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Microbiological Characteristics and Exopolysaccharides Yield of Yogurt Drink Stabilized with *Canna edulis* Ker Starch

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Original Research Article

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Abstract: The addition of stabilizer on the yogurt drink manufacturing affected the viability of *S. thermophilus* and *L.bulgaricus* as starter bacteria and could increase the exopolysaccharide yield of the final product. The effects of used Canna starch as a stabilizer in combination with carboxymethylcellulose (CMC) on microbiological characteristic and exopolysaccharide yield were investigated. The research design was used to complete randomized factorial design with two factor. Combination level of Canna starch/CMC levels added T0 (0.2%CMC) as a control, T1 (0.15% CMC + 0.025% canna), T2 (0.1% CMC + 0.05% canna), T3 (0.05% CMC + 0.075% canna), and T4 (0.1% Canna) as first factor and the storage time (1, 7, 14 and 21 days) as second factor. All data were analyzed by General Linear Model (GLM) and followed by Duncan's multiple range test (DMRT). This study resulted that yogurt drink stabilized with Canna starch has the better result than CMC on microbiological characteristic and exopolysaccharide yield. Yogurt drink samples stabilized with 0.2% Canna starch could maintain the viability of *L.bulgaricus* and *S.thermophilus* and could be resulted in the highest of exopolysaccharide yield.
Keywords: *Canna edulis* Ker, Exopoliscaharide, *Lactobacilus bulgaricus*, *streptococus thermophilus*.

INTRODUCTION

Yogurt drink is one type of fermented milk product that has been well known by Indonesian people. Health benefit that can be felt by the regularly consumed has a significant impact on the increase of products demand. Yogurt has promoting health benefits such as improving lactose digestion, reduce the cancer risk, lower blood cholesterol, gastrointestinal upsets protection and increase the immune respiratory [1]

The quality of yogurt drink should be well maintained. Addition of stabilizers has been commonly used in the process of yogurt manufacturing which has a purpose to maintain the quality of yogurt remains good while supplying the product to the consumer. Stabilizers were either made from chemical or natural compounds. However, recently many people prefer to apply the natural ingredients in the food product [2]

Canna edulis Ker plant was easy to cultivate in the tropical area mostly in southeastern Asia and southern China [3] . Canna rhizomes have long been used for food and as a source of commercial starch that contains 24.06% of amylose and 63.27% of amylopectin that the most useful function as a hydrocolloid. The application of canna as natural hydrocolloid on yogurt drink production have been studied [4] that 0.1% of Canna starch resulted in similar sensory quality compared with CMC.

The addition of stabilizer on the yogurt drink manufacturing was possible to increase the viability of *S. thermophilus* and *L.bulgaricus* as starter bacteria [2]. The viability of starter bacteria has a positive correlation with EPS yield of yogurt drink because LAB has the capability of producing exopolysaccharide. Therefore, this research aimed to investigate the microbial characteristic and EPS yield of yogurt drink stabilized with Canna starch.

MATERIALS AND METHODS

Yogurt drink was manufactured in Dairy Laboratory of Animal Science Department, National Pingtung University of Science and Technology, Taiwan.

Canna Starch Preparation

Indonesian Red *Canna edulis* Ker (aged 5-10 months) were purchased from local farmers in Malang, Indonesia. According to [5], Canna tubers washed, cut, then treated for 12 h with 1000 ppm of $Na_2S_2O_5$. The pieces of Canna were crushed and mixed with the ratio of 1:2 water, then squeezed for resulted solid fractions. The solid was added with 1:1 of water then precipitated for 12 hours. The formed starch was dried at 60°C for 12 hours, then milled and sieved with an 80 mesh to obtain powdered starch.

Preparation of yogurt drink

Sample was prepared into 5 group treatments including T0 (Raw cows milk + 15% skim milk powder (Fonterra Co., Auckland, New Zealand) + 0.2% CMC), T1 (Raw cows milk + 15% skim milk powder (Fonterra Co., Auckland, New Zealand) + 0.15% CMC + 0.025% Canna starch), T2 (Raw cows milk + 15% skim milk powder (Fonterra Co., Auckland, New Zealand) + 0.1% CMC + 0.05% Canna starch), T3 (Raw cows milk + 15% skim milk powder (Fonterra Co., Auckland, New Zealand) + 0.05% CMC + 0.075% Canna starch), and T4 (Raw cows milk + 15% skim milk powder (Fonterra Co., Auckland, New Zealand) + 0.1% Canna starch). Samples had been mixed thoroughly, then heated at 85°C for 30 min and cooled to 43°C. 2% v/v of yogurt starter culture including *S. thermophilus* 14086 and *L. Bulgaricus* 12297 (Chr. Hansen, Denmark) was inoculated into the sample. Acidified at 37°C until pH 4.6 was reached. Mixed with 1000 mL of 10 g/ 100 ml (w/ v) sugar solution, kept in the refrigerator at 4°C for 21 days then microbiology and exopolysaccharide yield were analyzed.

Microbiological characteristic

Microbiology analysis divided into three parts of methode such as viability of *Lactobacillus bulgaricus*, viability of *Streptococcus thermophilus*, and total yeast and mold.

Enumeration of *Lactobacillus bulgaricus* using the pour plate method

Lactobacillus bulgaricus was enumerated according to method of [6]. MRS agar was prepared by mixing MRS powder with water 62 g/ 1 L distilled water (Merc KgaA, Darmstadt, Germany). Agar was autoclaved followed by cooling to 45^{0}C. 1.0 mL of yogurt diluted (10^{-6}, 10^{-7}, and 10^{-8}) with sterilized NaCl was placed in a petri dish. Added 15 mL MRS agar then mixed by gently tilting and swirling the dish. The plates were left at room temperature until solid. Thereafter, the plates were inverted and placed in the incubator (37^{0}C) for 48 hours.

Enumeration of *Streptococcus thermophilus* using the pour plate method

Streptococcus thermophilus was calculated according to method of [6] . M17 agar was prepared by mixing M17 powder with water 37.25 g/ 950 mL distilled water (Becton Dickinson Co., 2284923; Sparks, USA). Agar was autoclaved followed by cooling to 45^{0}C then mixed with 50 mL of 10% w/ v sterilized lactose solution (Nacalai Tesque Inc., Kyoto, Japan). 1.0 mL of yogurt diluted (10^{-6}, 10^{-7}, and 10^{-8}) with sterilized NaCl was placed in a petri dish. Added 15 mL M17 agar then mixed by gently tilting and swirling the dish. The plates were left at room temperature until solid. The plates were inverted and placed in the incubator (37^{0}C) for 48 hours.

Enumeration of yeast and mold using the pour plate method

Yeast and mold were calculated according to method of [6] . Potato Dextrose Agar (PDA) was prepared by mixing PDA powder with water 39 g/ 1 L distilled water (Merc KgaA, VM746630632; Darmstadt, Germany). Agar was autoclaved followed by cooling to 45^{0}C. 1.0 mL of yogurt diluted (10^{-1}, 10^{-2}, and 10^{-3}) with sterilized NaCl was placed in a petri dish. Added 15 mL PDA agar then mixed by gently tilting and swirling the dish. The plates were left at room temperature until solid. The plates were inserted in the incubator (25^{0}C) for 72 hours.

Viability of *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, yeast, and mold were calculated as follows:

$$\text{CFU/mL} = \frac{\text{Number of colonie formed} \times \text{dilution factor of sample}}{\text{1.0 mL of sample}}$$

*CFU = Colony Forming Unit

Determination of Exopolysacharide yield

Exopolysaccharides (EPS) were extracted according to the method of [7] with slight modifications. First step was protein precipitated, an amount of 10 g yogurt sample treated with 20% trichloroacetic acid, then centrifuged at 2500 rpm at 4°C for 30 minutes (Hettich Centrifuger Universal 320R, Germany). The supernatant collected after centrifugation was treated with 95% absolute ethylic alcohol (1: 3) and left overnight at 4°C then centrifuged again at 2500 rpm at 4°C for 30 minutes. EPS precipitate was redissolved in distilled water and dialyzed with seamless cellulose tubing (Molecular Weight Cut Off of 13 kDa; Viskase Companies, Inc., USA) at 4°C for 24 hours against distilled water. The crude was diluted with distilled water to 50 mL in order

to determine the EPS yield by phenol-sulphuric acid method.

The quantitative determination of the EPS was made using the colorimetric phenolsulphuric method with glucose standard for total sugar dosage. The glucose standard curve was prepared, 100.0 mg glucose was dissolved in water to make 100 mL. Then 20, 30, 40, 50, 60, 70, 80, and 90 mg/ L glucose solution were made by diluting with distilled water to 2.0 mL, respectively. 1.0 mL of phenol solution 5% (v/ v) and 50 mL of sulphuric acid 95 % (v/ v) were added quickly and shake up. After 10 minutes standing, then measured using WPA Spectrawave – S800 Diode Array Spectrophotometer (Biochrom, Ltd., England) set to 490 nm of absorbance. 2.0 mL of distilled water used as a blank in the same conduct. The standard curve was drawn with concentration of EPS as the horizontal axis and absorbance values as the vertical axis (Figure 1).

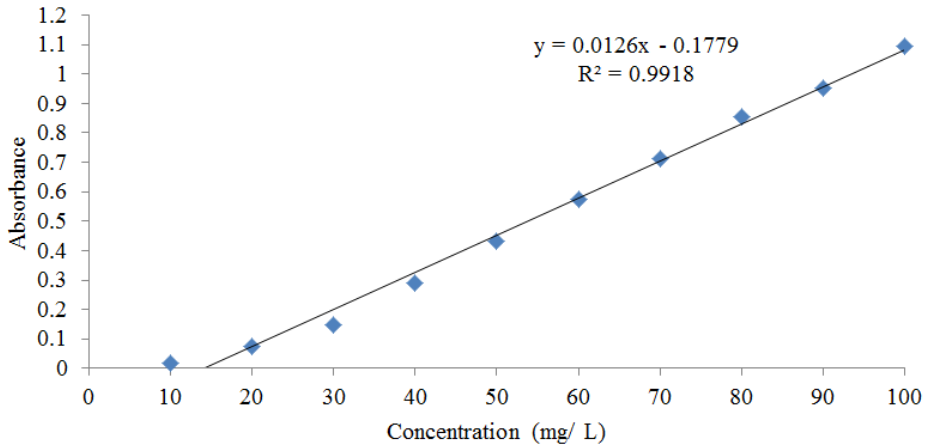

Fig-1: Glucose standard curve for EPS yield determination

For EPS yield determination, accurately 1000µL of EPS samples were added quickly with 1.0 mL of 5 % phenol solution and 5.0 mL of sulphuric acid 95 % (v/v) and shake up after 10 min standing, and then absorbance at 490 nm was measured. The concentration of EPS was determined in triplicate and 2.0 mL distilled water used as blank. The EPS content of each sample was calculated by the standard curve.

STATISTICAL DATA ANALYSIS

The data obtained from microbiological and exopolisacharide analyses of yogurt sample were analyzed using General Linear Model (GLM) of SPSS 1.6 software. When the data had a significant interaction, statistical analysis will continued by Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Microbiological characteristics

Viability of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*.

The results of *L. bulgaricus* viability in this experiments indicated that there was a significant difference ($P<0.05$) between the addition level of CMC and Canna starch (Figure 2). Total viability counts at the beginning of storage (8.21 ± 0.05 – 8.32 ± 0.03) log CFU/ mL and after 21 days of storage (7.51 ± 0.15 – 7.94 ± 0.31) log CFU/ mL, whereas increase was observed on 7th day (8.19 ± 0.26 – 8.66 ± 0.09) log CFU/ mL. The addition of 0.15% CMC + 0.025% Canna (T1) resulted the initial number of 8.23 ± 0.01 log CFU/ mL then significantly increased to 8.66 ± 0.09 log CFU/ mL after 7 days. Nevertheless, the addition of 0.1% Canna (T4) showed the stable result compared to the other treatments group with the highest average viability values of 8.27 ± 0.26 log CFU/ mL over 21 storage day. The addition of Canna starch was provided high levels of *L. bulgaricus* over 21 days cold storage period. It may cause by antioxidative properties of Canna starch that has the capability to reduce the redox potential (Eh) of yogurt and improved the viability of *L. bulgaricus.*

According to [8] when antioxidants such as cysteine or ascorbic acid were added, it will decrease the redox potential (Eh) of yogurt and increase the *L. bulgaricus* and *S. thermophilus* viability. Plant extract (PE) as a low-cost antioxidant have the capability to decrease the redox potential (Eh), increase the longevity, and improve the viability of anaerobic bacteria such as *L. bulgaricus* in yogurt product [9].

On the other hand, carbohydrate content (97.88%) in Indonesian native Canna starch [10] was affected the organic acid concentration of yogurt during storage [11]. stated that organic acids are the important factors of bacterial growth, when the level of organic acid was increased it will cause the reduction of *Lactobacillus spp* . The previous study by [6] explain that refrigerated storage of yogurt added with plant extract were significantly decreased the viable counts of *Lactobacillus* on 14th-day storage period [12], have

explained the interaction between *L. bulgaricus* and *S. thermophilus*, several amino acids such as histidine, glycine, valine, isoleucine, and leucine were produced by *L. bulgaricus* to stimulate the growth of *S. thermophilus*. While *S. thermophilus* produced formic acid that has a function as the growth stimulation of *L. bulgaricus*.

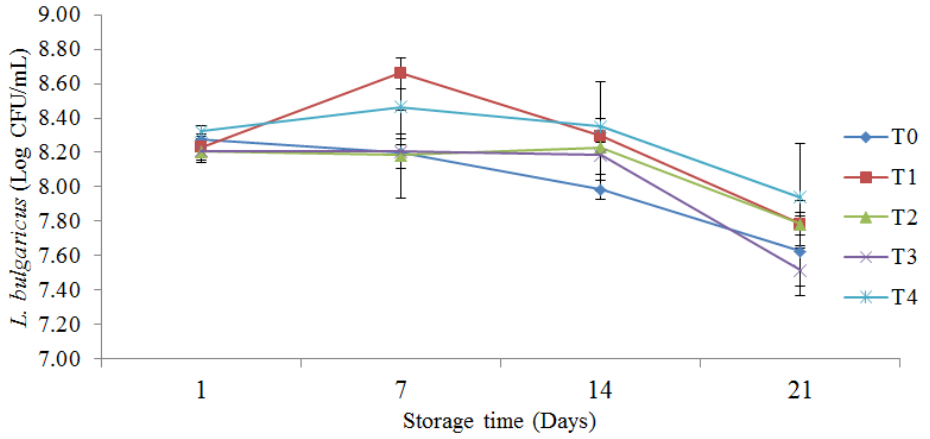

Fig-2: Interaction between addition level of Canna starch and CMC on L. bulgaricus viability during storage (p<0.05). Error bars represent standard deviation

Note : T0 (0.2% CMC), T1 (0.15% CMC + 0.025% Canna), T2 (0.1% CMC + 0.05% Canna), T3 (0.05% CMC + 0.075% Canna), and T4 (0.1% Canna). Samples were stored at 4^{o}C.

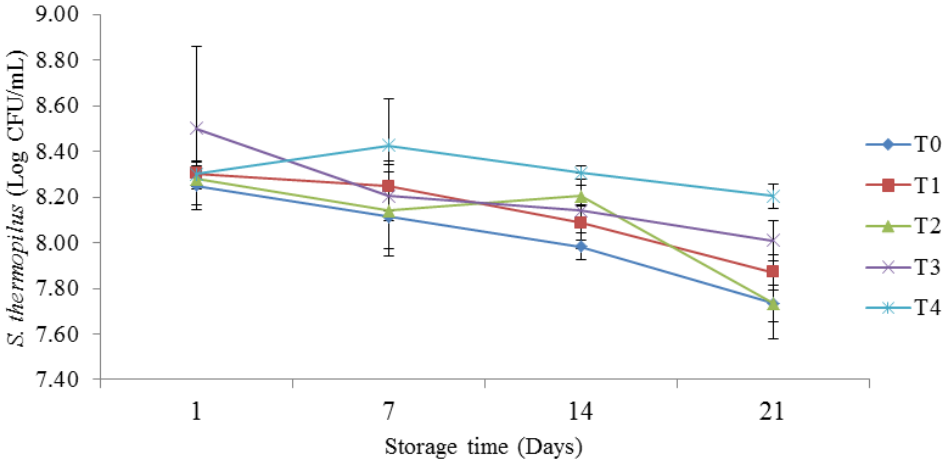

Fig-3: Interaction between addition level of Canna starch and CMC on *S. thermophilus* viability during storage (p<0.05). Error bars represent standard deviation

Note : T0 (0.2% CMC), T1 (0.15% CMC + 0.025% Canna), T2 (0.1% CMC + 0.05% Canna), T3 (0.05% CMC + 0.075% Canna), and T4 (0.1% Canna). Samples were stored at 4^{o}C.

S. thermophilus viability was significantly ($p<0.05$) affected by the additional level of Canna starch and CMC, and storage time (Figure 3). Yogurt drink with the addition of 0.05% CMC + 0.075% Canna starch (T3) resulted in the highest *S. thermophilus* counts (8.50 ± 0.36) log CFU/ mL at the beginning of the storage period then slowly decreased. *S. thermophilus* counts of yogurt added with 0.1% Canna starch (T4) tended to increase until day 7 of storage with the value (8.43 ± 0.20) log CFU/ mL then gradually decreased. The different trend of *S. thermophilus* counts was showed in yogurt with 0.1% CMC+0.05% Canna starch (T2). The viability of *S. thermophilus* decrease at 7 days then significant increase until 14 days (8.20 ± 0.05) log CFU/ mL, however significantly drop was happen at the end of storage (7.73 ± 0.15) log CFU/ mL.

The addition of Canna starch was presented sufficiently high levels of yogurt starter over 21 days cold storage period. It is hypothesized that Canna starch as complex carbohydrates was a good source for starter bacteria growth and metabolism. That is in agreement with [13] a potential source of prebiotic components was found in several complex carbohydrates such as sucrose, raffinose, stachyose, verbascose, oligosaccharides, and resistant starch. On the other hand, the chemical composition of Canna starch was

also contributed to the starter bacteria viability, data from Indonesia Directorate of Nutrition (1981) showed that Canna starch contains the high concentration of sodium, phosphorus, magnesium, potassium, iron, zinc, and calcium. The addition of minerals, vitamins, and amino acids will stimulate the growth of starter cultures in milk [14].

The experiment results are similar with [15] *S. thermophilus* viability of yogurt added with Korean traditional plant extracts showed to increase until 14 days of storage then decrease on the 28 days as final storage time, *S. thermophilus* showed more sensitive to the amount of lactic acid during the storage. According to [16] *S. thermophilus* growth and metabolism in yogurt were inhibited when stored over 3 and 4 weeks in cold temperature with pH condition around 4.3-4.5.

The experiment results of all treatment group showed that 7th and 14th storage days resulted the higher *L. bulgaricus* (LB) value than *S. thermophilus* (ST). The ratio value of Lb and St during 7 days storage were (8.20 : 8.11) log CFU/ mL (T0), (8.66 : 8.25) log CFU/ mL (T1), (8.19 : 8.14) log CFU/ mL (T2), (8.21 : 8.20) log CFU/ mL (T3), and (8.46 : 8.43) log CFU/ mL (T4) respectively. Wherease 14 days storage resulted the ratio value of Lb and St (7.98 : 7.98) log CFU/ mL (T0), (8.29 : 8.09) log CFU/ mL (T1), (8.23 : 8.20) log CFU/ mL (T2), (8.18 : 8.14) log CFU/ mL (T3), and (8.35 : 8.31) log CFU/ mL (T4) respectively.

There was a significant difference of interaction between treatment group on storage time ($p<0.05$), the addition of Canna starch with a different percentage will maintain the *L. bulgaricus* and *S. thermophilus* viability of yogurt drink over 21st storage days. Duncan Multiple Range Test (DMRT) at 5% significance level performed, yogurt drink added with 0.1% Canna starch with an average value of *L. bulgaricus* and *S. thermophilus* were 8.27 ± 0.26 and 8.31 ± 0.12 log CFU/ mL selected as the best treatment

Yeast and mold counts

All the yogurt samples showed no significant ($p>0.05$) difference in yeast and mold count (Table 1). Yeast and mold count of yogurt samples ranged from 0.00 to 2.40 log CFU/ mL. Yeast and mold were not detected at the beginning of storage until 7th days, respectively. Yogurt with the addition of 0.1% Canna starch (T4) had the lowest average yeast and mold count, over 21 storage days. However, the highest average yeast and mold count was showed by yogurt with the addition of 0.15% CMC + 0.025% Canna starch (T1). It can be seen clearly from Table 1 that the average counts of yeast and mold significantly increased ($p<0.05$) by 0.96 log CFU/ mL and 1.65 log CFU/ mL on 14 and 21 days of storage period, respectively.

The yeast and mold results in this experiment were related to the higher syneresis rate and lower pH. The increasing of syneresis rate indicated the higher water activity that is become a potential growth condition for yeasts and molds. According to [17] low pH has become the suitable conditions for the growth of yeasts that a major caused of spoilage of yogurt and fermented milk. According to [18] the reduction in potential oxygen and increase in acidity during post acidification process could be presented suitable growth condition for yeasts and molds then directly affected to increase the yeasts and molds population.

On the other hands, the added sugar in the manufacturing process of yogurt drink of this experiment could cause the yogurt easier to contaminated by yeast during storage. Yeast in yogurt can ferment sucrose and lactose as the major carbohydrates presence under refrigerated conditions. The common yogurts contained lactose around 4%, and around 5 and 10% sucrose concentration for fruit and flavored yogurt [19]. Sugar that added in this experiment contained glucose that could be fermented by yeast. According to [20] manufacturers sometimes use the invert sugar that contains glucose and fructose, while bacterial metabolism of lactose in milk may result in the small amounts of galactose. These three sugars, therefore, could also act as fermentable substrates for yeast growth.

Lactose is commonly sugar that available in yogurt. *Kluyveromyces marxianus var. lactis* or *K. marxianus var. Marxianus* is several kinds of yeast that could ferment the lactose. Both of these yeast species were easier to grow on the surfaces of uncleaned dairy industry equipment. Therefore, preventing contamination from yeast and mold can be done with applying the high hygiene standards. It is recommended that yogurt sales on the market should have a yeast and molds count less than 10 cfu/ g^{-1}. Some regulations already allowed the application of sorbic acid in yogurt product to prevent the growth of yeasts. Sorbic acid usually added as potassium sorbate with the level up to 300 mg/ kg^{-1} in fruit yogurt [21].

Table-1: Interaction between combination addition level of Canna starch and CMC on yeast and mold count during storage

Storage (Days)	Yeast and mold counts (Log CFU/mL)					Mean storage times
	T0	T1	T2	T3	T4	
1	ND	ND	ND	ND	ND	0.00 ± 0.00^{a}
7	ND	ND	ND	ND	ND	0.00 ± 0.00^{a}
14	0.87 ± 0.75	1.26 ± 0.24	1.16 ± 0.28	1.16 ± 0.28	0.33 ± 0.58	0.96 ± 0.53^{b}
21	2.08 ± 2.57	2.40 ± 1.53	1.06 ± 0.92	1.75 ± 1.74	0.97 ± 0.85	1.65 ± 1.51^{c}
Mean treatment	0.74 ± 1.45	0.92 ± 1.24	0.56 ± 0.71	0.73 ± 1.09	0.33 ± 0.60	

Note : T0 (0.2% CMC), T1 (0.15% CMC + 0.025% Canna), T2 (0.1% CMC + 0.05% Canna), T3 (0.05% CMC + 0.075% Canna), and T4 (0.1% Canna). Samples were stored at 4oC. Mean in the same row with different superscripts differ significantly (p>0.05).

There was no significant difference of interaction between treatment group on storage time (p>0.05), it means that the addition of Canna starch with a different percentage level did not affect to the yeasts and molds count of yogurt drink over 21st storage days.

Exopolysaccharides Yield (EPS)

The changes in exopolysaccharides (EPS) over 21 days of storage was presented in Figure 4. The initial EPS yield of all treatment group resulted in similar value was 81.19 mg/ L (T0), 80.71 mg/ L (T1), 82.48 mg/ L (T2), 80.92 mg/ L (T3), and 83.83 mg/ L (T4) respectively. Yogurts drink with the addition of Canna starch had significant increased (p<0.05) EPS yield on 7th and 14th storage days. During 7th storage days, EPS yield of the sample with Canna starch added was increased 17.85% (T1), 15.48% (T2), 21.12% (T3), and 19.39% (T4) were compared to control group that only increased 10.12%. While the highest EPS yield of all treatments were shown on 14 days. The value of each treatment were significantly increased with the higher Canna starch added that increased by 7.33 % to 95.96 mg/ L (T0), 7.90 % to 102.62 mg/ L (T1), 9.73 % to 104.52 mg/ L (T2), 18.06 % to 115.71 mg/ L (T3), and 25.16 % to 122.40 mg/ L (T4). However, at the end of storage days, there was not much change in comparison with 14 days for all treatment group.

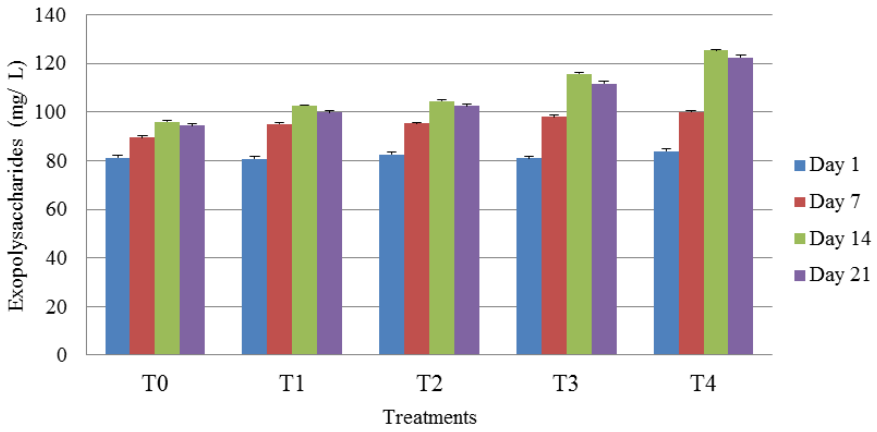

Fig-4: Interaction between addition level of Canna starch and CMC on exopolysaccharides yield during storage (p<0.05). Error bars represent standard deviation

Note : T0 (0.2% CMC), T1 (0.15% CMC + 0.025% Canna), T2 (0.1% CMC + 0.05% Canna), T3 (0.05% CMC + 0.075% Canna), and T4 (0.1% Canna). Samples were stored at 4°C.

The higher EPS yield of the yogurt drink with Canna starch added was related to the high amount of LAB. The carbohydrate content of Canna starch has a positive correlation with LAB growth and metabolism. Starch is the primary storage of polysaccharide that will affect the carbohydrate fermentation capability of lactic acid bacteria for produced exopolysaccharides. Indonesian native Canna starch contained 97.88% of carbohydrate (10)(Carolina and Ilmi, 2016).

According to [22] EPS characteristics produced by LAB such as EPS yield, rheological properties, structure, and monosaccharide composition were varied depends on LAB strain. The several factors such as pH, fermentation time, temperature, and growth medium influence the EPS synthesized by LAB [23]. *Streptococcus thermophilus* strains produce EPS from 50 to 350 mg/ L^{-1} with the optimum condition at 42°C and 6.8 of pH [24] and 45°C of temperature and pH 6.2 for Lactobacillus strains [25]. EPS with acidic polysaccharides that produced by *L. bulgaricus* OLL1073R-1 in yogurt resulted in advantageous immunological such as cold prevalence reduction,

influenza virus infections reduction, and enhancement the lymphocyte mitogenicity [26].

There was a significant difference of interaction between treatment group on storage time ($p<0.05$), the addition of Canna starch with a different percentage level will increase the EPS yield of yogurt drink over 21st storage days. The EPS yield was related to the yogurt drink stability during storage when the higher Canna starch was added, higher EPS yield will improve the viscosity and reduce the syneresis. Final product stability, mouthfeel, taste perception, and texture were significantly affected by EPS production [27]. EPS called as biopolymers may have the capability to improve the fermented milk texture and consistency [28] and play an essential role of syneresis prevention [29]. The EPS yield, EPS structure and EPS interactions with caseins micelles directly effect to rheological characteristics of the product [30].

CONCLUSION

Yogurt drink stabilized with Canna starch has the better result than CMC on microbiological characteristic and exopolysaccharides yield. Yogurt drink samples stabilized with 0.2% Canna starch showed could maintain the viability of *L.bulgaricus* and *S.thermophilus* while 0.2% of canna starch also resulted in the highest of exopolysaccharides yield.

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