mixed with different level of sucrose (2, 5 and 8%) and boiled water to a total volume of 100 ml. It was then incubated at 70°C for 15 to 20 min for brewing. The mixture was filtrated through filter paper and the supernatant was inoculated with 25 g/l of actively growing Kombucha culture, sealed with sterilized gas, and kept for different storage days (7, 14 and 21 days) at room temperature. The fermented liquids were centrifuged at 5000 g for 15 min (Battikh *et al.*,

2012; (Valiyan *et al.*, 2020). The resulting supernatants were autoclaved at 121°C under 15 psi for 15 min. The autoclaved beverages were used to evaluate the antibacterial activity.

Preparation of Bacterial Strains

The strains of the tested bacteria were two Gram-negative bacteria of *Escherichia coli* (PTCC 1338) and *Shigella dysenteriae* (PTCC 1188), and two Gram-positive bacteria of *Staphylococcus aureus* (PTCC 112) and *Bacillus cereus* (PTCC 1154). They were purchased from the Iranian Research Organization for Science and Technology (IROST) in a lyophilized form. Then, they were recovered in BHI[1] medium (Merck) for 24 h at 37°C in the microbiology laboratory of the Azadshahr branch, Islamic Azad University. The 24-hour culture of each bacterium was inoculated into Nutrient Broth medium (Merck) and it was incubated at 37°C to obtain turbidity equal to 0.5 McFarland=1.5×10^8 CFU/ml (Clinical and Laboratory Standards Institute [CLSI], 2018).

Evaluation of antibacterial activity by well method

Antibacterial activity of heated fermented beverages was evaluated based on agar well diffusion method. For this purpose, a bacterial suspension equivalent to 0.5 McFarland (1.5×10^8 CFU/ml) was prepared from all bacterial strains and a uniform culture was prepared from this suspension on the surface of the Mueller Hinton Agar medium (Merck). Then, wells with a diameter of 8.2 mm were created by using a cork borer. The heated fermented beverages for testing were poured into the wells, and plates were incubated at 37^0c for 24 to 48 h. Following that, the sensitivity and resistance of the each tested bacteria was determined by measuring the diameter of inhibition zone around the wells (CLSI, 2018; Sreeamulu *et al.*, 2000).

Statistical design and data analysis

Response surface methodology (RSM) is a statistical technique that determines the relationship between one or more response variables with several independent variables. This technique builds and designs different tests and models and evaluates the interactions between different parameters and can identify and measure optimal conditions with minimum number of experiments. It also offers a suitable formula for the desired response (Wang and Liu, 2008).

The statistical analysis of the data was performed using the Design-Expert software (Version 10.0.0, Stat-ease Inc., Minneapolis, MN, USA). The experimental design of CCD is shown in Tables 1 and 2. The variables were coded according to Eq. (1):

$$x_i = (X_i - X_0)/\Delta X \quad (1)$$

where x_i= (dimensionless) coded value of the variable X_i, X_0= the value of X_i at the center point, and ΔX= the step change. The behavior of the system was explained by the following second-degree polynomial equation:

$$Y=\beta_0 + \sum_{i=1}^{4}\beta_i x_i + \sum_{i=1}^{4}\beta_{ii}x^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{ij}x_i x_j \quad (2)$$

where Y is response, β_0, β_i, β_{ii} and β_{ij} are constant coefficients and x_i represents the coded independent variables.

Additionally, the regression analysis and the coefficients were calculated. The fit of the regression model was checked by the adjusted coefficient of determination (R^2_{Adj}). The two-dimensional graphical representation of the system behavior called the response surface was used to describe the individual and cumulative effects of the variables as well as the mutual interactions between the variables on the dependent variable. A statistically significant and the suitable polynomial response surface models of each tested bacteria inhibition zone response variable for each tea were detected.

Results and discussion

A summary of different runs of experiments and their respective values is shown in Table 3. The runs contain 36 treatments related to

1 Brain Heart Infusion

different fermentation conditions, namely 3 levels of fermentation time (7, 14 and 21 days) and 3 levels of sucrose concentration (2, 5 and 8%) on antibacterial activity of four heated kombucha beverages based on black tea, green tea, lemon verbena and peppermint against four bacterial pathogens including *S. dysenteriae, E. coli, S. aureus* and *B. cereus*. The results of antibacterial activity are based on the diameter of inhibition zone and the mean of three replicates. In addition to these results, the predicted values of the prediction models obtained by the software are presented (Table 3).

Table 1- Code and levels of variables chosen for the trails

	Symbols		Levels*				
Factors	**coded**	**Uncoded**	**-α**	**-1**	**0**	**+1**	**+α**
Sucrose concentration (%)	x_1	X_1	2	2	5	8	8
Storage day	x_2	X_2	7	7	14	21	21
	x_3	X_3		1.30	1.90	2.50	

*$x_1 = (X_1\text{-}5)/3$; $x_2 = (X_2\text{-}14)/7$; α=1

Table 2- Levels of categorical factor (Nominal) chosen for the trails

Name	Type	Level	L1	L2	L3	L4
Kind of herbal tea	Nominal	4	Black tea	Green tea	Lemon tea	peppermint

Effect of independent variables on the antibacterial activity of heated kombucha beverages against *E. coli*

According to the analysis of variance, it was found that, except for the type of herbal tea, the variables of sucrose concentration and fermentation time had a significant effect (P<0.0001) on the antibacterial activity of heated kombucha beverages against *E. coli* (P<0.001). Also, the interaction effects of sucrose concentration with fermentation time and sucrose concentration with type of herbal tea at 99.9% and fermentation time with type of herbal tea at 99% had significant effects on the antibacterial activity of heated kombucha against this Gram-negative bacteria (Table 4).

Figure. 1 (a, b, c & d) shows the positive effect of the independent variables of the present study on increasing the diameter of inhibition zone of *E. coli* in all fermented beverages prepared with different herbal teas. With increasing sucrose concentration from 2 to 8% and fermentation time from 7 to 21 days, the antibacterial activity of heated fermented beverages increased (Figure 1). As can be seen in Figure. 1, this effect is particularly more evident in heated kombucha beverage prepared with peppermint and lemon verbena (Figure 1 c & d). This means that these beverages have shown greater antibacterial activity against *E. coli* than fermented beverages prepared from black and green tea.

Also the prediction models obtained by the software are presented in Table 4. In this models, R-sq is 0.78 which is a fairly good value and Adeq-Precision value is greater than 4, indicating the model's desirability, so the greater parameter (16.92) indicates the navigation of these models.

Effect of independent variables on the antibacterial activity of heated kombucha beverages against *S. dysentriae*

Analysis of variance of mean diameter of inhibition zone of *S. dysenteriae* in heated fermented beverages showed that all independent variables including sucrose concentration, fermentation time and type of herbal tea and their interactions had significant effect on the antibacterial activity of heated kombucha beverages (P<0.001).

Figure 2 (a, b, c & d) shows the positive effect of increasing sucrose concentration and storage time on increased antibacterial activity (increase in diameter of inhibition zone) of heated kombucha beverages against *S. dysenteriae*. This effect is particularly significant in beverages prepared with lemon

verbena and peppermint and kombucha beverages prepared with lemon verbena in 8% sucrose and fermentation time of 21 days have the highest antibacterial activity against *S. dysenteriae* (Figure 2. c). The prediction models obtained by the software (Table 4) as for *S. dysentriae*, showes R-sq of 0.78 and Adeq-Precision of 16.1, which indicating the models desirability and indicates the navigation of these models (Table 4).

Table 3- Central composite design arrangement of process variable and actual values and predicted values of responses

treatment	Sucrose con. (%)	storage day	kind of tea†	Inhibition zone of bacteria (mm)*							
				E. coli		*S. dysenteriae*		*S. aureus*		*B. cereus*	
				Actual values	Predicted values	Actual values	Predicted values	Actual values	Predicted values	Actual values	Predicted values
1	2	7	BT	0.00	0.73	9.33	7.17	0.00	5.75	0.00	7.55
2	2	7	GT	0.00	4.95	0.00	6.98	10.67	10.11	14.33	13.28
3	2	7	LT	9.33	5.48	11.00	10.60	13.67	10.44	17.00	15.26
4	2	7	PT	8.33	4.50	13.00	10.52	8.33	4.35	8.67	5.88
5	2	14	BT	8.67	10.68	11.33	11.23	8.67	9.92	16.00	14.08
6	2	14	GT	15.00	13.16	22.00	15.75	12.67	11.46	23.33	18.54
7	2	14	LT	0.00	-0.19	9.00	10.60	8.67	5.00	8.67	3.70
8	2	14	PT	8.33	7.95	13.00	12.20	8.67	11.78	11.00	14.39
9	2	21	BT	10.00	12.40	14.00	17.61	12.00	14.53	15.00	21.33
10	2	21	GT	0.00	1.44	11.00	13.13	12.00	13.18	15.00	13.62
11	2	21	LT	0.00	1.27	10.00	8.39	12.67	13.43	12.00	13.85
12	2	21	PT	0.00	-2.59	9.67	7.45	11.00	9.65	11.67	10.33
13	5	7	BT	9.00	8.03	15.00	14.60	13.00	12.72	9.67	11.12
14	5	7	GT	8.67	9.82	8.67	10.75	13.67	14.18	11.33	13.82
15	5	7	LT	8.33	7.92	9.00	10.71	13.00	11.61	15.00	12.78
16	5	7	PT	8.67	6.19	15.33	12.78	16.00	14.31	10.67	8.11
17	5	14	BT	8.56	7.98	11.00	11.41	14.22	13.37	12.33	11.57
18	5	14	GT	9.00	9.99	11.00	10.69	13.67	15.62	13.67	14.73
19	5	14	LT	0.00	-0.47	8.67	7.78	11.00	7.73	13.00	8.27
20	5	14	PT	11.00	4.19	10.33	10.43	16.67	14.15	16.33	15.17
21	5	21	BT	0.00	5.17	17.00	16.88	11.67	16.54	14.33	18.31
22	5	21	GT	0.00	4.35	8.33	10.58	0.00	3.88	0.00	4.93
23	5	21	LT	12.67	10.97	17.33	14.12	13.67	11.51	15.67	14.31
24	5	21	PT	12.33	13.90	18.67	21.47	15.00	15.11	19.67	19.93
25	8	7	BT	0.00	0.72	9.33	10.10	0.00	2.09	0.00	1.09
26	8	7	GT	10.00	9.31	15.67	14.54	11.67	10.93	13.00	12.94
27	8	7	LT	16.33	14.19	23.33	22.78	18.00	15.73	24.00	21.05
28	8	7	PT	0.00	-3.27	11.67	9.32	9.33	5.32	11.00	5.92
29	8	14	BT	0.00	1.29	10.67	9.91	10.00	11.13	11.33	12.37
30	8	14	GT	0.00	2.15	13.00	14.30	11.67	12.91	13.00	15.07
31	8	14	LT	0.00	3.05	8.67	11.67	0.00	2.42	0.00	4.30
32	8	14	PT	11.33	9.56	13.00	13.16	9.33	9.44	15.00	13.23
33	8	21	BT	14.00	12.38	18.00	18.45	11.67	12.42	17.00	18.41
34	8	21	GT	0.00	0.93	9.67	10.75	0.00	1.57	0.00	2.18
35	8	21	LT	10.33	9.40	16.00	13.13	11.00	9.80	15.67	13.59
36	8	21	PT	14.00	14.18	19.33	19.32	16.00	13.99	23.33	21.25

†BT: Black tea, GT: Green tea, Lemon tea: LT, PT: Peppermint tea, *Data are means of 3 replicates

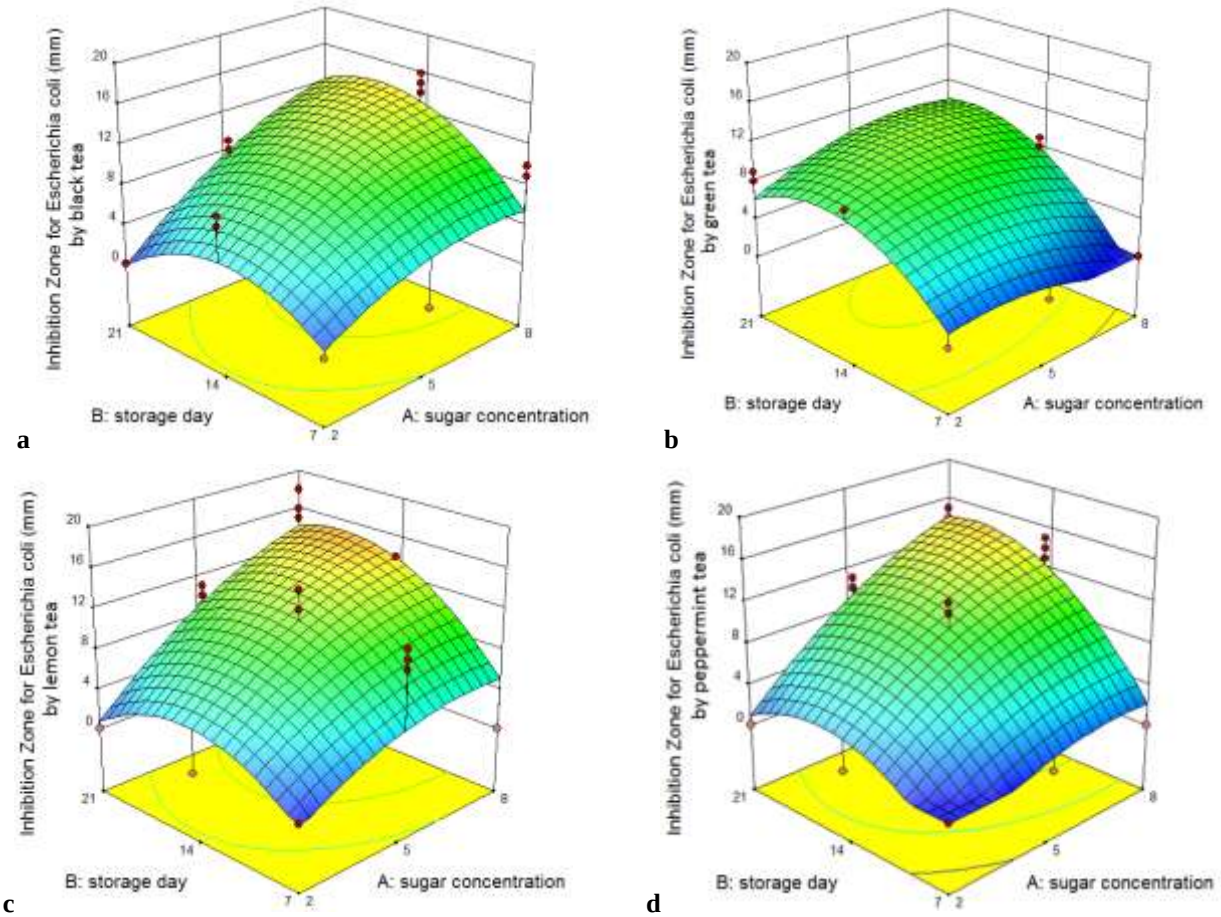

Fig. 1. Inhibition zone of *Escherichia coli* in heated Kombucha beverages (Prepared by black tea (a), green tea (b), lemon verbena (c) and by peppermint (d))

Effect of independent variables on the antibacterial activity of heated kombucha beverages against *S. aureus*

Analysis of variance of mean diameter of inhibition zone of *S. aureus* in heated beverages showed that among the independent variables, sucrose concentration and type of herbal tea had significant effect on antibacterial activity of kombucha beverages against *S. aureus* ($P<0.001$). In addition, in analysis of the interaction effects, sucrose concentration with fermentation time, sucrose concentration with herbal tea type and fermentation time with herbal tea type were significant at levels of 99%, 99.9% and 99%, respectively (Table 4). Among the heated kombucha beverages, beverage prepared with lemon verbena at 8% sucrose showed the highest antibacterial activity against *S. aureus* (Figure 3. c).

R-sq of 0.736, Adeq-Precision of 15.14 and the prediction models obtained by the software for *S. aureus*, indicate the desirability and the navigation of these models (Table 4).

Effect of independent variables on the antibacterial activity of heated kombucha beverages against *B. cereus*

In investigation of effective variables on antibacterial activity of heated fermented beverages against *B. cereus*, variance analysis results in Table 4 showed that among the independent variables, only sucrose concentration had significant effect on the antibacterial activity of heated beverages against *B. cereus* ($P<0.001$). Also, the interaction between sucrose concentration with fermentation time and sucrose concentration with herbal tea type had significant effect on this antibacterial activity ($P<0.001$) and with increasing sucrose concentration and fermentation time in all heated fermented beverages an increase in antibacterial activity

(increase in diameter of inhibition zone) was evident (Figure 4. a, b, c & d). This effect is particularly significant in fermented beverages prepared with lemon verbena, peppermint and black tea.

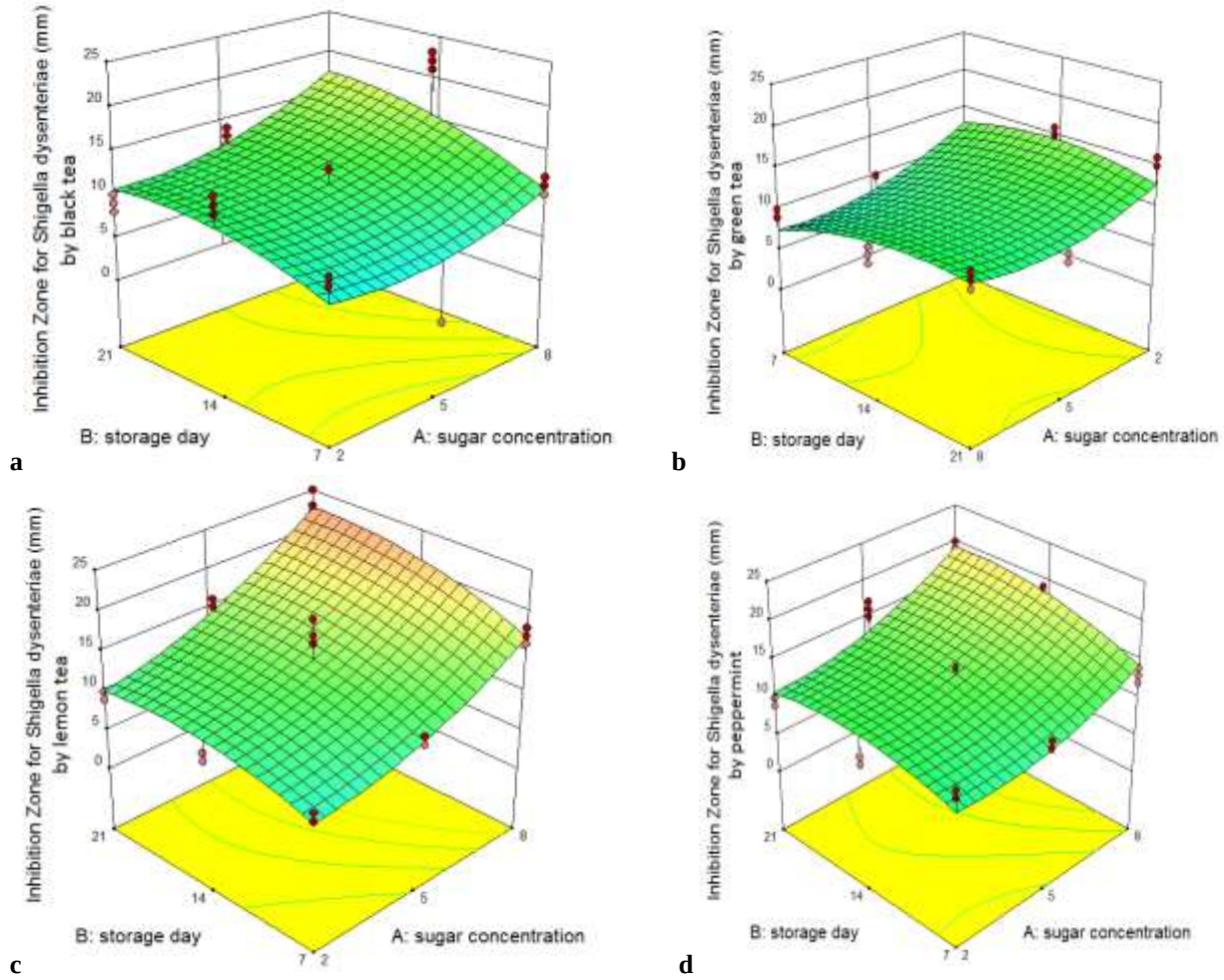

Fig. 2. Inhibition zone of *Shigella dysenteriae* in heated Kombucha beverages (Prepared by black tea (a), green tea (b), lemon verbena (c) and by peppermint (d))

The prediction models obtained by the software for *B. cereus* in Table 4, with R-sq of 0.732 and Adeq-Precision of 15.63 indicate the desirability and navigation of these models (Table 4).

In general, increasing sucrose concentration had significant effect on antibacterial activity of heated kombucha beverages against all tested bacteria and increased antibacterial activity of these beverages. Increasing of fermentation time had significant effect on antibacterial activity of heated beverages against *E. coli* and *S. dysentriae*. Also type of herbal tea had significant effect on antibacterial activity of heated kombucha beverages against *S. aureus* and *S. dysentriae*. Batikh et al. (2012) showed the antibacterial activity of various analogs of Kombucha after 21 days of fermentation. Moreover, a direct correlation between fermentation time with an increase in the production of acetic acid and organic acids and the diameter of the inhibition zone was observed in the study by Talawat et al. (2006).

Increased antibacterial activity with increasing fermentation time was also reported by Sreeramulu et al. (2000). This implies that antimicrobial components are microbial metabolites produced by the bacteria and yeast responsible for fermentation in the kombucha beverage and in the fermentation process.

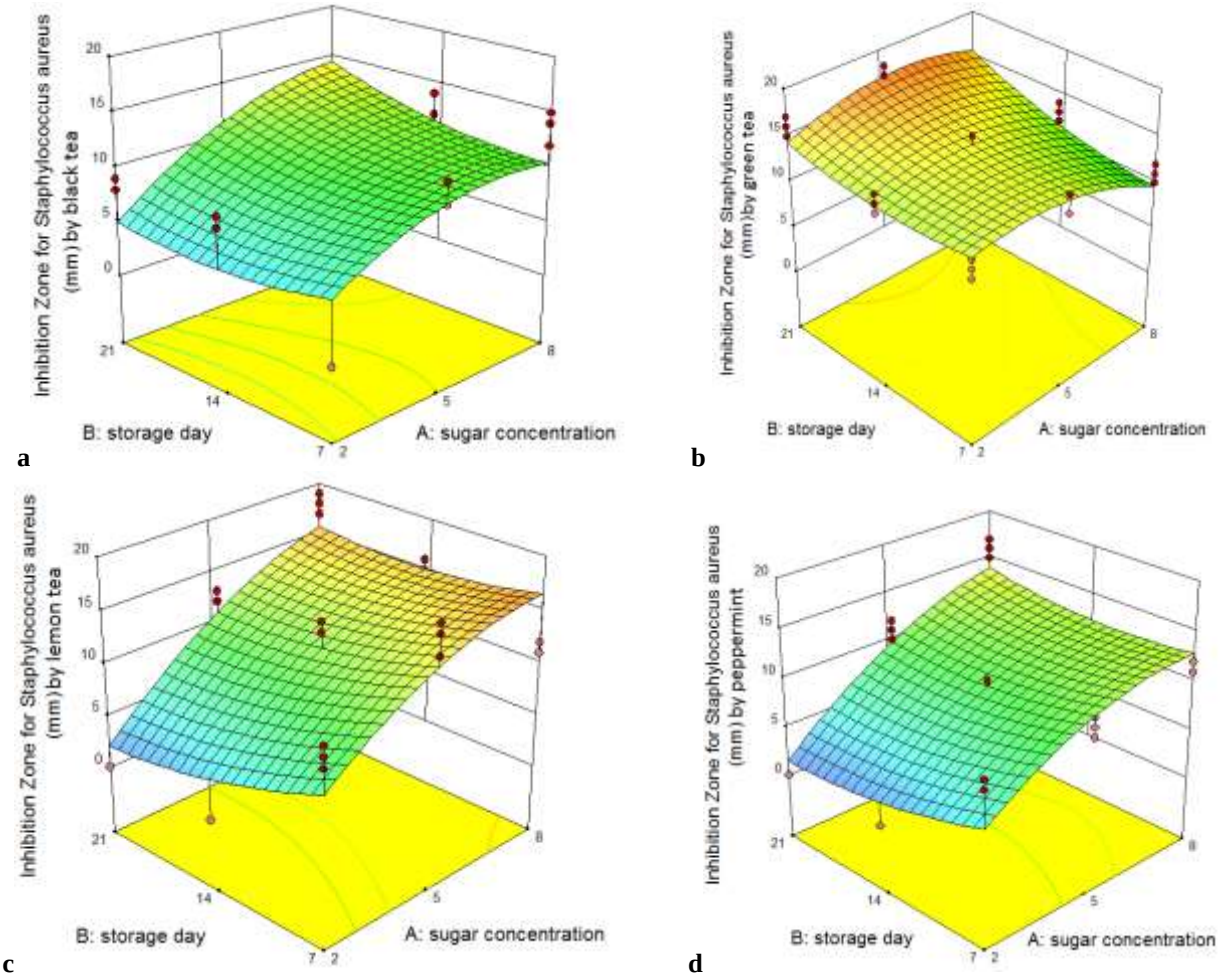

Fig. 3. Inhibition zone of *Staphylococcus aureus* in heated Kombucha beverages (Prepared by black tea (a), green tea (b), lemon verbena (c) and by peppermint (d))

Table 4- P-value and other parameters extracted from analysis of variance table

	Escherichia coli	*Shigella dysenteriae*	*Staphylococcus aureus*	*Bacillus cereus*
Source	**p-value**			
Model	2.09E-25***	2.722E-19***	4.293E-21***	7.675E-21***
A:sucrose concentration	1.68E-17***	1.751E-11***	1.254E-17***	3.551E-23***
B:storage day	1.96E-15***	1.802E-07***	0.796	0.892
C:kind of tea	0.197	1.035E-05***	1.081E-08***	0.6961
AB	4.02E-06***	0.020456642*	0.0028**	3.347E-06***
AC	4.63E-07***	4.839E-12***	1.546E-09***	9.111E-08***
BC	0.0083**	0.182145761	0.00154**	0.4029
A^2	0.0015**	0.0006231***	0.00048***	0.00958**
B^2	4.23E-11***	0.002975**	0.0683	0.72511
Lack of fit	8.369E-41***	2.743E-29***	1.844E-28***	2.484E-31***
R-Sq.	0.788907	0.71048	0.736382	0.732911
Adj R-Sq.	0.75713	0.666897	0.696698	0.692704
Pred R-Sq.	0.715423	0.617704	0.62945	0.625955
Adeq Precision	16.92704	16.10733	15.14196	15.63691

*** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$

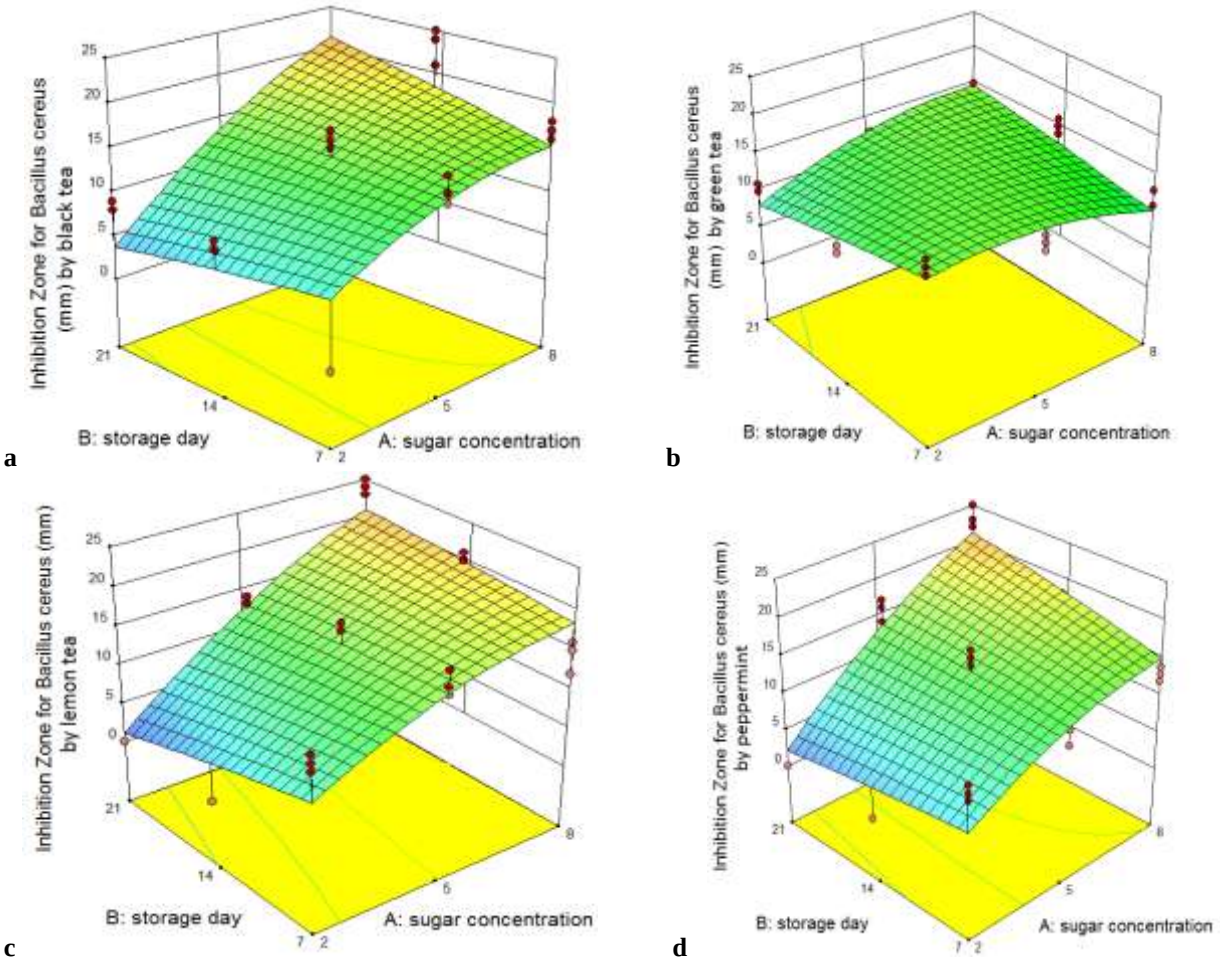

Fig. 4. Inhibition zone of *Bacillus cereus* in heated Kombucha beverages (Prepared by black tea (a), green tea (b), lemon verbena (c) and by peppermint (d))

Also this is related to the basis of production of this fermented beverage and its strong dependence on the amount of sucrose available. The sucrose in tea is metabolized to glucose and fructose by the microorganisms in kombucha. At first, Glucose was converted by yeasts into ethanol and carbon dioxide and then ethanol was changed into acetic acid and other organic acids by acetic acid bacteria (Antibacterial activity of these beverages are attributed to these compounds). Considering the need of microorganisms in kombucha for a carbon source (sucrose), it is concluded that the increase in antibacterial activity was associated with the increase in sugar concentration (Jayabalan *et al.*, 2014; Batikh *et al.*, 2011; Talawat *et al.*, 2006; Sreeramulu *et al.*, 2000; Haizhen *et al.*, 2008).

Since the fermented beverages studied were heated and the protein and enzyme compounds (Antibacterial activity is thought to be related to them) have been eliminated, is strengthened the relationship between antibacterial activity with organic acids and their dependence on sucrose consumption and organic acid production.

Prediction of fermentation conditions to achieve the best antibacterial activity of heated kombucha beverages

One of the capabilities of the surface response method is to search for optimal conditions for optimal response and to introduce the best formula and conditions for achieving optimal results with minimum number of tests. In this regard, the relationships presented in Table 5 represent the best predictive model for calculating the diameter of

inhibition zone of the tested bacteria, which is specifically expressed for each herbal tea.

Table 5- Final equation for inhibition zone of bacteria for each herbal tea

Kind of bacteria	Kind of tea	Final Equation
E . coli	**black tea**	-15.0386+ 2.191358* A+2.160714* B+0.093254* A*B- 0.20525* A^2- 0.08617* B^2
	green tea	-14.2423+ 0.728395* A+ 2.565476* B+ 0.093254* A*B -0.20525* A^2- 0.08617* B^2
	lemon verbena	-17.5941+ 2.339506 B*A 2.311508* B+ 0.093254* A*B- 0.20525* A^2- 0.08617* B^2
	Peppermint	-21.8164+ 2.302469* A+ 2.525794* B+ 0.093254* A*B- 0.20525* A^2- 0.08617* B^2
S. dysenteriae	**black tea**	3.37345679- 1.841049383* A+ 1.096230159* B+ 0.04265873* A*B+ 0.211419753* A^2- 0.033446712* B^2
	green tea	14.26234568- 3.359567901* A+ 0.826388889* B+ 0.04265873* A*B+ 0.211419753* A^2- 0.033446712* B^2
	lemon verbena	2.651234568- 0.896604938* A+ 1.016865079* B+ 0.04265873* A*B+ 0.211419753* A^2- 0.033446712* B^2
	Peppermint	6.00308642- 1.581790123* A+ 0.953373016* B+ 0.04265873* A*B+ 0.211419753* A^2- 0.033446712* B^2
S. aureus	**black tea**	4.86882716+ 2.617283951* A- 0.755952381* B+ 0.057539683* A*B- 0.223765432* A^2+ 0.020975057* B^2
	green tea	14.09104938+ 1.24691358* A- 0.621031746* B+ 0.057539683* A*B- 0.223765432 * A^2 +0.020975057 * B^2
	lemon verbena	7.924382716+ 3.302469136* A- 1.10515873* B+ 0.057539683* A*B- 0.223765432* A^2+ 0.020975057* B^2
	Peppermint	4.979938272+ 3.098765432* A- 0.970238095* B+ 0.057539683* A*B- 0.223765432* A^2+ 0.020975057* B^2
B. cereus	**black tea**	4.472222222+ 2.541666667* A- 0.368055556* B+ 0.118055556* A*B- 0.208333333* A^2- 0.005102041* B^2
	green tea	15.0462963+ 0.708333333* A- 0.487103175* B+ 0.118055556* A*B- 0.208333333* A^2- 0.005102041* B^2
	lemon verbena	6.083333333+ 2.930555556* A- 0.606150794* B+ 0.118055556* A*B- 0.208333333* A^2- 0.005102041* B^2
	Peppermint	2.305555556+ 2.782407407* A- 0.360119048* B+ 0.118055556* A*B- 0.208333333* A^2- 0.005102041* B^2

Table 6 shows the highest antibacterial activity of heated kombucha beverages against each of the tested bacteria. The highest antibacterial activity against *S. dysentriae* with a diameter of the inhibition zone of 23 mm was observed in fermented beverage prepared with lemon verbena at the sucrose concentration of 8% and fermentation time of 20 days. Also, preparation of heated kombucha beverages based on peppermint and black tea created the highest antibacterial activity against *B. cereus*, (mean of diameter of inhibition zone of 21 mm) at the sucrose concentration of 8% and in 21 days of fermentation time. The best fermentation conditions for obtaining the highest antibacterial activity against *S. aureus*, with the highest diameter of the inhibition zone of 17 mm, was seen in the beverage prepared with green tea, the sucrose concentration of 5.5% and the fermentation time of 21 days. Finally, the highest antibacterial activity against *E. coli*, with the highest diameter of the inhibition zone of 15 mm, was observed in heated kombucha beverage prepared with lemon verbena, sucrose concentration of 8% and in 21 days of fermentation time. Other

results obtained from Tables 6, and 3 indicate the higher resistance of *E. coli* to heated beverages, compared to the other tested bacteria. The resistance of *E. coli* to kombucha beverage has been reported by others (Battikh *et al.*, 2012).

Cell wall resistance of Gram-negative bacteria to inhibitors such as antimicrobial chemicals, herbal compounds, extracts, essential oils, antibiotics, etc. is related to lower permeability of the outer membrane of these bacteria and the presence of lipopolysaccharide in the cell wall as well as the periplasmic space of these bacteria, which restricts the entry of antimicrobial agents into the bacterial cell (Hayouni *et al.*, 2007; Russel, 1991; Burt., 2004). Although *S. dysenteriae* and *E. coli* are both Gram-negative and from the family Enterobacteriaceae, significant sensitivity of *S. dysentriae* to heated kombucha beverages was interesting. In studies of antibacterial activity of honey samples, this sensitivity was attributed to specific differences of each microorganism species to the antibacterial activity of honey sample (Tumin *et al.*, 2005; Ceyhan and Ugur, 2001; Taormina *et al.*, 2001; Nzeako and Hamdi, 2000).

Table 6- Individual preparation (fermentation conditions) for heated kombucha beverages in order to achieve the maximum inhibiton zone of each bacteria

Bacteria	Sucrose concentration (%)	Storage day	Kind of tea	inhibition zone (mm)
S. dysenteriae	8.0	20	lemon tea	23
B. cereus	8.0	21	black tea or peppermint	21
S. aureus	5.5	21	green tea	17
E. coli	8.0	18	lemon tea	15

In general, heated kombucha beverages showed significant antibacterial activity against the tested bacteria. It has also been reported in similar studies (Velicanski *et al.*, 2007, 2014; Battikh *et al.*, 2011; Sreeramulu *et al.* 2000).

In the study of the antimicrobial activity of various kombucha beverage analogues, showed that the antimicrobial activity of heated beverages remained stable by 56% decrease, and beverages prepared with lemon verbena (*L. citriodora*), peppermint (*M. piperita*) and fennel (*F. vulgare*) were among the heated beverages that showed the highest antimicrobial activity. Due to the sensitivity of proteins and enzymes to heat, they suggested that the antimicrobial activity of heated fermented beverages was not due to heat-sensitive compounds. (Battikh *et al.*, 2012).

The production of protein metabolites and enzymatic compounds of antimicrobial nature by microorganisms found in kombucha beverages along with the acidic pH and high content of acetic acid and other organic acids in these beverages are mechanisms thought for antimicrobial activity in these fermented beverages (Sreeramulu *et al.*, 2000; Greenwalt *et al.*, 1998). The type of herbal tea used in the preparation of kombucha beverages can also cause the antimicrobial activity of these beverages due to the presence of antimicrobial compounds in the plant's natural constituents.

Due to the high sensitivity of proteins and enzymes to the heat and activity of these compounds at high pH, the antibacterial activity of heated beverages cannot be attributed to the production and the presence of these compounds with the heat sensitive protein nature and the high content of acetic acid and other organic acids in the fermented beverages and the presence of the intrinsic antimicrobial compounds of the plants used can justify the antibacterial activity of the beverages.

Sreeramulu et al. (2000) studied the antimicrobial activity of the kombucha beverage, reported antibacterial activity against *E. coli*, *S. sonnei*, *S. typhimurium*, *S. enteritidis* and *C. jejuni*, even after heat treatment. Due to the presence of this antibacterial activity even in neutralized beverages, the researchers attributed the antibacterial activity of this fermented beverage to the presence of antimicrobial compounds other than acetic acid

and protein compounds. Of course, numerous studies have also identified acetic acid as the dominant compound of the antimicrobial activity of kombucha beverages (Velicanski *et al.*, 2014; Steinkraus *et al.*, 1996; Cetojevic Simin *et al.*, 2008). Acetic acid and other organic acids can cause antimicrobial activity by acidifying the cytoplasm and by accumulating the separated acid anion into toxic amounts (Mani-Lopez *et al.*, 2012).

In addition to the acidic pH and high content of acetic acid and other organic acids, the presence of antibacterial compounds inherent in the used plant in preparation of beverages can be effective on this activity. For example, compounds such as phenylpropanoid with verbascoside (the most abundant compounds) and other compounds such as iridoid, verbenalin, along with flavonoids, luteolin, and aryenine, have been identified in the plant of lemon verbena (Bilia *et al.*, 2008). In the present study, the highest antibacterial activity against *E. coli* and *S. dysenteriae* was observed in heated kombucha beverages prepared with lemon verbena (at 8% sucrose concentration and fermentation time of 18 and 20 days) (Table 6). Heated kombucha beverage prepared with green tea (at 5.5% sucrose concentration and 21 days fermentation time) showed the highest antibacterial activity against *S. aureus* (Table 6). This effect may be due to natural compounds in green tea such as catechins including epigallocatechin galate, epigallocatechin, epicatechin gallate, epicatechin and galocatechin and other compounds (An *et al.*, 2004; Noormandi & Dabaghzadeh, 2015). In the study of the quality of kombucha beverages prepared with various herbal teas, from beverages prepared with the green tea was reported the best quality and the highest antibacterial activity against *S. aureus* and *E. coli* (Primiani *et al.*, 2018). Also increased antimicrobial activity of green tea has been reported due to epimerization of the tea catechins under the heating conditions (Kim *et al.*, 2007).

Also, the highest antibacterial activity against *B. cereus* was observed in two heated kombucha beverages, prepared with black tea and peppermint (at 8% sucrose concentration and 21 days fermentation time) (Table 6). In the study of the biochemical and antibacterial activities of the essential oil of peppermint leaves, the most important constituents of the essential oil of peppermint leaves collected from Iran were identified as, alpha terpinen, isomentone, trans carveol, betacariophyllene and piperidinone oxide and were confirmed the antibacterial activity of peppermint (*Mentha piperita*), against *E. coli* and *S. aureus* (Yadegarinia *et al.*, 2006).

Catechins are the most important constituents in tea to which the biological effects of tea are related to them. Catechins comprise at least four major phenolic compounds, including epigallocatechin, epicatechin gallate, epigallocatechin gallate, and epicatechin. The most common in green tea is epigallocatechin gallate, which accounts for about 50% of tea catechins. The compounds of tea catechins may change during fermentation and oxidation processes. For example, tea catechins changed to thearubigins and theaflavins during fermentation. Usually, green tea catechins have a content of about 13 to 30%, which in black tea is about 5% instead, the content of oxidized phenolic compounds is 25% in black tea. Since black tea undergoes extensive fermentation, its catechin composition differs from green tea. Therefore, greater amounts of natural tea catechins are usually preserved in green tea, which undergoes less fermentation. The mechanism of the effect of tea catechins on the antibacterial activity might due to the effect of these compounds on the cell membrane and cell wall of bacteria (Song and Seong, 2007).

The high affinity of tea catechins to the cell wall components of bacteria causes their antibacterial activity. Differences in susceptibility of *S. aureus* and Gram-negative bacteria to catechins are related to this binding (Yoda et al., 2004). This antibacterial activity is strengthened by the replacement of the gallate group of catechins (Stapleton *et al.*, 2004).

Antimicrobial effect of tea catechins against *B. cereus* at nanomolar levels even higher than tetracycline and vancomycin have been

reported to promote the use of green tea in the treatment of food poisoning (Friedman *et al.*, 2006).

Conclusion

The results showed significant antibacterial activity of heated kombucha beverages against the gastrointestinal pathogenic bacteria. Increasing sucrose concentration and fermentation time increased the antibacterial activity of these beverages. The highest antibacterial activity against *E. coli* and *S. dysenteriae* was observed in the heated fermented beverage prepared with lemon verbena. Kombucha beverages prepared with green tea showed the highest antibacterial activity against *S. aureus* and the highest antibacterial activity against *B. cereus* was observed in two heated kombucha beverages prepared with black tea and peppermint. The antibacterial activity of heated beverages cannot be attributed to the production and presence of compounds with a heat-sensitive protein nature, and the high content of acetic acid and other organic acids in the beverages and the presence of the inherent antimicrobial compounds of the plants used may justify the antibacterial activity of the fermented beverages.

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Declaration of interest

All authors declare that they had no conflicts of interest.

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بررسی فعالیت ضدباکتریایی نوشیدنی‌های کامبوچا حرارت دیده، تهیه شده با چند دمنوش گیاهی به‌وسیله روش سطح پاسخ

فاطمه ولیان[۱]- هادی کوهساری[۲*]- ابوالفضل فدوی[۱]

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چکیده

ترکیبات و فعالیت‌های بیولوژیکی نوشیدنی کامبوچا به نوع دمنوش گیاهی، غلظت ساکارز و زمان تخمیر بستگی دارد. این مطالعه با هدف بررسی تأثیر شرایط مختلف آماده‌سازی بر فعالیت ضدباکتریایی نوشیدنی‌های حرارت دیده کامبوچا توسط روش سطح پاسخ (RSM) انجام شد. چهار نوع دمنوش گیاهی شامل چای سیاه، چای سبز، به لیمو و نعناع فلفلی با سه غلظت ۲، ۵ و ۸ درصد ساکارز تهیه شدند و با کشت فعال کامبوچا تلقیح گردیدند. پس از ۷، ۱۴ و ۲۱ روز، نوشیدنی‌ها اتوکلاو شدند و فعالیت ضدباکتریایی آنها علیه چهار باکتری شامل *اشریشیا کلی*، *شیگلا دیسانتری*، *استافیلوکوکوس اورئوس* و *باسیلوس سرئوس* با روش چاهک ارزیابی شد. برای بررسی اثر غلظت ساکارز، زمان تخمیر و نوع دمنوش گیاهی بر فعالیت ضدباکتریایی نوشیدنی‌های حرارت دیده از RSM استفاده شد. نتایج نشان داد که افزایش غلظت ساکارز اثر قابل توجهی بر فعالیت ضد باکتریایی نوشیدنی‌های حرارت دیده علیه همه باکتری‌های آزمایش شده داشت. افزایش زمان تخمیر تأثیر معناداری بر فعالیت ضدباکتریایی نوشیدنی‌های حرارت دیده علیه *اشریشیا کلی* و *شیگلا دیسانتری* داشت. نوع دمنوش گیاهی تأثیر قابل توجهی بر فعالیت ضدباکتریایی علیه *استافیلوکوکوس اورئوس* و *شیگلا دیسانتری* داشت. بیشترین فعالیت ضدباکتریایی علیه *اشریشیا کلی* و *شیگلا دیسانتری* در نوشیدنی‌های تهیه شده با به‌لیمو مشاهده شد. نوشیدنی‌های تهیه شده با چای سبز بیشترین فعالیت ضد باکتریایی را در برابر *استافیلوکوکوس اورئوس* نشان دادند. بیشترین فعالیت ضدباکتریایی علیه *باسیلوس سرئوس* در نوشیدنی‌های حرارت دیده تهیه شده با چای سیاه و نعناع مشاهده شد. به‌طور کلی، نتایج این تحقیق فعالیت ضدباکتریایی قابل توجه نوشیدنی‌های کامبوچا حرارت دیده را علیه باکتری‌های مورد آزمون نشان داد.

واژه‌های کلیدی: اثر ضدمیکروبی، نوشیدنی تخمیری، شرایط تخمیر، غلظت ساکارز، زمان تخمیر

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Full Research Paper

Improvement of antioxidant and emulsifying properties of *Cajanus cajan*'s protein hydrolysate by glycosylation through maillard reaction

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Abstract

The aim of the present study was to use the Maillard reaction as a means to glycosylate protein hydrolysates obtained from *Cajanus cajan* and to evaluate the effects of this chemical modification on antioxidant and emulsifying properties. Chemical properties, amino acid composition, and molecular weight distribution of the hydrolysates were evaluated. Glucose, galactose, and maltodextrin in the ratios of 1:2, 1:1, and 2:1 (hydrolysate: sugar, dry weight basis) were used for glycosylation. Antioxidant activity was evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and nitric oxide scavenging. The sonication technique was used to prepare the (oil/water) emulsions. The droplet size distribution and zeta potential of the emulsions were measured during 4 days of storage. Results showed that glycosylation by glucose in the ratio of 2:1 increased DPPH scavenging activity from 37.96% to 85.53% and nitric oxide inhibition activity from 14.50% to 54.83%. Although glycosylation improved emulsifying stability of glycosylated hydrolysates compared to non-glycosylated hydrolysates, no significant difference was observed between the three examined sugars.

Key words: Glycosylation, Maillard reaction, Protein hydrolysate, Antioxidant, Emulsion

Introduction

Cajanus cajan or pigeon pea is a legume from the family *Papilionoideae* is mostly cultivated in tropical and subtropical countries. This legume is known to have a good nutritional quality (amino acid profile) and a high amount of hydrophobic amino acids (Saxena *et al*., 2010).

Consumer demands for plant-based ingredients have led the food and cosmetic industries to focus on the attempts towards the replacement of animal proteins with plant protein (Kutzli *et al.*, 2020). Although the quality of the plant proteins is lower than animal proteins, enzymatic hydrolysis can improve some of the biological and functional

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properties of the plant proteins (Wouters *et al*., 2016). On the other hand, small peptides produced due to the hydrolysis process can cause some new problems in food systems; such as instability or inducing allergenicity (Wouters *et al.*, 2016). In this regard, protein hydrolysate modification might be a proper means to improve the quality (e.g. antioxidants and emulsifying properties), and decreasing the possibility of making allergens.

Glycosylation is a chemical modification and a process of adding sugar to a protein by enzymatic or chemical reactions such as the Maillard reaction. It has been shown that glycosylation has a positive effect on antioxidant (Hou *et al*., 2013; Wang *et al*., 2020), biological (Jeewanthi *et al*., 2015), functional properties (Jongh and Broersen, 2012; Wang *et al*., 2020), and the flavor (Hong *et al*., 2016) of protein hydrolysates. However, Plant-based protein and peptide glycosylation are not fully studied in the literature (Kutzli *et al.*, 2020).

Maillard reaction is defined as a "series of non-enzymatic chemical reactions between carbonyl compounds (mainly carbohydrates) and amino compounds from natural resources". Maillard reaction is a natural and non-toxic reaction that is mostly used to improve biological and functional properties of proteins and protein hydrolysates (Karnjanapratum *et al*., 2018; Li *et al*., 2016; Lie *et al*., 2014; Liu *et al*., 2020; Zhang *et al.*, 2018). This reaction has a complicated effect on the protein surface, protein structure, and surface hydrophobicity. The type of sugar and amino acid and the reaction conditions are important factors affecting the biological and functional properties of final product (Anzani *et al*., 2019; Kutzli *et al*., 2020; Wang *et al.*, 2020).

To the best of our knowledge, the functionality of *Cajanus cajan*'s protein hydrolysate as a food emulsifier, and the modification of this protein hydrolysate has not been studied.

The aim of the present study was to use the Maillard reaction as a means to glycosylate *Cajanus cajan*'s protein hydrolysates and to evaluate the effect of this chemical modification on antioxidant and emulsifying properties.

Materials and methods

Cajanus cajan was purchased from a farm in Azarshahr, East Azerbaijan Province, Iran. Alcalase enzyme (from *Bacillus licheniformis* with proteolytic activity of 2.4 (AU/ml) was purchased from Sigma-Aldrich (Spain). Commercial cod liver oil was kindly provided by Maritex A/S, subsidiary of TINE, BA (Sortland, Norway) and stored at -40°C until use. The fatty acid composition (major fatty acids only) of the fish oil used was C16:0, 9.5%; C16:1, 8.7%; C18:1, 16.3%; C20:1, 12.6%; C20:5, 9.2% and C22:6, 11.4%. The tocopherol content of the fish oil was: alpha-tocopherol, 200± 3 µg/g oil; beta-tocopherol, 5± 1 µg/g oil; gamma-tocopherol, 96± 3 µg/g oil and delta-tocopherol, 47± 1 µg/g oil. The peroxide value (PV) of the fish oil used was 0.38± 0.04 meq/kg oil. All other chemicals and solvents used were of analytical grade.

Protein extraction

Protein extraction was performed according to Akintayo et al. (1999) with some modifications. A suspension (1:10 w/v; pH 11) of *Cajanus cajan*'s sieved flour in distilled water was prepared. The suspension was stirred for 1 h at room temperature and stored at 4°C overnight, then centrifuged for 20 min at 6000 rpm at 4°C. The supernatant was collected and its pH was adjusted to 3 (by 0.1 N HCl) and centrifuged at the same conditions as the previous step. The pellet was freeze-dried (FDB-5503, Operon, South Korea) and stored at -20°C until use.

Protein hydrolysis

Protein concentrate (freeze-dried pellet from the previous section) solution (5% w/v) was hydrolyzed using Alcalase enzyme at pH 8 (adjusted by 0.1 N NaOH) by enzyme concentration of 2.47%. The reaction took place in a shaker incubator (48.35°C, 3.26 h, 200 rpm), on the next step, the solution was transferred to 85°C water bath for 10 min to stop the reaction. The solution was centrifuged

for 20 min at 6000 rpm at room temperature. The supernatant was freeze-dried and stored at -4°C for further experiments (Meshginfar *et al*., 2014).

Chemical analysis

The moisture content was measured by drying the samples at 105°C to reach the constant weight (AOAC, 2005). The protein content was measured by the Kjeldahl method and using a protein factor of 6.25 (AOAC, 2005). Ash content was measured by pre-drying the sample and using a 600°C furnace until obtaining white ash after 24 h (AOAC, 2005). The lipid content of the samples was measured by Soxhlet extraction (AOAC, 2005).

Amino acid composition of protein concentrate and hydrolysate

Cajanus protein concentrate and protein hydrolysate were completely hydrolyzed and derivatized by EZ: faast Amino Acid Kit (Phenomenex, Torrance, Ca, USA) with 6 M HCL for 1 h at 110°C. Then, the hydrolyzed samples were neutralized and purified by solid-phase extraction sorbent tip and derivatized. The samples were injected into an Agilent HPLC 1100 (Santa Clara, CA, USA) coupled to Agilent Ion Trap MS. Separation was done at 35°C on 250× 3.0 mm Zebron ZB-AAA column (Phenomenex, Torrance, CA, USA) using a gradient of 68- 83% 10 mM ammonium formate in methanol and 10 mM ammonium formate in water by 0.5 ml/min flow rate. An external standard mixture was used to identify the compounds (García-Moreno *et al*., 2016).

Chemical score of protein concentrate and hydrolysate

The chemical score is based on the essential amino acid profile (g 100 g^{-1}) in a standard protein that is described by FAO/WHO and is calculated as follows (Raftani Amiri *et al*., 2016):

$$Chemical\ score = \frac{Essential\ amino\ acid\ in\ test\ protein}{Essential\ amino\ acid\ in\ standard\ protein} \quad (1)$$

Molecular weight distribution of hydrolysate

To measure the molecular weight distribution of the hydrolysate, a size exclusion chromatography (SEC) with FPLC ÄKTA system (Amersham Biosciences, Uppsala, Sweden) and Superdex Peptide 10/300GL column (GE Healthcare, Uppsala, Sweden) was used. The temperature of the column was 25°C and the mobile phase was ammonium acetate buffer at pH 8. 100 µl of the sample (5 mg/ml, filtered by 0.2 µm filter) was injected and eluted at flow rate of 0.25 ml/min, and the absorbance was measured at 280 nm. Cytochrome c (12.3 kDa), aprotinin (6.5 kDa), Gly_3 (189 Da), and Gly (75 Da) were used as molecular weight marker (Gringer *et al*., 2016).

Glycosylation

Freeze-dried protein hydrolysates and one of the dry sugars glucose, galactose, or maltodextrin (DE 20) were mixed (1:2, 1:1, and 2:1, dry weight base), distilled water was added to the dry mixture, and the pH was adjusted to 7.5 (by adding 0.1 N NaOH or HCl). The final concentration of the hydrolysate in each solution was 20 mg/ml. One solution was prepared without sugar as control. The ten prepared solutions were shaken for 2 h at room temperature and stored at 4°C overnight without shaking to complete the hydration process. On the next day, the solutions were incubated in a 90°C water bath for 8 h. Finally, the solutions were cooled down with cold water and used for antioxidant assays (Mulcahy et al., 2016).

DPPH scavenging activity

DPPH solution (0.1 ml, 0.1 mM in 95% ethanol) was added to 0.1 ml of each sample solution (from the previous step) and incubated in the dark at room temperature for 60 min. Any particle in solutions was removed by centrifugation (10 min, 6000 rpm, room temperature) before the end of incubation time. The negative control was prepared in the same way without the test compounds, and conjugated samples were compared to the non-conjugated hydrolysate. The absorbance of the samples was measured at 517 nm and DPPH

scavenging activity was calculated as described by Farvin et al. (2014):

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (2)$$

Nitric oxide inhibition activity

60 µl of sample solution was mixed with 60 µl sodium nitroprusside in PBS buffer (0.025 M) and incubated at ambient temperature for 150 min. The negative control was prepared in the same way without the test compounds, and conjugated samples were compared to the non-conjugated hydrolysate. After incubation, the same volume of Griess reagent was added to the samples, and absorbance was measured at 546 nm. The nitric oxide inhibition activity was calculated as described by Tsai et al. (2007) using the following formula:

$$\%Inhibition = \frac{Absorbance\ of\ the\ control - Absorbance\ of\ the\ sample}{Absorbance\ of\ the\ control} \times 100 \quad (3)$$

Fourier transform infrared spectroscopy

The FTIR spectra of the freeze-dried protein hydrolysate and glycosylated protein hydrolysates were recorded by FTIR spectrophotometer (Bruker Tensor II FTIR, Bruker corporation, US) from 400- 4000 cm^{-1} at a resolution of 4 cm^{-1} (Li *et al*., 2016).

Preparation of emulsions

To prepare the 5% fish oil-in-water emulsions, the samples with the highest antioxidant activity were selected as aqueous phase and prepared as described in glycosylation section. Sodium caseinate solution (0.2% aqueous phase) was used as a control. The pH of all samples was adjusted to 7-7.5. After the addition of the oil phase to each aqueous phase, ultraturrax (Polytron, PT 1200 E, 18000 rpm, 30 sec) was used to partially emulsify the solution. The samples were vortexed to break any produced foam. A sonicator (Microson XL2000, probe P1, 75% amplitude, 30 sec, 2 passes) was used for emulsification. The vortex step was repeated after the sonication. The prepared emulsions were covered and stored at room temperature for further experiments (García-Moreno et al., 2018).

Droplet size distribution

The droplet size distribution of the samples was measured by laser diffraction in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, UK) at days 0, 1 and 3 of storage at room temperature. Emulsions were diluted in the system's circulating water (3000 rpm). The laser obscuration was 8-12%. Results were reported as volume mean diameter ($D_{4,3}$) (García-Moreno *et al*., 2016).

Zeta potential

Diluted solution (2:1000) of emulsions in distilled water was prepared at day 1 and the zeta potential of the solutions was measured by using the Zetasizer Nano ZS system (Malvern Instruments Ltd., Worcestershire, UK) at 20°C (García-Moreno *et al*., 2016).

Statistical analysis

All experiments were done in triplicate. ANOVA analysis was carried out. Differences were significant at $p<0.05$ .Results were reported as mean± standard deviation. Microsoft Office Excel (2010) was used for data management and graph generation, and SPSS (16.0) for statistical analysis.

Results and discussion

Chemical analysis

The chemical composition of *Cajanus cajan*'s flour, protein concentrate, and protein hydrolysate is shown in Table 1. The protein content of the concentrate was 73.85% that is 52.4% more than the protein content of the flour and shows the efficiency of the protein extraction process. The low amount of lipids in the flour is advantageous because no defatting step using chemical solvents is needed to remove the excess lipids. The lipid content decreased further during the protein extraction and hydrolysis processes, which increases the oxidative stability of protein concentrate and hydrolysate (Ovissipour *et al.*, 2012).

Increases in protein content of protein hydrolysate is due to the increased solubility of the proteins during the hydrolysis and the removal of the non-protein components by centrifugation. Ash content also increases due to the addition of HCl and NaOH to adjust the pH for hydrolysis (Halim and Sarbon, 2017). Water content is reduced because of freeze-drying of the protein concentrate and hydrolysate.

Table 1- Chemical composition of *Cajanus cajan*'s flour, protein concentrate and protein hydrolysate.

Sample	Protein (%)	Lipid (%)	Moisture (%)	Ash (%)
***Cajanus cajan*'s flour**	21.45±3.2[a]	4.21±0.1[a]	11.35±0.1[a]	2.60±0.0[a]
Protein concentrate	73.85±2.8[b]	1.87±0.2[b]	5.24±0.1[b]	0.94±0.1[b]
Protein hydroysate	89.41±2.4[c]	0.53±0.1[c]	2.32±0.1[c]	3.41±0.0[c]

a-c in each column indicates the significant differences ($p<0.05$)

Amino acid analysis of protein concentrate and hydrolysate

Amino acid composition of *Cajanus cajan*'s protein concentrate and protein hydrolysate is shown in Table 2. The amino acid composition is a key factor affecting the antioxidant properties of the protein hydrolysate. Histidine, tyrosine, methionine, and lysine are reported to be antioxidant amino acids (Ng *et al*., 2013), moreover, hydrophobic amino acids can improve the antioxidant activity of the hydrolysates (de Queiroz *et al*., 2017; Halim and Sarbon, 2017). The amount of hydrophobic amino acids in protein hydrolysate was 216.13 mg/g protein which helps the peptides to be solubilized in lipids and interact with hydrophobic radicals and polyunsaturated fatty acids (Yu and Tan, 2017). Aromatic amino acid content in protein hydrolysate was 68.26 mg/g protein.

Table 2- Amino acid composition of protein concentrate and protein hydrolysate.

Amino acids	Protein concentrate mg/g protein	Protein hydrolysate mg/g protein	Recommended values by FAO* mg/g protein	Chemical score of protein concentrate	Chemical score of protein hydrolysate
Arginine	73.94±13.85	67.75±20.72	-	-	-
Histidine	16.02±0.87	18.16±2.34	16	1.00±0.05	1.13±0.15
Isoleucine	24.82±0.63	25.73±6.04	30	0.82±0.02	0.85±0.20
Leucine	43.81±4.51	42.97±9.30	61	0.71±0.0.7	0.7±0.15
Lysine	38.48±4.83	53.99±22.35	48	0.80±0.10	1.12±0.46
Phenylalanine	34.91±2.64	35.58±3.34	-	-	-
Methionine	9.93±0.45	8.46±2.09	23	0.43±0.00[a]	0.36±0.09
Threonine	15.87±3.59	14.85±6.26	25	0.63±0.14	0.59±0.25
Valine	38.92±5.23	36.71±10.55	40	0.97±0.13	0.91±0.26
Alanine	21.41±2.77	22.29±6.01	-	-	-
Aspartic acid	52.86±11.68	60.60±21.93	-	-	-
Tyrosine	17.12±1.22	17.83±2.06	-	-	-
Glutamic acid	73.18±14.32	105.43±33.41	-	-	-
Glycine	20.21±4.70	20.73±0.03	-	-	-
Proline	24.41±2.86	23.66±4.62	-	-	-
Serine	25.59±4.85	28.73±13.52	-	-	-

*Recommended amino acid scoring pattern for adolescents and adults. FAO 2011. Tryptophan was not analyzed by the method applied.

Chemical scores of Histidine, Lysine and Valine had significant differences ($p< 0.05$).

These amino acids can donate proton to the radicals and stabilize the peptide molecule structure through resonance. Phenylalanine also is a radical scavenger (Sampath Kumar *et al*.,

2011). Furthermore, higher amount of histidine, as a metal chelator and hydrogen donor, can improve the antioxidant properties of the protein concentrate (Ng *et al*., 2013). Glutamic acid, arginine, aspartic acid and lysine were found in highest amount. Hydrolysis, depending on the exposure of protein to enzyme, can increase or decrease the amount of hydrophobic amino acids (Ng *et al*., 2013). Hydrolysis of the *Cajanus cajan*'s protein caused a significant ($p<0.05$) increase in lysine and glutamic acid, but did not considerably change the amount of the other amino acids.

As the antioxidant properties of the Maillard products are influenced by both amino acid and sugar, knowing the frequency of amino acids can provide us with an insight into the properties of the final products. It has been shown that a higher amount of alkaline amino acids such as Arginine and Lysine lead to Maillard products with higher free radical scavenging activities (Shen *et al*., 2018). As the *Cajanus cajan*'s protein hydrolysate is proved to be rich in the two mentioned amino acids, considerable antioxidant activity was expected

The chemical score is used to estimate the nutritive value of the examined protein in comparison to essential amino acids in a standard protein recommended by FAO. Results showed that the limiting amino acids in *Cajanus cajan*'s protein concentrate and protein hydrolysate are methionine, and threonine (Table 2). Other amino acids are almost adequate. Hydrolysis did not have significant negative effect on chemical score (except for Valine), and significantly ($p<0.05$) increased the chemical score of histidine and lysine. According to FAO (2011), both *Cajanus cajan*'s protein concentrate and protein hydrolysate met the nutritive value requirements for adults.

Molecular weight distribution of hydrolysate

Figure 1 shows the size exclusion chromatogram of *Cajanus cajan*'s protein concentrate and protein hydrolysate at 280 nm. The amount of large and small peptides in the protein concentrate is almost similar and very low because no enzyme was used during the protein extraction.

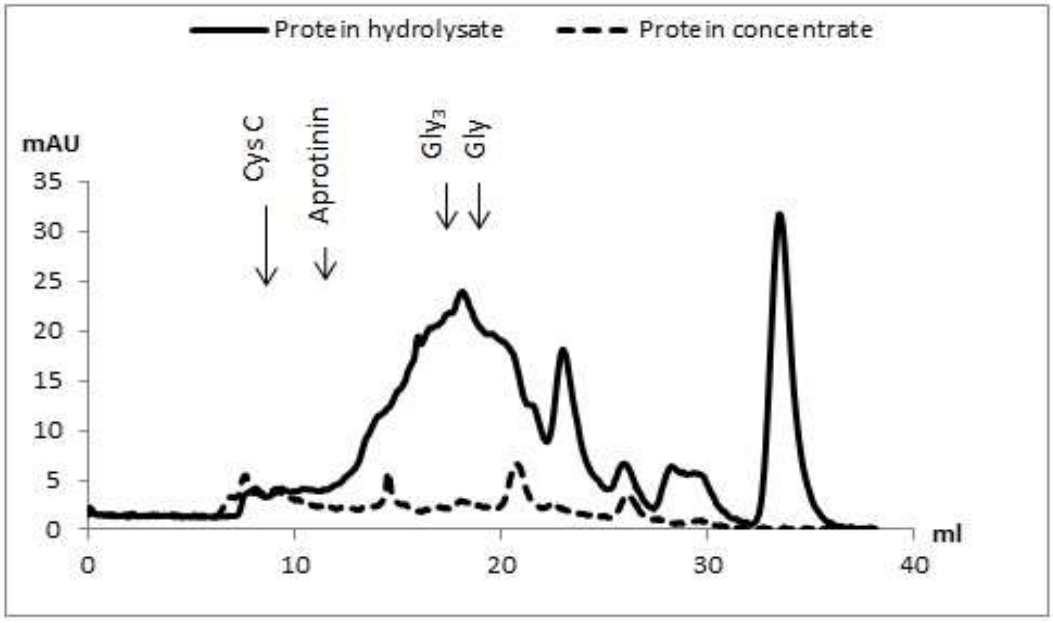

Fig. 1. Size exclusion chromatogram of protein concentrate and protein hydrolysate (by Alcalase)

These peptides were produced due to the acid and base application during the protein extraction. On the other hand, the difference in peptide sizes for protein hydrolysate was distinct. Most of the peptides in protein hydrolysate were smaller than the standards by having a molecular weight between 6.5 kDa (aprotinin) and 75 Da (Glycine). This is because

of the effect of Alcalase on peptide bonds with a wide range of specificity (García-Moreno *et al*., 2016). The peaks that are appeared beyond 20 ml are related to the retained peptides on the column because of hydrophobic interactions and higher affinity to the column and measuring their molecular weight is not feasible (Gringer *et al*., 2016).

Peptides with lower molecular weight often have higher antioxidant activities (Zou et al., 2016). Gringer et al. (2016) found that peptides with molecular weights below 10 kDa in marinated herring (*Clupea harengus*) salt brine had remarkable antiradical activity, reducing power and metal chelating activity.

Antioxidant activity

Figure 2 shows the DPPH scavenging activity and the nitric oxide inhibition activity of glycosylated and non-glycosylated hydrolysates from *Cajanus cajan*. DPPH scavenging assay is a useful method to evaluate the ability of various compounds in scavenging the free radicals (Kedare and Singh, 2011). As DPPH is a free radical, higher DPPH scavenging means the higher antioxidant potential of the sample.

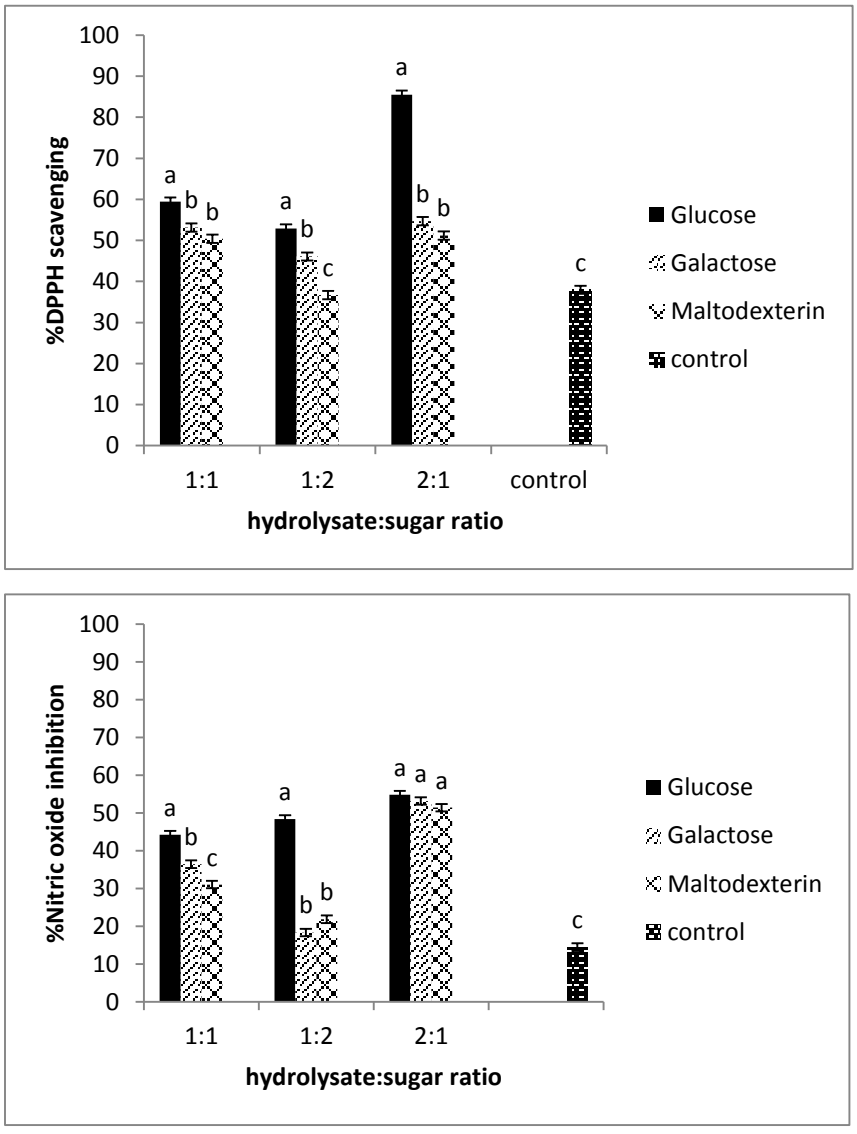

Fig. 2. Antioxidant activity of *Cajanus cajan*'s protein hydrolysate (control) and its glycosylated hydrolysates. A-c indicates the significant difference between sugars in each ratio ($p<0.05$)

As it is shown in Figure 2, the addition of different sugars (glucose, galactose, and maltodextrin) and different hydrolysate to sugar ratios (1:2, 1:1, and 2:1) leads to different antioxidant activities for the samples. Results showed that the addition of sugars increased the DPPH scavenging activity of glycosylated protein hydrolysates compared to the non-glycosylated protein hydrolysate (except for DPPH scavenging of the hydrolysate to maltodextrin in the ratio of 1:2). The hydrolysates: glucose conjugates had higher DPPH scavenging activity in all three ratios and the ratio of 2:1 (hydrolysate: glucose), showed the highest DPPH scavenging activity. Karnjanapratum et al. (2017) reported the same ratio as the most effective ratio in the DPPH scavenging activity in glycosylated gelatin hydrolysates. Our results showed that glycosylation by glucose in the ratio of 2:1 increased the DPPH scavenging activity from 38.0% to 85.5%. This might be due to the effect of higher concentrations of sugar which provides more reducing groups and influences the degree of browning and produces Maillard products with higher antioxidant activities.

Nitric oxide is a free radical that can participate in the oxidative processes (Tsai *et al.*, 2007). The results for the nitric oxide inhibition activity was similar to the DPPH scavenging activity, where adding glucose in 2:1 ratio showed the highest nitric oxide inhibition activity (54.8%) compared to the non-glycosylated hydrolysate (14.5%).

Liu et al. (2014) reported that increasing the protein concentration against glucose, led to increase the antioxidant activity. They also stated that high molecular weight compounds produced during the Maillard reaction have a major contribution to the antioxidant activity of the product mixture. Hydroxyl groups and intermediate reductants can also possess antioxidant properties (Rao *et al*., 2011). Glycosylation, by changing the structure of the hydrolysate and addition of OH groups, have an important effect on the antioxidant activity of the glycosylates (Lie *et al*., 2014). Browning compounds produced in the Maillard reaction are the main radical scavengers (Wang *et al*., 2011). Gottardi et al. (2014) stated that DPPH scavenging activity is based on hydrogen and electron transfer that is dependent on sugar type as a hydrogen donor or acceptor. Different sugars react with different speeds, which in turn have an important effect on the production of the final products in the Maillard reaction (Oliviera *et al*., 2016). Moreover, the degree of glycosylation increases when the size of carbohydrates decreases (Niu *et al*., 2011). Zhang et al. (2018) found similar results and stated that glycosylation of *Morchella esculenta* by Maillard reaction can improve antioxidant activity and generate novel sources of antioxidant and functional foods.

Fourier transform infrared spectroscopy

Although molecular vibration of numerous atoms makes spectroscopic analysis of the polymeric molecules difficult, FTIR spectroscopy is a useful way of establishing peptide: sugar conjugate structures based on the absorption of radiation from atoms vibrations and shows the chemical composition and conformational structure of compounds (Liu *et al.*, 2014). According to the results from antioxidant properties assays, hydrolysate: glucose conjugate (2:1) had the highest antioxidant activity. Therefore, glycosylation of hydrolysate with glucose was evaluated by FTIR.

Characterization of physical stability of the emulsions

Functional properties of proteins and peptides can be altered by Maillard reaction to produce food ingredients with different applications; this is the most studied subject in the field of protein and peptide conjugates (Oliviera *et al.*, 2016). To evaluate the emulsifying properties of the glycosylated protein hydrolysates, the droplet size and zeta potential of fish oil emulsions prepared with sample solutions, as the aqueous phase, were measured (Table 4). In all emulsions, droplet size increased from day 0 to day 3. On day zero, the droplet size of the emulsions prepared with hydrolysate and its conjugates was not significantly different ($p<0.05$) compared to

emulsions produced with sodium caseinate as control; except for hydrolysate: maltodextrin conjugate which had a larger droplet size. This means that apart from hydrolysate: maltodexterin conjugate, the other emulsifiers had the same emulsifying ability. It could be as a result of the similarity of the amino acid profile of the hydrolysate and molecular weight of the glucose and galactose that makes their emulsifying ability to be similar. Furtheremore, the difference between maltodextrin conjugate emulsifying ability with other samples might be due to the difference of the sugar characteristics.

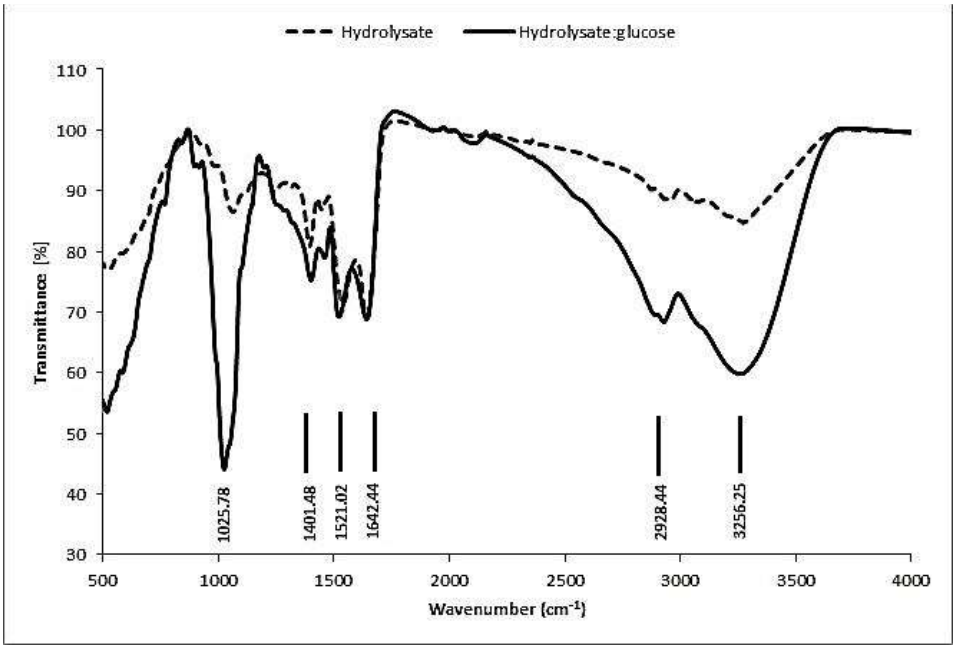

Fig. 3. FTIR spectrums of solid *Cajanus cajan*'s hydrolysate and its glucose conjugate

Table 3- Peak assignment for the FTIR spectrums

Frequency (cm^{-1})	Assignment
1025.78	C-O Stretching vibration
1404.48	C-N Stretching vibration
1521.02	N-H Plane bending and the C-N stretching vibration
1643.44	C=O Stretching vibration
2928.44	-C-H [sp^3] Antisymmetric stretching vibration
3256.25	-O-H Stretching vibration

Table 4- Droplet size and zeta potential of fish oil emulsions stabilized with different sugars

Emulsion	Droplet size [$D_{4,3}$(µm)]			Zeta potential (mV)
	Day 0	Day 1	Day 3	
Hydrolysate	1.7 ± 0.0^{a}	2.8 ± 0.4^{a}	7.8 ± 1.7^{a}	-43.4 ± 2.7^{a}
Hydrolysate: glucose	1.1 ± 0.6^{a}	2.3 ± 0.2^{a}	4.3 ± 0.2^{b}	-43.2 ± 1.6^{a}
Hydrolysate: galactose	1.5 ± 0.5^{a}	4.2 ± 0.1^{b}	5.6 ± 0.2^{b}	-44.6 ± 1.9^{a}
Hydrolysate: maltodextrin	3.7 ± 0.3^{b}	4.5 ± 0.7^{b}	5.2 ± 0.2^{b}	-44.0 ± 1.8^{a}
Sodium caseinate	0.9 ± 0.1^{a}	1.0 ± 0.1^{c}	8.5 ± 6.8^{c}	-42.2 ± 1.1^{a}

a-c in each column indicates the significant differences ($p<0.05$)

As it was mentioned, a high amount of hydrophobic amino acids in the hydrolysates (Table 2) can help the emulsifying ability and stability. Therefore, it could be anticipated that

the added sugar might make any possible difference in the emulsion stability. During the storage, the behavior of the emulsions was different. Sodium caseinate showed the smallest droplet size at day 0 but the largest droplet size at day 3. On day 1, there was no significant difference between hydrolysate and hydrolysate: glucose emulsions, and between hydrolysate: galactose and hydrolysate: maltodextrin emulsions, however, the difference between these two groups was significant ($p<0.05$) (droplet size of hydrolysate and hydrolysate: glucose< hydrolysate: galactose and hydrolysate: maltodextrin). On day 3, there was no significant difference between different conjugates, but the difference between the droplet size of emulsion stabilized with hydrolysate, and emulsions stabilized with conjugates was significant ($p<0.05$). This result shows that glycosylation has had a positive effect on the emulsion stability of the *Cajanus cajan*'s protein hydrolysate, and that glycosylation also improved the emulsion stability by preventing droplet size increase due to coalescence or flocculation. All of the emulsions had a white appearance without phase breaking or creaming (Figure 4).

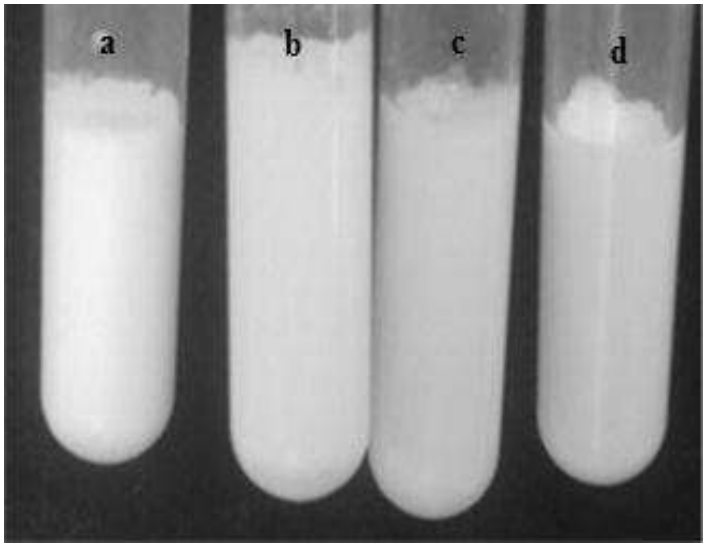

Fig. 4. The appearance of emulsions at day 3. a) Protein hydrolysate b) hydrolysate: glucose c) hydrolysate: galactose d) hydrolysate: maltodextrin

Table 5- Particle size comparison of fish oil emulsions stabilized with different sugars

	Emulsion	Particle size (µm)		
		0.1-1	1-10	10-100
Day 0	Hydrolysate	4.56 ± 0.01^{ab}	0.59 ± 0.00^{b}	0.16 ± 0.01^{a}
	Hydrolysate: glucose	4.65 ± 0.03^{a}	0.59 ± 0.02^{b}	0.08 ± 0.07^{a}
	Hydrolysate: galactose	4.43 ± 0.05^{bc}	0.83 ± 0.16^{a}	0.15 ± 0.09^{a}
	Hydrolysate: maltodextrin	4.30 ± 0.14^{c}	0.82 ± 0.11^{a}	0.22 ± 0.07^{a}
Day 1	Hydrolysate	3.97 ± 0.02^{ab}	1.22 ± 0.02^{b}	0.32 ± 0.05^{ab}
	Hydrolysate: glucose	4.07 ± 0.10^{a}	1.13 ± 0.10^{b}	0.29 ± 0.01^{b}
	Hydrolysate: galactose	3.85 ± 0.01^{bc}	1.24 ± 0.07^{ab}	0.42 ± 0.04^{a}
	Hydrolysate: maltodextrin	3.6 ± 0.15^{c}	1.36 ± 0.02^{a}	0.44 ± 0.12^{a}
Day 3	Hydrolysate	3.21 ± 0.1^{a}	1.54 ± 0.06^{c}	0.87 ± 0.11^{a}
	Hydrolysate: glucose	3.30 ± 0.07^{a}	1.74 ± 0.08^{b}	0.63 ± 0.13^{b}
	Hydrolysate: galactose	3.27 ± 0.06^{a}	1.84 ± 0.02^{ab}	0.57 ± 0.05^{b}
	Hydrolysate: maltodextrin	3.17 ± 0.05^{a}	1.91 ± 0.08^{a}	0.61 ± 0.00^{b}

a-c in each column for each day indicates the significant differences ($p<0.05$).

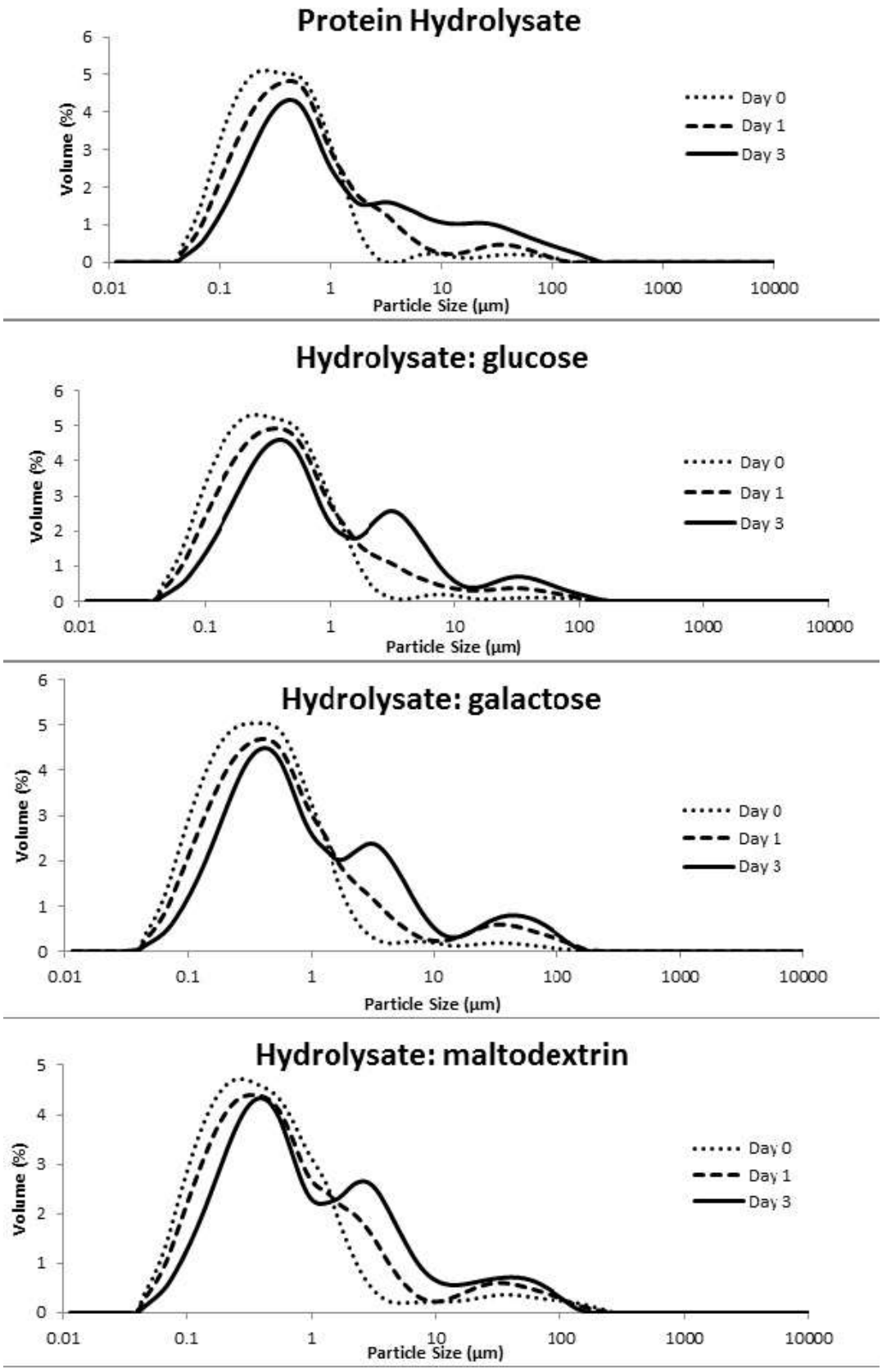

Fig. 5. Particle size distribution graphs

During the emulsification, the hydrolysate is absorbed into the interface, and the conjugated sugar helps the colloidal stability by thickening the aqueous phase (Ru *et al.*, 2009). The molecular mass, load, structure, and reaction time of the sugar and hydrolysate are important for their emulsifying properties (Kato, 2002). Moreover, the hydrophilic- hydrophobic balance of the conjugate played a critical role here (Olivier, 2006). Furthermore, glycosylation indirectly improved the emulsifying properties by increasing the solubility (Kutzli *et al*., 2020). Our results are in agreement with Li et al. (2016), Xue et al. (2017) and Kutzli et al. (2020) who reported that the glycosylation has a positive effect on emulsifying properties.

The surface charge of the emulsifier is important for stability of the emulsion (Li *et al.*, 2016), and measuring the zeta potential provides useful information about the charge of proteins and hydrolysates (Wouters *et al*., 2016). The zeta potential of the emulsions was not changed by glycosylation and all samples showed highly negative zeta potentials between -42 to -45. The induced repulsive forces can prevent the emulsions from flocculation, and maintain stability because the stability is related to the steric and electrostatic repulsions (Lam and Nikerson, 2013). There was no significant difference between zeta potential of the emulsions stabilized with different sugar conjugates ($p>0.05$). This is in agreement with the study of Li et al. (2016).

Figure 5 shows the particle size distribution graphs from mastersizer. These graphs demonstrate the changes in particle size very well. In each graph, a shift to the right during the storage indicates that the size of particles is increasing because of the flocculation and/ or coalescence during the time. All graphs had a large peak between 0.1 and 1 μm. During the storage, smaller peaks between 1 to 10 and 10 to 100 μm were observed. The number of droplets with more than 100 μm increased in the emulsions stabilized by protein hydrolysate, and on day 3, The emulsion stabilized by glycosylated hydrolysates significantly ($p<0.05$) had the lowest amount of such droplets (Table 5).

Conclusions

Evaluation of antioxidant and emulsifying properties of *Cajanus cajan*'s protein hydrolysate and effect of glycosylation by glucose, galactose, and maltodextrin showed that glycosylation has a significant positive effect on the antioxidant and emulsifying properties of the hydrolysates and glucose was the most effective sugar in the present study. FTIR results also proved glycosylation and production of Maillard reaction products. Further experiments are needed to identify the structure of active compounds. The practical application of hydrolysates and glycosylated hydrolysates in real food products should be investigated.

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نشریه پژوهشهای علوم و صنایع غذایی ایران
جلد ۱۷، شماره ۶، بهمن- اسفند ۱۴۰۰، ص. ۱۵۲-۱۳۷

بهبود ویژگی‌های آنتی‌اکسیدانی و امولسیفایری پروتئین‌های هیدرولیزشده نخود کاجان (*Cajanus cajan*) توسط گلیکوزیلاسیون با واکنش میلارد

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تاریخ پذیرش: ۱۴۰۰/۰۷/۰۷

چکیده

هدف این مطالعه استفاده از واکنش میلارد به‌عنوان ابزاری برای گلیکوزیلاسیون پروتئین‌های هیدرولیزشده حاصل از نخود کاجان (*Cajanus cajan*) و ارزیابی این اصلاح شیمیایی بر ویژگی‌های آنتی‌اکسیدانی و امولسیفایری بود. ویژگی‌های شیمیایی، ترکیب آمینواسیدی و توزیع وزن مولکولی پروتئین‌های هیدرولیز شده مورد بررسی قرار گرفت. از گلوکز، گالاکتوز و مالتودکسترین در نسبت‌های ۱ به ۲، ۱ به ۱ و ۲ به ۱ (پروتئین هیدرولیزشده به قند، وزن خشک) برای گلیکوزیلاسیون استفاده شد. فعالیت آنتی‌اکسیدانی از طریق دو آزمون فعالیت مهار رادیکال آزاد ۱و ۱- دی‌فنیل-۲- پیکریل هیدرازیل (DPPH) و مهار نیتریک‌اکسید بررسی شد. امولسیون‌ها (روغن/ آب) توسط روش سونیفیکاسیون تهیه شدند. توزیع اندازه ذرات و پتانسیل زتا امولسیون‌ها طی ۴ روز نگهداری اندازه‌گیری شد. گلیکوزیلاسیون با گلوکز در نسبت ۲ به ۱ مهار DPPH را از ۳۷/۹۶٪ به ۸۵/۵۳٪ و مهار نیتریک‌اکسید را از ۱۴/۵۰٪ تا ۵۴/۸۳٪ افزایش داد. همچنین گلیکوزیلاسیون توسط هرکدام از قندها پایداری امولسیون‌ها را افزایش داد و تفاوت معناداری بین نوع قند مورد استفاده مشاهده نشد.

واژه‌های کلیدی: گلیکوزیلاسیون، واکنش میلارد، پروتئین هیدرولیزشده، آنتی‌اکسیدان، امولسیون

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Full Research Paper

The effects of commercial mixed-strain starter cultures on the chemical and sensory characteristics of UF-Feta cheese analogue during ripening

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Abstract

UF- Feta cheese is mostly produced from bovine milk and is usually consumed fresh or only after a short period of ripening (60 days). In this research, the influence of commercial starter cultures (SafeIT 2, FRC- 65 and R- 704) and ripening time (0- 60 days) on chemical (total solids, fat, protein, ash, salt, acidity, pH), biochemical (pH 4.6, TCA, PTA-soluble nitrogen, acid degree value) and sensory (color and appearance, aroma, texture, flavor and total acceptance) characteristics of UF- Feta cheese analogues was investigated. According to our results, the starter culture types were known to have a significant effect ($P\leq 0.05$) on pH, %salt, %protein, and pH 4.6- soluble nitrogen of cheeses, whereas the other chemical properties were not affected by them. Ripening time only significantly ($P\leq 0.05$) influenced %acidity, pH, %salt, acid degree value (meq acid 100 g^{-1} fat), %protein and %proteolysis products of samples. Also, the starter culture and ripening time did not affect the sensory properties significantly, excluding color and appearance, however, the produced cheeses from SafeIT 2 had higher sensory scores compared with the others containing FRC- 65 and R- 704 cultures.

Keywords: Analogue cheese, Lactic acid bacteria, Ripening time, Starter culture.

Introduction

The widespread use of lactic acid bacteria (LAB) in manufacturing fermented dairy products such as cheese, yogurt and cultured milk have been reported. Cheese, as one of the most important commercial fermented milk products, has received much scientific attention in recent years. The appearance, flavor, texture and overall acceptance of cheeses are considerably affected by LAB (Grattepanche *et al*,. 2007, Martínez-Cuesta *et al*,. 2001, Wouters *et al*,. 2002). LAB strongly influence cheese ripening process by lactic acid production, decrease in oxidation/reduction (OR) potential, autolysis and its associated release of intracellular enzymes, like protease and lipase, into the curd (Di Cagno *et al*,. 2003, Vernile *et al*,. 2008, Zárate *et al*,. 1997).

Cheese ripening is a complex process in which extensive microbiological, biochemical

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and physical changes occur continuously. Proteolysis and lipolysis are the two main biochemical reactions that occur during the cheese ripening period. Indigenous milk proteases and lipases, microbial enzymes and residual chymosin are responsible for these transformations. Peptides and free amino acids (FAAs) release during proteolysis directly and degradation of these compounds to related acids, amines, thiols, etc. indirectly affect the sensory properties of cheeses such as aroma, flavor and texture. During ripening, the lipolysis results in increased free fatty acid (FFA) concentrations, which in turn affects the textural and sensory characteristics of cheese samples. Hydrolysis of fat is especially important in soft cheeses (van Kranenburg *et al,*. 2002).

Many industrially utilized dairy starter cultures have a highly proteolytic activity (Korhonen and Pihlanto 2006). The sensory properties of cheeses are improved by LAB as a result of casein catabolism and produced peptides and FAAs (Beresford and Williams 2004). According to the literature, LAB applied as starter cultures have generally weak lipolytic activity (Larráyoz *et al,*. 2001, Sarantinopoulos *et al,*. 2001, Avila *et al,*. 2007), however, their high number or extended ripening time can release high amounts of FFA (Gobbetti *et al,*. 1997c, Santillo *et al,*. 2007, Sheehan *et al,*. 2009). LAB can also produce lactic acid even during ripening which is the principal cause of lowering the pH (Wang *et al,*. 2012).

Ultrafiltration (UF) technique has been widely used for the manufacture of soft cheese varieties, especially for UF-Feta cheese. This cheese, which is very popular in Iran, is mostly produced from bovine milk and is typically consumed fresh or only after a short period of ageing (60 days). UF-Feta cheese has a maximum shelf life of 2 months at refrigerated temperature (Iran Standard no. 12736).

In general, different dairy and non-dairy proteins, types of fats or edible oils, various types of starches and water are the most important ingredients in analogue cheeses formulations (Guinee, 2007). Inaccessibility to fresh milk or low milk production in some areas, lower price and variety of formulations are the most important reasons for extension of cheese analogues. Dairy (Milk protein concentrate (MPC), Whey protein concentrate (WPC), skim milk powder (SMP)) and non-dairy (soy, peanut) protein sources, dairy fat (cream, butter and butter oil) and vegetable oil or fat, salt and water are the main constituents which are used in the production of UF-Feta cheese analogue. The chemical compositions of this type of cheese are as follows: a minimum of 35% (w/w) total solids (TS), a minimum of 10% (w/w) protein, 10- 25%, 25- 45% and 45-60% (w/w, on dry basis) fat for low-, semi- and full fat types, respectively, a maximum of 3% salt, 0.5- 2% acidity (lactic acid) and a maximum of pH 5.2. (Iran Standard no. 12736). Besides the short ripening time, the use of vegetable fats and proteins in UF-Feta cheese analogues formulations is also described to enhance the texture and flavor problems in these cheeses. No data have previously been reported in the literature on the improvement of sensory properties of these cheeses during ripening. Therefore, the aim of this study was to determine whether the sensory characteristics of UF-Feta cheese analogue could be improved using starter cultures. On this basis, three commercial starter cultures (SafeIt 2, R-704 and FRC-65) were used and their effects on different properties of cheeses were evaluated during ripening.

Materials and methods

Calcium chloride (food-grade) was obtained from Kemira Agro Ltd. (Helsinki, Finland). MPC-75 (75% protein, 1.5% fat, 10.9% lactose, 7.6% ash, 5% water) was prepared from Milei GmbH (Stuttgart, Germany). WPC-35 (35% protein, 3% fat, 50.2% lactose, 7.2% ash, 4.6% water), SMP (skim milk powder) (36% protein, 1.35% fat, 50.8% lactose, 7.85% ash, 4% water) and butter (82% fat, 0.49% protein, 16% water) were purchased from a local dairy factory (Pegah, Mashhad, Iran). Margarine (80% fat, 0.5% protein, 18% water) was supplied by Behineh Wazin Co. (Mahgol^{TM}) (Tehran, Iran). Full fat soy flour (38% protein, 18% fat, 15% soluble carbohydrate, 15% non-soluble

carbohydrate, 13.7% water) was obtained from Soyan Toos Co. (Mashhad, Iran). Three freeze-dried mixed cultures of SafeIt 2 (*Lactococcus lactis* subsp. *cremoris, L. lactis* subsp. *lactis, Streptococcus thermophiles, Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lb. helveticus*), FRC-65 (*Lb. delbrueckii* subsp. *bulgaricus, St. thermophiles, L. lactis* subsp. *lactic* and *L. lactis* subsp. *cremoris*) and R-704 (*L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*) (Chr. Hansens, Denmark) were used as starter. Fromase® 2200 TL, as fungal rennet, was prepared from DSM Co., The Netherlands.

Cheese manufacture

The formulation of cheese samples was based on the optimized formulation obtained from the previous study (Gholamhosseinpour *et al,*. 2014). In the optimal cheese formulation, the amounts of MPC, WPC, soy milk and margarine were 9.13%, 3%, 15% and 7.65%, respectively. The production of cheese samples was also performed according to the method as described in detail by Gholamhosseinpour et al. (2018). Chemical, biochemical and sensory analyses of cheese samples were carried out on days 3, 20, 40 and 60 after manufacturing.

Compositional analyses

Samples of cheese were analyzed in triplicates for total solids matter by oven-drying (AOAC 2005), fat content by the Gerber butyrometer technique (BSI 1989), ash by incineration at 550ºC (AOAC 935.42, 2005), salt concentration by the Mohr method (IDF 1988), acidity by titration method (AOAC 920.124, 2005) and protein content by the standard micro-Kjeldahl method (AOAC 920.123, 2005) using the Kjeltec Auto 1030 Analyzer. The pH of a 1:1 slurry of cheese in distilled water (Al-Otaibi and Wilbey 2006) was also measured with a Metrohm pH meter (Model 691, Herisau, Switzerland) after calibrating with fresh standard buffers at pHs 4 and 7.

Biochemical assays

Proteolysis

The concentrations of pH 4.6-soluble nitrogen (pH 4.6-SN), trichloroacetic acid-soluble nitrogen (TCA-SN) and phosphotungstic acid-soluble nitrogen (PTA-SN) were measured according to Messens et al. (1999), Reis et al. (2000) and Tavaria et al. (2003) with some modifications, respectively. The nitrogen contents of these fractions were determined using the standard micro-Kjeldahl technique (AOAC 920.123, 2005). The contents of pH 4.6-SN, TCA-SN and PTA-SN were expressed per unit mass of total nitrogen (TN).

Lipolysis

A slight modification of Nunez et al. (1986) method was used for evaluating the lipolysis degree of samples by assaying the Acid Degree Value (ADV). All Biochemical determinations were performed in triplicate.

Sensory evaluations

Sensory evaluation was done on cheese samples after 3, 20, 40 and 60 days of production by ten trained panelists. The 2× 2× 2 cm pieces of cheese were prepared and their codes were randomly determined. The panelists assessed cheese samples in a random order and washed their mouth before testing of each sample. Color and appearance, aroma, texture, flavor and total acceptance of cheese samples were evaluated based on a five-point hedonic scale (1=very poor; 5=excellent).

Statistical analyses

A complete randomized design was used to evaluate the effect of starter type and ripening time on the response variables. Analysis of variance (ANOVA) was carried out using the Minitab VERSION 16.2.3 statistical software package (MINITAB Inc., USA).

Results and discussion

Chemical Properties

Table 1 shows the average values of the main compositional criteria of cheeses for the experimental treatments. The average amount of TS in cheeses prepared with different starter cultures was varied from 35.88% to 35.92%. According to our results, the TS content of the resultant cheese samples was not significantly affected by the various starter cultures. This is

consistent with the results of Hynes et al. (2003) and Hayaloglu et al. (2005), who stated that total solids content of cheese samples was not significantly affected by the type of starter. Changes in the amount of total solids during the ripening period of cheeses was between 35.83 to 35.94%. It was also observed that the amount of TS of each cheese samples was not significantly different during ripening. Miočinović et al. (2011) also did not observe any significant effect of ripening time on TS content. It should also be noted that there are contradictory reports about the effect of ripening time on TS content in different cheese varieties. Aly (1995), Azarnia et al. (1997), Al-Otaibi and Wilbey (2004) and Lopez et al. (2007) reported that with increasing ripening time, the amount of TS increased. They stated that the high temperature of curd preservation, which affects the hydration of casein, led to increase in the degree of syneresis due to increasing salt concentration in the curd; water evaporation during storage and low water absorption in cheeses with low pH could be the causes of this increase. Inversely, Milci et al. (2005) and Shahab Lavasani et al. (2012) reported a decrease in TS content during ripening. The migration of water-soluble proteins and peptides from the curd into the surrounding brine, the lipolysis and the transfer of FFAs from the block of cheese to brine have been suggested as the most important reasons for this decrease.

The amount of fat in cheeses containing different starter cultures ranged from 15.96 to 16.08% and at different ripening times between 15.94 to 16.11%. Based on our results, there was no significant impact of type of starter, ripening time and their interactions on the concentration of fat in cheese samples. Hynes et al. (2003) also reported no significant effect of starter type on the amount of fat in different cheeses. Various results have also been reported about changes in the concentrations of fat in different cheeses during ripening. Milci et al. (2005) and Shahab Lavasani et al. (2012) observed that as the ripening time increased, the fat content of the cheese samples decreased significantly. Fat hydrolyzed to FFA and volatile compounds has been stated as the main reason for this reduction. In contrast, Aly (1995) observed that the fat content of Feta cheese increased during ripening and stated that the decrease in moisture with time is the reason. Furthermore, Karami et al. (2009b) observed no significant differences in the fat concentrations of cheeses.

While the titratable acidity of cheeses was not significantly influenced by various starters, this parameter was significantly increased ($p\leq0.05$) during ripening for all samples so that the acidity from 0.95% on day 3 increased to 1.1% on day 60 (at the end of ripening). This increase in acidity has been attributed to the lactate formation and also produce free fatty and amino acids by lipolysis and proteolysis, respectively (Özer *et al*,. 2003, Souza and Saad 2009). Lactic acid catabolism and its entry into brine as well as ammonia production can reduce the acidity at the end of the ripening period in some cheeses. The interaction between the two factors (starter type and time of ripening) was not also significant. Depending on starter type, Hayaloglu et al. (2005) and Hayaloglu et al. (2013) reported both significant and non-significant effects of the starters on acidity of different cheeses.

The pH of the cheeses prepared with R-704 and FRC-65 starter, didn't show any significant differences. However, the pH of both these cheeses was significantly ($p\leq0.05$) different from those prepared with SafeIt 2 starter. Hayaloglu et al. (2005) did not find significant impacts of starters on the pH of various cheeses. The pH of the cheeses also differed significantly ($p\leq0.05$) over the ripening period. The pH trend of cheeses decreased after 40 days of production due to the conversion of lactose to lactic acid by LAB. Later on, an increase was observed until the end of the 60th day of ripening which could be as a result of the production of ammonia (common end product of amino acid catabolism) combined with the metabolism of lactic acid by yeasts and molds (Gobbetti *et al*,. 1997a, Gobbetti *et al*,. 1997b, Hayaloglu *et al*,. 2007). The two factors had no significant interaction effect on the pH.

Table 1- The effects of starter culture type, ripening time and their interactions on chemical properties of UF-Feta cheese analogues

Chemical and biochemical properties	Starter type	Ripening time (day)				Mean
		3	20	40	60	
Total solids (%)	SafeIt 2	35.83± 0.29	36.00± 0.00	35.67± 0.29	36.00± 0.00	35.88± 0.16
	R-704	36.00± 0.00	35.83± 0.29	36.00± 0.00	35.83± 0.29	35.92± 0.10
	FRC-65	36.00± 0.00	35.83± 0.29	35.83± 0.29	36.00± 0.00	35.92± 0.10
	Mean	35.94± 0.10	35.89± 0.10	35.83± 0.17	35.94± 0.10	
Fat content (%)	SafeIt 2	16.00± 0.00	16.00± 0.00	16.17± 0.29	16.17± 0.29	16.08± 0.10
	R-704	16.00± 0.00	16.00± 0.00	15.83± 0.29	16.00± 0.00	15.96± 0.08
	FRC-65	16.00± 0.00	16.00± 0.00	15.83± 0.29	16.17± 0.29	16.00± 0.14
	Mean	16.00± 0.00	16.00± 0.00	15.94± 0.20	16.11± 0.10	
Titratable acidity (% lactic acid)	SafeIt 2	0.93± 0.02	1.00± 0.00	1.03± 0.02	1.09± 0.02	1.01± 0.07
	R-704	0.96± 0.00	1.00± 0.00	1.02± 0.02	1.11± 0.02	1.02± 0.06
	FRC-65	0.95± 0.02	1.00± 0.00	1.03± 0.02	1.09± 0.02	1.02± 0.06
	Mean	0.95^{C}± 0.01	1.00^{B}± 0.00	1.02^{B}± 0.00	1.10^{A}±0.01	
pH	SafeIt 2	4.70± 0.01	4.67± 0.01	4.63± 0.01	4.76± 0.01	4.69^{A}± 0.05
	R-704	4.68± 0.01	4.65± 0.00	4.63± 0.02	4.73± 0.01	4.67^{B}± 0.05
	FRC-65	4.68± 0.02	4.65± 0.01	4.63± 0.01	4.75± 0.03	4.68^{B}± 0.06
	Mean	4.69^{B}± 0.01	4.66^{C}± 0.01	4.63^{D}± 0.00	4.75^{A}± 0.01	
Ash (%)	SafeIt 2	2.78± 0.19	2.78± 0.19	2.67± 0.00	2.67± 0.00	2.73± 0.06
	R-704	4.78± 0.19	2.67± 0.00	2.67± 0.00	2.67± 0.00	2.70± 0.05
	FRC-65	2.78± 0.19	2.78± 0.00	2.67± 0.19	2.78± 0.19	2.75± 0.05
	Mean	2.78± 0.00	2.74± 0.06	2.67± 0.00	2.71± 0.06	
Salt (%)	SafeIt 2	1.64^{de}± 0.00	1.66^{cd}± 0.03	1.76^{a}± 0.00	1.76^{a}± 0.00	1.70^{A}± 0.06
	R-704	1.60^{ef}± 0.02	1.69^{bc}± 0.02	1.69^{bcd}± 0.02	1.76^{a}± 0.00	1.68^{B}± 0.07
	FRC-65	1.56^{f}± 0.02	1.65^{cd}± 0.02	1.72^{ab}± 0.02	1.76^{a}± 0.00	1.67^{B}± 0.09
	Mean	1.60^{D}± 0.04	1.67^{C}± 0.02	1.72^{B}± 0.03	1.76^{A}± 0.00	
Protein (%)	SafeIt 2	10.60^{ab}± 0.02	10.47^{def}± 0.03	10.42^{fg}± 0.03	10.52^{bcd}± 0.03	10.50^{A}± 0.07
	R-704	10.58^{abc}± 0.02	10.45^{def}± 0.03	10.40^{fg}± 0.02	10.51^{cde}± 0.02	10.49^{A}± 0.08
	FRC-65	10.61^{a}± 0.02	10.45^{def}± 0.04	10.35^{g}± 0.04	10.44^{ef}± 0.03	10.46^{B}± 0.11
	Mean	10.60^{A}± 0.01	10.46^{C}± 0.01	10.39^{D}± 0.04	10.49^{B}± 0.05	
pH 4.6-SN/TN (%)	SafeIt 2	5.46^{d}± 0.00	5.74^{d}± 0.13	6.37^{c}± 0.27	6.83^{ab}± 0.00	6.10^{A}± 0.62
	R-704	4.00^{e}± 0.11	5.83^{d}± 0.08	6.75^{abc}± 0.03	6.99^{ab}± 0.07	5.89^{B}± 1.36
	FRC-65	3.07^{f}± 0.00	6.62^{bc}± 0.27	7.02^{a}± 0.03	7.01^{ab}± 0.06	5.93^{B}± 1.92
	Mean	4.18^{D}± 1.20	6.06^{C}± 0.48	6.71^{B}± 0.33	6.94^{A}± 0.10	
TCA-SN/TN (%)	SafeIt 2	1.59± 0.00	1.71± 0.00	1.77± 0.14	1.81± 0.08	1.72± 0.10
	R-704	1.55± 0.04	1.67± 0.05	1.79± 0.04	1.84± 0.00	1.7± 0.13
	FRC-65	1.58± 0.02	1.67± 0.05	1.79± 0.04	1.88± 0.05	1.73± 0.13
	Mean	1.57^{C}± 0.02	1.68^{B}± 0.02	1.78^{A}± 0.01	1.84^{A}± 0.04	
PTA-SN/TN (%)	SafeIt 2	0.07± 0.00	0.13± 0.00	0.21± 0.03	0.27± 0.01	0.17± 0.09
	R-704	0.07± 0.00	0.13± 0.01	0.19± 0.02	0.26± 0.01	0.16± 0.08
	FRC-65	0.07± 0.00	0.14± 0.01	0.20± 0.01	0.28± 0.02	0.17± 0.09
	Mean	0.07^{D}± 0.00	0.13^{C}± 0.01	0.20^{B}± 0.01	0.27^{A}± 0.01	
ADV (meq acid 100 g^{-1} fat)	SafeIt 2	0.11± 0.01	0.13± 0.03	0.15± 0.01	0.18± 0.01	0.14± 0.03
	R-704	0.11± 0.01	0.14± 0.02	0.16± 0.03	0.18± 0.01	0.15± 0.03
	FRC-65	0.10± 0.01	0.14± 0.00	0.16± 0.01	0.19± 0.01	0.15± 0.04
	Mean	0.11^{C}± 0.00	0.14^{B}± 0.01	0.16^{B}± 0.01	0.18^{A}± 0.01	

Means within a same row or column with different superscript lowercase and capital letters indicate significant differences ($p \leq 0.05$).

The amount of ash in samples containing different starter cultures varied between 2.70 to 2.75% and at different ripening times ranged from 2.67 to 2.78%. The results showed that the effect of starter, time of ripening and the interaction between them on % ash content of cheeses was not significant. Al-Otaibi and Wilbey (2004) and Hayaloglu et al. (2005) also reported that the starter type and ripening time did not significantly affect the ash content of cheeses.

The results obtained from the study showed that the effect of starters, ripening time and their interaction on the salt level was significant ($p\leq0.05$). The salt content of samples was 1.6%, 1.67%, 1.72% and 1.76% on 3, 20, 40 and 60-day, respectively. Azarnia et al. (1997) stated that this increase may be due to the loss of water as well as the gradual salt penetration from brine into the cheese during ripening. According to some authors (Hynes *et al*,. 2003, Muir *et al*,. 1996), the starter effect on the salt concentrations of various cheeses was not significant, while Hayaloglu et al. (2013) reported the significant effect of starter type on the salt content of Gokceada cheese. Different results have been reported about changes in the concentration of salts during ripening. No significant increase was observed by Azarnia et al. (1997) and Shahab Lavasani et al. (2012) in the salt concentration of cheeses during ripening but Karimi et al. (2012) stated a non-significant decrease in the salt concentration. Also, in some cases, there was no noticeable change in salt content during ripening (Al-Otaibi and Wilbey 2004, Karami *et al*,. 2008).

Proteolysis

According to Table 1, the content of protein in the samples was significantly ($p\leq0.05$) affected by the type of starter, ripening time and the interaction between them. The amount of protein in the cheeses decreased gradually up to 40 days of ripening and afterwards increased continuously until the end of the ripening process. The initial decrease (up to 40 days of ripening) in protein content can be attributed to the proteolysis, whereas the subsequent increase may be due to the water evaporation and the increase in TS.

The pH 4.6-SN is used as a ripening extension index in cheese, which is a measure of proteolytic activity and reflects the amount of proteins and peptides that are soluble in water at pH value of 4.6, the isoelectric point of caseins (de Oliveira Carneiro *et al*,. 2020). The obtained results (Table 1) indicated that the concentration of pH 4.6-SN/TN was significantly ($p\leq0.05$) affected by the starter type, time of ripening and their interaction, however, no significant difference was found between the concentrations of pH 4.6-SN/TN in cheese samples prepared with R-704 and FRC-65 starters. The effect of starter culture on pH 4.6-SN/TN is related to the type of strain and its thermophilic and mesophilic nature. Increase in the pH 4.6-SN/TN ratio during ripening of UF-Feta cheese has been also observed in previous studies (Karami *et al*,. 2009b, Fathollahi *et al*,. 2010, Miočinović *et al*,. 2011). As the time of ripening increased, an increase in the amount of pH 4.6-SN/TN from 4.18% on day 3 to 6.94% on day 60, was observed. The comparison of 3-days cheeses was showed that the pH 4.6-SN/TN content of samples prepared with SafeIT 2 starter was higher than that of the others, which this could be due to the presence of *Lb. helveticus* in SafeIT 2 starter and its autolytic activity. Hannon et al. (2006) also stated that the lysis of *Lb. helveticus* in UF-cheese was started from the beginning of ripening but the onset and the extent of lysis were dependent on the strains and species used. Release of intracellular peptidases following autolysis of starter and nonstarter lactic acid bacteria (NSLAB) further contributes to proteolysis in cheese (Pillidge *et al*,. 2003).

The cheese ripening depth index (TCA-SN/TN) indicates the amount of low molecular weight nitrogenous substances accumulated during ripening period, that remains soluble in a 12% TCA solution (de Oliveira Carneiro *et al*,. 2020). According to the results, the level of TCA-SN/TN was significantly influenced by ripening time ($p\leq0.05$), whereas the starter type and its interaction with ripening time had not significant effects (Table 1). The study of

Hayaloglu et al. (2013) showed that a significant influence of starter type on TCA-SN/TN ratio can result from either species diversity of starter culture or from process conditions. No significant differences were found in TCA-SN/TN content between the cheese samples prepared with various starter cultures, although, its concentration in the cheeses made with SafeIt 2 and FRC-65 starters was higher than those of cheeses made with R-704 starter, which may be due to the presence of thermophilic bacteria (*Lb. helveticus*, *Lb. delbrueckii* subsp. *bulgaricus* and *St. thermophiles*) in two first starter cultures and their higher proteolytic activity. The overall proteolytic activity of lactobacilli has been found higher than that of *Lactococcus lactis* because lactobacilli possess additional peptidases and because their peptidases have higher expression levels. The presence of streptococcal peptidases indicated that these species also play a role in peptide degradation (Manso *et al*,. 2005). The TCA-SN/TN level of cheeses increased significantly ($p \leq 0.05$) with ripening time which is in accordance with previous studies (Al-Otaibi and Wilbey 2005, Azarnia *et al*,. 1997, Pezeshki *et al*,. 2011). The highest increase in the level of this fraction was observed during the first 40 days of ripening. Casein hydrolysis during ripening has been expressed as the main cause of the increase in the amount of TCA-SN/TN in different samples.

The free amino acid index, represented by the PTA-SN/TN ratio, reflects the further hydrolysis of small peptides into dipeptides, tripeptides, and free amino acids (Lacroix *et al*,. 2010). Similar to the trend described for TCA-SN/TN, the starter type and its interaction with ripening time had no significant impact on PTA-SN/TN ratio but the value of PTA-SN/TN increased significantly throughout ripening ($p \leq 0.05$) so that its level increased from 0.07% at 3 days to 0.27% at the end of ripening (Table 1). This increase is due to the hydrolysis of casein. The study of Hayaloglu et al. (2005) on Turkish white-brined cheese also showed that the type of starter had no significant effect on the amount of PTA-SN/TN. Also, other studies conducted on different types of cheeses (i.e., semi-hard cheese from high protein UF milk retentate, Feta, and low-fat UF) suggested that the ripening time led to increased level of PTA-SN/TN in cheese (Moatsou *et al*,. 2002, Miočinović *et al*,. 2011). The SafeIt 2 and FRC-65 starter cultures produced higher concentrations of PTA-SN than the R-704 starter in cheeses, which may be explained by the higher proteolytic activity or by the faster autolysis of thermophilic lactobacilli of two first starters. The earlier lysis of thermophilic bacteria accelerates the release of intracellular enzymes into the cheese matrix and thus increases proteolysis rate (Pappa *et al*,. 2006). Daly et al. (2010) reported that the rapid autolysis of *Lb. helveticus* during ripening and liberation of active peptidases in cheese causes increase in free amino acids and peptides concentration in whey.

Lipolysis

The presence of lipases in cheese may originated from the milk, rennet preparation, primary starter cultures, adjunct starter cultures, NSLAB and exogenous lipase preparations (Collins *et al*,. 2004). The results showed that the acid degree values (ADVs) significantly ($p \leq 0.05$) increased in all cheeses during ripening, while starter type and its interaction with time had not significant effect on ADVs (Table 1). Hayaloglu et al. (2005) observed that the use of various starter cultures caused different levels of lipolysis in Turkish white-brined cheese. As it is seen from Table 1, the ADV ranged from 0.11 at 3 d. to 0.18 at 60 d of ripening. The highest increase in ADV was also observed in the first 20 days of ripening. Georgala et al. (2005) and Atasoy and Türkoğlu (2009) also studied different cheeses and found that the level of ADV increased during ripening. Shahab Lavasani et al. (2012) Stated that the increase in ADV during ripening of Lighvan cheese was due to proteolysis and post-acidification. Also, the reason for the slowing down of lipolysis at the end of the ripening period of some cheeses is the inhibitory effect of increasing the concentration of salt and free fatty acids on the activity of

lipase (Azarnia *et al*,. 1997, Shahab Lavasani *et al*,. 2012).

Sensory properties

According to Table 2, only ripening time and its interaction with starter culture had significant effects ($p \leq 0.05$) on color and appearance scores of cheeses, whereas the type of starter culture did not show significant influence. The panelists awarded highest scores to 3 day old cheeses containing FRC-65 cultures and lowest scores to 60 day old cheeses made with starter R-704. The scores of color and appearance of cheeses prepared with SafeIt 2 and FRC-65 cultures decreased continually with storage time whereas these scores in cheeses made with starter R-704 increased during the first 40 days of ripening and then decreased thereafter. Hayaloglu et al. (2005) did not observe significant impacts of starters on the color and appearance of cheese. With regard to the ripening time, some reports found that this variable had no significant effects on appearance characteristics of cheeses (Milci *et al*,. 2005, Miočinović *et al*,. 2011).

Table 2- The effects of starter culture type, ripening time and their interactions on sensory properties of UF-Feta cheese analogues

Sensory properties	Starter type	Ripening time (day)				Mean
		3	20	40	60	
Colour & appearance	SafeIt 2	$4.50^{ab}\pm0.53$	$4.40^{ab}\pm0.52$	$4.20^{ab}\pm0.42$	$4.00^{ab}\pm0.67$	4.28±0.22
	R-704	$4.10^{ab}\pm0.32$	$4.40^{ab}\pm0.52$	$4.50^{ab}\pm0.53$	$3.90^{b}\pm0.57$	4.23±0.28
	FRC-65	$4.70^{a}\pm0.48$	$4.20^{ab}\pm0.42$	$4.10^{ab}\pm0.32$	$4.10^{ab}\pm0.32$	4.28±0.29
	Mean	$4.43^{A}\pm0.31$	$4.33^{A}\pm0.12$	$4.27^{AB}\pm0.21$	$4.00^{B}\pm0.10$	
Texture	SafeIt 2	4.50±0.71	4.40±0.52	4.30±0.67	4.20±0.42	4.35±0.13
	R-704	4.30±0.48	4.10±0.57	4.20±0.79	4.00±0.67	4.15±0.13
	FRC-65	4.30±0.48	4.30±0.48	3.60±0.70	4.00±0.47	4.05±0.33
	Mean	4.37±0.12	4.27±0.15	4.03±0.38	4.07±0.12	
Aroma	SafeIt 2	4.10±0.88	4.30±0.48	3.80±0.79	4.10±0.74	4.08±0.21
	R-704	4.00±1.15	3.80±0.63	4.00±0.67	4.10±0.57	3.98±0.13
	FRC-65	4.40±1.26	4.20±0.63	3.40±1.07	3.80±0.79	3.95±0.44
	Mean	4.17±0.21	4.10±0.26	3.73±0.31	4.00±0.17	
Flavor	SafeIt 2	4.10±0.74	4.00±0.47	4.00±1.05	4.00±0.67	4.03±0.05
	R-704	4.10±0.32	3.60±0.70	3.80±0.79	4.50±0.71	4.00±0.39
	FRC-65	4.20±0.63	4.00±0.82	3.70±0.95	3.80±0.63	3.93±0.22
	Mean	4.13±0.06	3.87±0.23	3.83±0.15	4.10±0.36	
Total acceptance	SafeIt 2	4.10±0.74	4.00±0.00	4.00±0.67	3.90±0.74	4.00±0.08
	R-704	3.30±1.06	4.00±0.67	3.60±0.84	4.00±0.67	3.73±0.34
	FRC-65	3.80±0.79	3.70±0.67	4.60±0.52	4.00±0.67	4.03±0.40
	Mean	3.73±0.40	3.90±0.17	4.07±0.50	3.97±0.06	

Means within a same row or column with different superscript lowercase letters indicate significant differences ($p \leq 0.05$).

Means within a same row or column with different superscript capital letters indicate significant differences ($p \leq 0.05$).

According to our results, the cheese samples prepared with SafeIt 2 starter cultures were more spreadable and less hard (softer), therefore, these cheeses received higher scores for texture compared to the cheeses made with two other starter cultures, although the differences were not significant ($p>0.05$) (Table 2). Katsiari et al. (2002) and Hayaloglu et al. (2005) also reported no significant changes in texture scores of cheeses made with different starter cultures. Ripening time, also, had no significant effect on texture scores. The decrease in moisture content during the ripening was the reason for the decrease in the

texture scores of the cheeses. Ghods Rohani et al. (2010), Karami et al. (2009a) and Miočinović et al. (2011) found no significant differences in texture scores during ripening, while Karimi et al. (2012) observed a significant increase in texture scores up to 30 days of ripening.

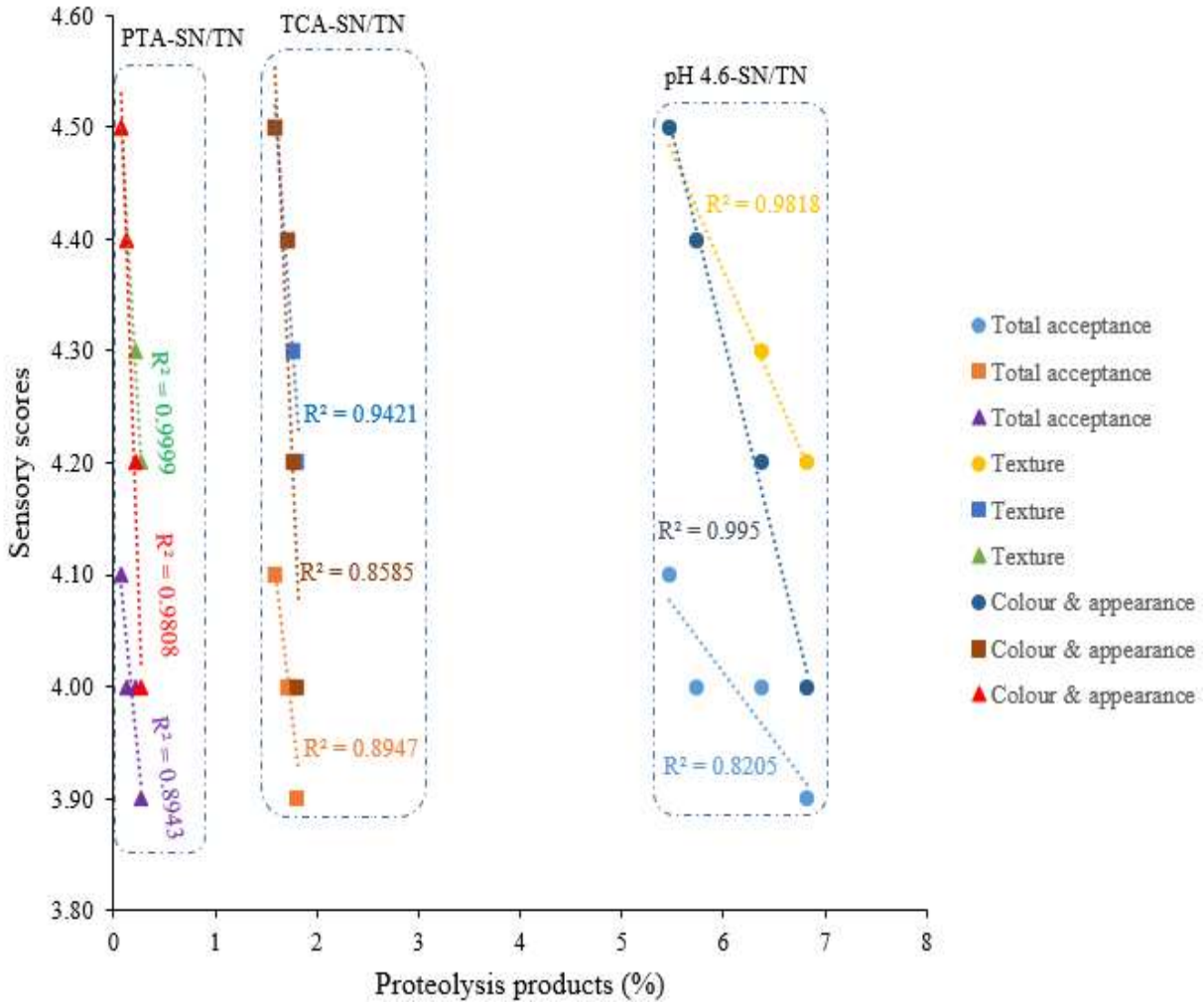

Fig. 1. Correlations between sensory scores (colour and appearance, texture and total acceptance) and proteolysis products (pH 4.6-SN/TN, TCA-SN/TN and PTA-SN/TN) of cheeses produced with SafeIt 2 starter culture.

Starter type, time of ripening and their interactions had no significant effect on aroma scores of cheeses (Table 2). The Hayaloglu et al. (2005) study also showed that the type of starter did not have a significant effect on the aroma scores of the cheese samples. Also, the results of the studies of Milci et al. (2005) and Miočinović et al. (2011) (up to 35 days of ripening) showed no significant differences for aroma scores of cheeses during ripening.

The flavor scores of cheeses were not significantly ($p>0.05$) influenced by starter type, ripening time and their interaction (Table 2). As it can be observed, the mean flavor scores of cheeses made with SafeIt 2 starters was higher than that of the cheeses prepared with R-704 and FRC-65 starter cultures. This could be due to the higher proteolytic activity of SafeIt 2 starters, leading to an increase in FAAs. Gomez et al. (1999) and Hayaloglu et al,. (2005) also did not report any significant impact of the starter type on the flavor scores of different cheeses. The effect of ripening time on the flavor scores of the cheeses was also not

found to be significant (Karami *et al,*. 2009a (from 20 d to 60 d of ripening), Ghods Rohani *et al,*. (2010) and Alonso *et al,*. (2011)). However, some studies showed a significant increase in flavor scores of different cheeses with ripening (Karimi *et al,*. (2012) (up to 60 d of ripening) and Shahab Lavasani *et al,*. (2012)).

As can be seen in Table 2, during the ripening period of 60 days, the total acceptance scores of cheeses produced from different starter cultures did not differ significantly ($p>0.05$). Similar to flavor scores, the mean total acceptance scores of cheeses produced with SafeIt 2 starters was also higher than that of the cheeses prepared with R-704 and FRC-65 starter cultures. In the study of Hayaloglu et al. (2005), no differences in total acceptance scores were also observed among the cheeses manufactured with different starter cultures. Karami et al. (2009a) found that the total acceptance scores of cheeses were not influenced by ripening time (from 20 d to 60 d), although these differences were significant in comparison to 3 d of ripening. In contrast to our findings, significant increase in total acceptance scores with time of ripening has been also reported ((Milci *et al,*. (2005); Karimi *et al,*. (2012) (up to 30 d of ripening) and Shahab Lavasani *et al,*. (2012)).

Good correlations were observed between sensory scores (color and appearance, texture and total acceptance) and proteolysis products (pH 4.6-SN/TN, TCA-SN/TN and PTA-SN/TN) of cheeses produced with SafeIt 2 starter culture (Figure 1), while no such correlations were found in the cheeses produced from the two other cultures (R-704 and FRC-65). Determination coefficients of mentioned attributes of cheeses containing SafeIt 2 starter were greater than 82%.

Conclusion

According to our results, pH, %salt, %protein and pH 4.6-SN were the only chemical characteristics that were affected by starter type and among them, pH 4.6-SN, as the main proteolysis product, was more important. However, the starter type had not significant effect on the sensory properties of cheeses, but the cheeses made with SafeIT 2, because of the presence of thermophilic and mesophilic strains and higher mean levels of proteolysis and lipolysis products, had higher sensory scores than those of cheeses produced from FRC-65 and R-704 cultures. Furthermore, high correlations (R^2= 0.8205- 0.9999) were found between some of the sensory properties and proteolysis products of cheeses produced from SafeIt 2 starter, while there were no such correlations for the cheeses containing R-704 and FRC-65. Therefore, for the manufacturing of UF-Feta cheese analogues, as the fresh unripened cheeses, the use of mixed commercial starter cultures contained combination of thermophilic and mesophilic strains are suggested instead of only mesophilic starter cultures. Ripening time, however, had no significant effects on %TS, %fat and %ash of cheeses, but the other chemical characteristics were influenced significantly ($p\leq0.05$). The sensory scores also were non-significantly reduced during ripening. From the results obtained in the present work, it may be concluded that the use of SafeIT 2 starter is preferred. It should be noted that the SafeIt 2 starter also has higher phage resistance stability than the two other starters.

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اثر کشت‌های آغازگر سویه‌مخلوط تجاری بر ویژگی‌های شیمیایی و حسی پنیر فتای فراپالایش آنالوگ طی دوره رسیدگی

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چکیده

پنیر فتای فراپالایش پنیری است که عمدتاً از شیر گاو تولید شده و معمولاً به‌صورت تازه و یا پس از یک دوره کوتاه رسیدگی (۶۰ روز) مصرف می‌گردد. در این پژوهش، اثر کشت‌های آغازگر تجاری (SafeIT 2، FRC - 65 و R- 704) و زمان رسیدگی (صفر تا ۶۰ روز) بر ویژگی‌های شیمیایی (مواد جامد کل، چربی، پروتئین، خاکستر، نمک، اسیدیته، pH)، بیوشیمیایی (ازت محلول در pH ۴/۶، اسید تری‌کلرواستیک و اسید فسفوتنگستیک، عدد اسیدی) و حسی (رنگ و ظاهر، آروما، بافت، طعم و پذیرش کلی) پنیر فتای فراپالایش آنالوگ بررسی گردید. بر اساس نتایج حاصله، نوع کشت آغازگر بر میزان pH، نمک، پروتئین و ازت محلول در pH= ۴/۶ نمونه‌های پنیر اثر معنی‌داری داشت ($P\leq 0.05$)، در حالی که سایر خواص شیمیایی تحت تاثیر معنی‌دار آن قرار نگرفت. اثر زمان رسیدگی نیز تنها بر میزان اسیدیته، pH، نمک، عدد اسیدی، پروتئین و محصولات پروتئولیز نمونه‌ها معنی‌دار بود. همچنین، کشت آغازگر و زمان رسیدگی بر خصوصیات حسی نمونه‌های پنیر، به‌جز رنگ و ظاهر، اثر معنی‌داری نداشتند، هرچند پنیرهای تولید شده از کشت آغازگر SafeIT 2 در مقایسه با نمونه‌های تولید شده از کشت‌های FRC- 65 و R- 704 امتیازات حسی بالاتری داشتند.

واژه‌های کلیدی: پنیر آنالوگ، باکتری‌های اسید لاکتیک، زمان رسیدگی، کشت آغازگر.

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بسم الله الرحمن الرحیم

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با شماره پروانه ۱۲۴/۸۴۷ و درجه علمی – پژوهشی شماره ۳/۱۱/۸۱۰ / ۸۸/۵/۱۰ از وزارت علوم، تحقیقات و فناوری

جلد ۱۷ شماره ۶ بهمن- اسفند ۱۴۰۰

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