Potency of Chitosan and Chitooligochitosan (COS) from Marine Shrimp Shells as Prebiotics for *Streptococcus thermophillus* and *Lactobacillus bulgaricus* Probiotics

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Abstract

Lately, chitosan as a result of chitin deacetilation has known as potencial compound as food industry, pharmacy and environmental agents. The weakness of chitosan is the low water solubility and high viscosity. The size reduction by cutting the β-1, 4 glicosidic bond to improve the bioactivity is the alternative solution. This research aims to find out the addition of chitosan and COS for the Streptococcus thermophillus FNCC – 0041 and Lactobacillus bulgaricus FNCC – 0040 probiotics bacteria culture. S. thermophillus and L. bulgaricus were cultured in the MRS Broth media with addition of Commercial Chitosan (CC), Commercial Oligochitosan (COC), Self-production Chitosan (PC) and Self-production Oligochitosan (POC) in different doses (0.05; 0.1; and 0.2 mg.ml^{-1}). Percentage DD on those treatments was similar. The spectra vibration of The FT-IR analysis of PC and POC were fit to the CC and COC. In chitosan treatments, the best growth of S. thermophilus was reached at 0.2 mg/mL PC, while in chitooligochitosan was from 0.20 mg/mL COC treatments, respectively. The addition of different type of chitosan have a significant effect ($p<0.05$) to the growth of S. thermophillus, but have not resulted the significant effect to the L. bulgaricus ($p>0.05$) growth. So, therefore the addition of chitosan and COS as prebiotics for the probiotics were in dose dependant manner. Compare to chitosan commercial, our chitosan production have a good potency to be developed.

***Keywords**: chitosan, oligochitosan, prebiotics, probiotics*

INTRODUCTION

Chitosan is generated from chitin deacetilation that produce some good benefits due to its physicochemistry and biological aspects (Islam *et al*., 2020) in terms of food industry, pharmacy and environemental agent (Arias *et al*., 2018). The weakness of this compound is due to the low water solubility with high viscosity (Tanasale *et al*., 2019). The common strategy is by cutting the β-1, 4 glicosidic bond (Akbari-Alavijeh *et al*., 2020) and the product called chitooligosaccharide (COS).

Poeple used to get COS by physical treatments such as thermal heating (Yudiati *et al*., 2018; Rizfa *et al*., 2020) enzimatic methods (Lee *et al*., 2008) and chemical reagents such as Hidrogen peroxide (H_2O_2) (Qin *et al*., 2002; Rizfa *et al*., 2020). Tanasale *et al*. (2019) reported that COS product by using H_2O_2 has the molecular weight of 4.2 x 10^3. The degree of polimerisation was around 2 – 20 monomers (Liaqat & Eltem, 2018) with higher bioactivity (Xia *et al*., 2011).

Research by Lee *et al*. (2002) revealed that *Lactobacillus* sp. and *Bifidobacterium bifidium* growth were stimulated by enzymatic COS, pursuing that COS had a prebiotic function in order to support acid lactic bacteria growth (Ismail *et al*., 2020). Furhtermore, Soleimani *et al*. (2017) was also supported this facts. They claimed that COS was triggered the prebiotics growth. Rizfa *et al*. (2020) stated that alginate oligosaccharide (AOS) depolimerization with H_2O_2 was able to

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improve the antioxidant activity. While, Yudiati *et al*. (2020) was also reported that AOS fulfilled the needs of probiotic commercial bacteria to grow. On the other hand, the exploration of polimerized COS with H_2O_2 had not been fully explored yet. Based on this fact, the study on the chitosan and COS potency in different doses and the relation on *S. thermophillus* dan *L. bulgaricus*. growth were conducted.

MATERIALS AND METHODS

Commercial chitosan originally from shrimp shells' waste were obtained from *Chitosan Shrimp Shell*, Monodon Group Bogor. *Lactobacillus bulgaricus* FNCC – 0041 and *Streptococcus thermophilus* FNCC – 0040 were purchased from Microbiology Laboratory, Center of Food Study, Gadjah Mada University.

Shrimp shell was diluted in 3.5% NOH at 10:1 (V/W) and followed by heating up at 65°C (2 hrs), and cooled down. The solution was then rinsed with aquadest until pH neutral. The samples was then dried at 100°C. The deproteinated sample was then diluted with 1 N HCl at 15:1 (V/W) and continued by heated up for 30 mns. This then followed by rinsing with aquadest (neutral pH) and was dried up for 100°C to reach the constant weight (Tanasale *et al*., 2019).

Chitosan extraction was done by diluted chitin in 60% NaOH ini 20:1 (V/W) for 6 hrs at 80-100°C. The neutral filtrate was then filtered and the dried up at 100°C to get the constant weight. The depolimerisation was conducted by this procedur. Four grams of chitosan was diluted in 200 mL of 0.5% acetic acid and 10 mL of H_2O_2 10% was then added. The solution was mixed and reacted at 100°C (2 hrs) and were cooled and managed until pH 7.0 by adding NaOH 10 N until the hidrolysate gel has performed (Tian *et al*., 2004). Precipitated was then conducted by adding alcohol isopropyle (3:1). The pellet was then dried up in the oven at 40°C for the constant weight (Uju *et al*., 2018). The dried chitosan was then grounded with mortar.

The confirmation of chitosan and COS charaterisation was done by Fourier Transform Infrared Spectrophotometer (Tanasale *et al*., 2019). Chitosan deacetylation (DD) was determined by base line methods based on this following equation (Khan *et al*., 2002).

Lactobacillus bulgaricus FNCC–0041 and *Streptococcus thermophilus* FNCC–0040 was suspended in MRS *Broth* (*de Man, Rogosa and Shape Broth*) media and incubated for 48 hrs at 37°C.

The effectiveness of prebiotics (chitosan and COS) was done by observing the growth of probiotic bacteria in the MRS Broth media with chitosan and COS addition. The 2 mL bacteria suspension (10^6 cfu/ml) was put into erlenmeyer. Each erlenmeyer was completed with magnetic stirrer and 40 mL of MRS Broth containing 0.05; 0.1 and 0.2 mg/ml chitosan and COS (Yang *et al*., 2011). This then followed by taking 5 mL bacterial suspension aseptically at 0, 5, 10, 15, 20, 25, dan 30 hrs and then cultured in MRS Agar (*de Man, Rogosa and Shape Agar*) to get the total plate count. The bacterial specific growth rate (µ/hari) was determined at logarithmic phase based on this formula (Pramono *et al*., 2003)

The bacterial growth peak was determined based on the observation of exponentialy phase until dead phase with linear regression Y = ax + ab (Rosmania & Yanti, 2019). Data analysis was done by ANOVA with 5% level of sinificance. The analysis was using Microsoft Excel 2019 software to find out the influence of chitosan and COS to the cultured organisms.

RESULTS AND DISCUSSION

One of Chitsan and COS Charaterisation is the deacetyltion degree. Generally, the deacetylation degree (DD) of CC, PC and COC, POC were similar i.e. 77.5%, 85% and 64%, 62.5%, respectively (Figure 1).

The percentage of DD ini self-production and commercial chitosan were in the range of permittable chitosan, which is >70% (Dompeipen *et al*., 2016). Our production of chitosan was only left of 22.5% of acetyl group. The analysis of FT-IR in all treatments showed the similar signal vibration

in 400 - 4000 cm^{-1} wave number. There were the existence of O-H stretching at 3430 cm^{-1}, C-H aliphatic (2919,69 cm^{-1}), N-H groups at 1618,65 cm^{-1} and C-O streching (1088,23 cm^{-1}). The characterisation of functional groups is presented in Figure 2 and Table 1.

The oxidative degradation in depolimerization process using hydrogen peroxide was enable to cut off the polychain polysaccharide in random ways and lead to the degradation of particle size. So, therefore, the production of new compound with lower molecular weight was appeared (Tian *et al.*, 2004, Rizfa *et al*., 2020). The thermal heating application, in fact, speed up the hydrogen peroxyde decomposition which produce hydrogen ions and anion hydroxyle. Those ions will attempt for further reaction and producing the highly reactive hycroxyle forms (OH) (Qin *et al.*, 2002).

The effect of different concentration of CC in *S. thermophillus* FNCC – 040 and *L. bulgaricus* FNCC – 041 growth can be seen in Figure 3 a and b. Meanwhile, the effect of PC in the growth of *S. thermophillus* and *L. bulgaricus* is presented in Figure 4 a and b.

The effect of different concentration of commecial oligochitosan (COC) in *S. thermophillus* and *L. bulgaricus* growth can be seen in Figure 5 a and b. While, the effect of self production oligochitosan (POC) in the growth of *S. thermophillus* and *L. bulgaricus* are presented in Figure 6 a and b.

From those figures, it can be seen that growth of two lactic acid bacteria was normal and expressed in quadratically curve which start as a lag, continued to exponentially, stationary phase and lastly with dead phase. The statistical analysis confirmed that that the addition of CC, PC, COC and POC in two different lactic acid bacteria reached the different results. The growth of *S. thermophillus* was significantly different ($p<0.05$), while *L. bulgaricus* was non significantly different. This facts is also denoted in Table 2.

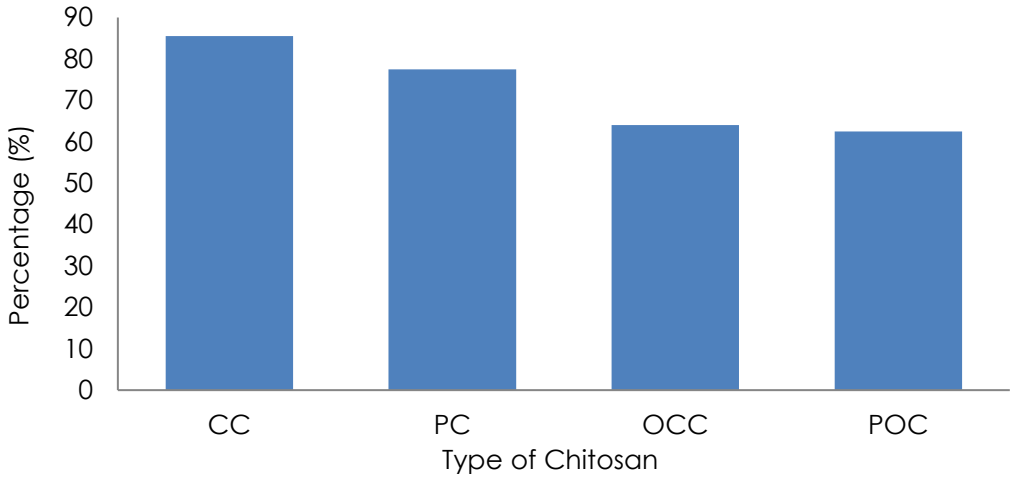


Figure 1. The deacetylation degree of different type of chitosan and COS

Table 1. Characterisation of different type of chitosan

Sampel	Wavenumber cm^{-1}			
	O-H *stretching*	C-H aliphatic	N-H	C-O *stretching*
CC	3405.5	2953	1639.85	1073.49
COC	3444.02	2899	1633.78	1071.36
PC	3430.68	2919.69	1618.65	1088.23
POC	3406.23	2927.02	1631.28	1072.68

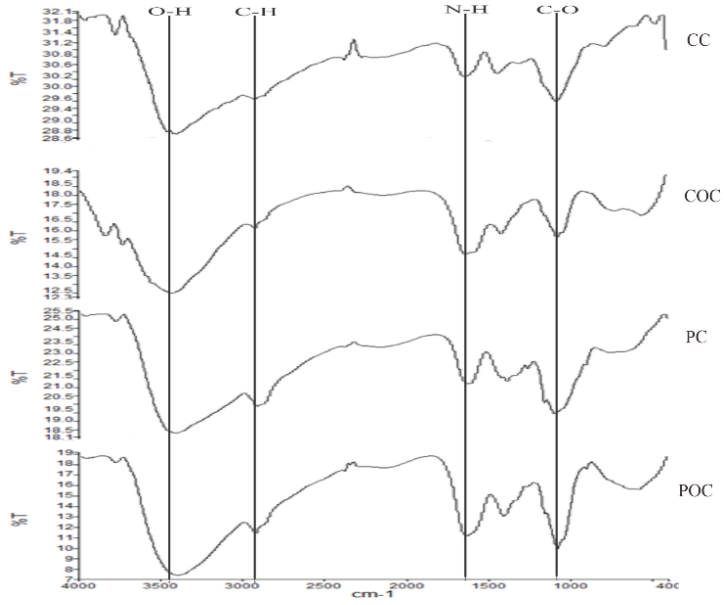
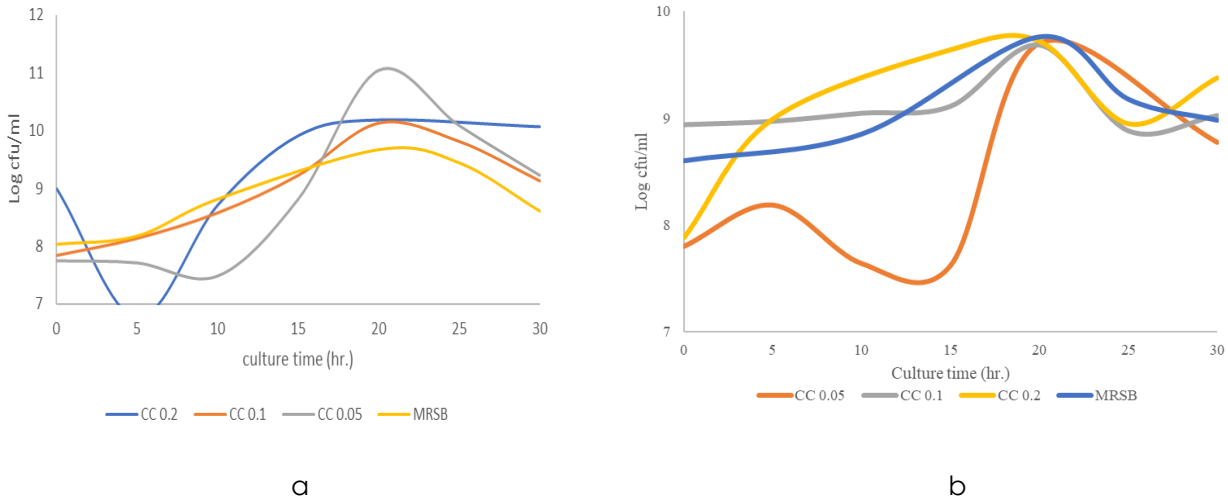


Figure 2. FT-IR spectra vibration of different type of chitosan (400-4000 cm^{-1})



a b

Figure 3. (a) Growth of S. thermophillus FNCC – 040 and (b) L. bulgaricus FNCC – 041 in different concentration of commercial chitosan

The Effect of Chitosan on the Growth of *S. thermophillus* and *L. bulgaricus*

Chitosan is a low solubility compound and its application for probiotics was done by fermentation process from gut microflora (Tang *et al*., 2020). Soleimani *et al*. (2017) stated that the chitosan application at 6.5 mg/mL had a good potency as prebiotics compounds due to the ability of *Lactobacillus* to grow. Additionally, Asim *et al*. (2020) pursuing that the antibacterial activity of chitosan influence by some factors such as the type of chitosan, the deacetialation degree, pH and other physicochemically characters. Chitosan was able to immobilise the bacteria by providing the nutritional requirements and as a matrix. This will keep the viability of bacteria (Oktarina *et al.* (2017). Our present results from Figure 3-6 and Table 2, supported this finding, though, in detail, the results from different treatments show a slight differences.

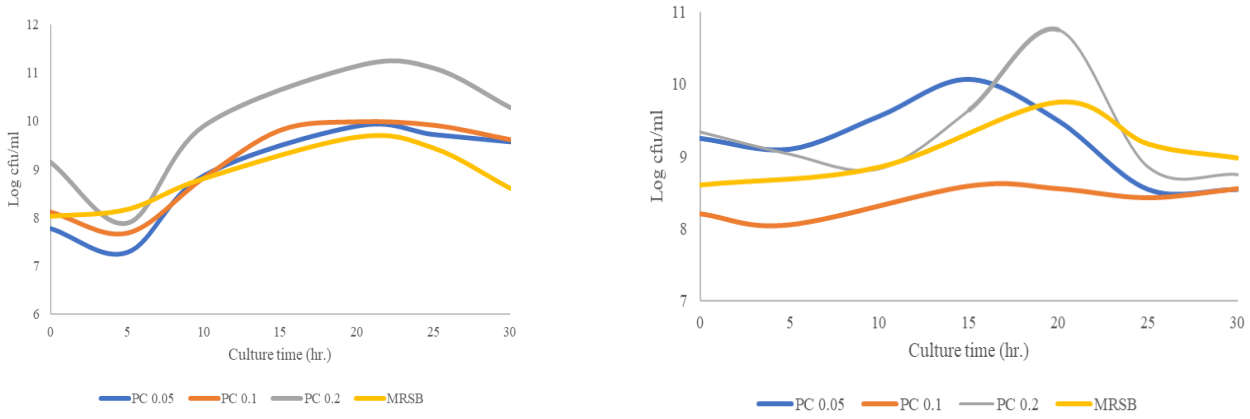


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Figure 4. (a) Growth of *S. thermophillus* FNCC – 040 and (b) *L. bulgaricus* FNCC – 041 at different concentration of self-production chitosan

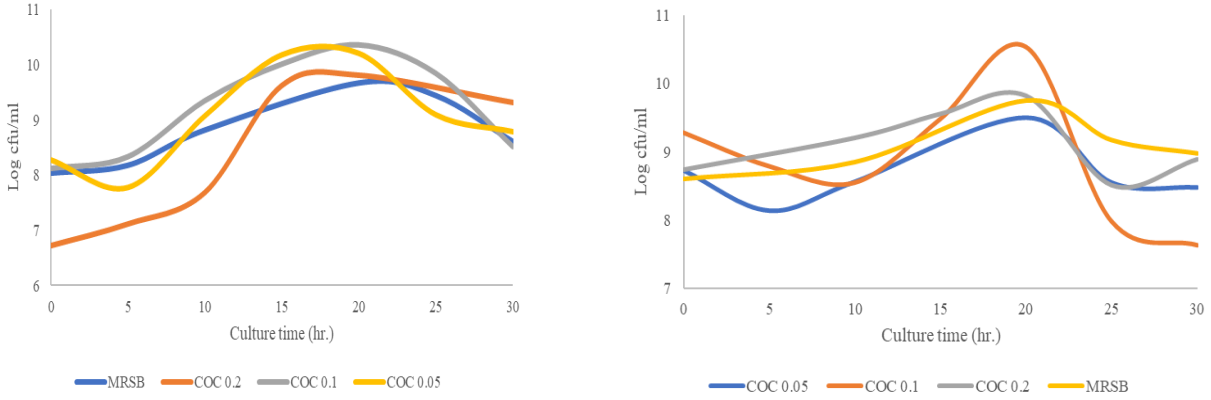

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Figure 5. (a) Growth of *S. thermophillus* FNCC – 040 and (b) *L. bulgaricus* FNCC – 041at different concentration of commercial oligochitosan

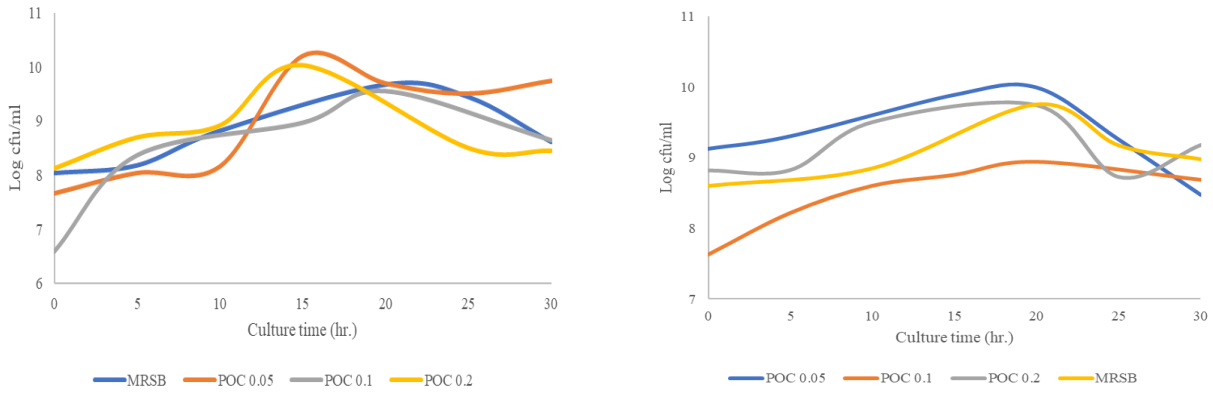

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Figure 6. (a) Growth of *S. thermophillus* FNCC – 040 and (b) *L. bulgaricus* FNCC – 041 at different concentration of self-production oligochitosan

Table 2. Time of growth peak, cell density and specific growth rate of *S. thermophillus* and *L. bulgaricus* (Log cfu/ml) at different treatment of chitosan

Treatment	*S. thermophillus* FNCC – 0040			*L. bulgaricus* FNCC – 0041		
	Peak of growth (hrs)	Log cfu/ml	SGR (µ/hrs)	Peak of growth (hrs)	Log cfu/ml	SGR (µ/hrs)
CC 0.05 mg/ml	20.13	10.03 ± 0.56	0.30 ± 0.43	21.41	8.82 ± 0.87	0.13 ± 0.19
CC 0.10 mg/ml	20.64	9.70 ± 0.49	0.25 ± 0.25	19.82	9.28 ± 0.43	0.03 + 0.42
CC 0.20 mg/ml	23.48	10.05 ± 0.47	0.95 ± 0.41	16.76	9.86 ± 0.04	0.27 ± 0.46
PC 0.05 mg/ml	21.22	9.87 ± 0.16	1.18 ± 1.32	14.98	8.93 ± 1.12	0.07 ± 0.21
PC 0.10 mg/ml	21.24	10.15 ± 0.02	0.04 ± 0.08	17.33	8.03 ± 0.73	0.38 ± 0.35
PC 0.20 mg/ml	22.65	10.53 ± 0.80	0.78 ± 1.11	19.91	9.74 ± 0.94	0.07 ± 0.28
COC 0.05 mg/ml	17.54	9.72 ± 0.58	1.37 ± 0.32	19.08	8.65 ± 0.73	0.51 ± 0.79
COC 0.10 mg/ml	19.72	9.69 ± 0.90	0.46 ± 0.73	18.89	9.60 ± 0.89	0.11 ± 0.14
COC 0.20 mg/ml	20.61	9.40 ± 0.43	0.02 ± 0.03	19.12	9.26 ± 0.72	0.01 ± 0.12
POC 0.05 mg/ml	15.44	10.20 ± 0.01	0.05 ± 0.07	18.53	9.54 ± 0.54	0.14 ± 1.21
POC 0.10 mg/ml	21.15	9.14 ± 0.46	0.13 ± 0.40	21.09	8.51 ± 0.47	0.28 ± 0.34
POC 0.20 mg/ml	17.48	9.58 ± 0.52	0.42 ± 0.56	18.17	8.92 ± 0.74	0.31 ± 0.76
MRS *Broth*	19.69	9.29 ± 0.38	0.61 ± 0.81	19.32	0.83 ± 0.87	0.10 ± 0.81

The *S. thermophillus* and *L. bulgaricus* culture in MRS broth-chitosan added media showed the positive growth. The best growth chitosan showed from PC 0.2 mg/ml treatment aginst *S. thermophillus* (136.2 x 10^9 cfu/mL). This data suggested that the prebiotics bacteria used this chitosan as their nutrition an certain concentration, similarly our previous findings with alginate (Yudiati *et al*, 2020). In fact, the DD from our self-production chitosan was 77.5%, which is lower than commercial chitosan (85%). The higher percentage DD produce the higher antibacterial activity (Jung *et al.,* 2010). Yucel-Falco *et al*. (2017) informed that lactic acid bacteria was sensitive to the antimicrobial activity in chitosan. Lee *et al.* (2002) confirmed that chitosan polymers at higher concentration (0.16-0.31%) showed the inhibition of *Lactobacillus* sp. and *S. thermophillus* growth culture. Furthermore, Li & Zhuang. (2020) was also reported the mechanism of chitosan as antibacterial. The action is bonding the negative chemical structure of chitosan with bacterial cell receptor which damage the cell membrane. This will trigger the bacterial cell compound and lead the bacterial death. At a higher concentration, chitosan will be protonated and the cell bacterial surface will be coated to prevent the damage of inner cell bacteria and the positively charged bacteria will be rejected vice versa. So, therefore, this were resulted the prevention of the agglutination forms.

The MRS broth-chitosan added media was significantly improve the *S. thermophillus* growth, but not in *L. bulgaricus*. Li *et al*. (2009) informed that the type and dose of probiotic is highly influenced to the effectiveness of nutrition utilization. Probiotics is in fact have the selectivity characters to the prebiotics, including chitosan. This prebiotics can be utilized as nutrition to stimulate growth and cell metabolism of lactic acid bacteria (Pan *et al.,* 2009; Yudiati *et al*., 2020). On the other hand, it is postulated that *L. bulgaricus* was not utilised the chitosan as their nutrient source and tend to be selective in terms of the prebiotic usage for the similar manners.

The effect of chitooligosaccharide on the growth of *S. thermophillus* and *L. bulgaricus*

Chitooligosaccharide is water soluble compound, with polimeration degree <20 and have the low molecular weight ie. 3.9 kDa (Liaqat & Eltem, 2018). COS has proven to have the better prebiotic activity compared to chitosan and marketable standard inuline (Ismail *et al.*, 2020). Moreover, Fernandes *et al*. (2012) have also stated that chitooligosaccharide is classified

as the weak prebiotics compared to other oligosaccharides particularly fructooligosaccharide and galactooligosaccharide.

Chitooligosaccharide consists of 18 fold higher sugar reduction compared to chitosan (Ismail *et al.* 2020). This is also act as the good carbon source for beneficial microbiota growth in intestinum (Kong *et al.* 2014). Lee *et al.* (2002) was also reported that chitooligosaccharide able to stimulate the lactic acid bacteria namely *Lactobacillus* sp. and *Bifidobacterium bifidium*. The supplementation of chitoologosaccharide in food was able to enhance the growth and density of bacteria from genus *Bacteroides, Faecalibacterium, Alistipes*, and *Prevotella* (Liu *et al.,* 2020). Supporting to this reports, Liang *et al.* (2013) was also stated that chitooligosaccharide enable to stimulate the *Lactobacillus paracasei* BCRC12193 and *Lactobacillus kefir* BCRC1401 growth. Type and dose of probiotics usage is highly dependant to its efficiency.

The *S. thermophillus* and *L. bulgaricus* culture in MRS *Broth* media chitooligosaccharide addicted showed the different bacterial growth. *S. thermophillus* culture in media MRS Broth media with chitosan oligosaccharide commercial at 0.1 mg/ml showed the highest stationary phase (225.3 x 10^8 cfu/ml). In accordance to Li & Zhuang (2020) chitosan has the higher antibacterial activity than chitosan oligomer. Moreover, the similar authors were also informed that the bactericidal effect of COS is higher in gram positive bacteria rather than gram negative bacteria. Furthermore, the chitooligosaccharide was significantly abled to inhibit the growth of *Lactobacillus* sp. and *S. thermophillus* at relatively high concentration (0.63%) (Lee *et al.* 2002)and also in intestinum (Liu *et al.,* 2020).

The MRS broth media addicted-chitooligosaccharide showed the increment of *S. thermophillus* FNCC – 0040 at significantly growth and unsignificantly growth on *L. bulgaricus* FNCC – 0041. Research by Lee *et al.* (2002), confirmed that chitooligosaccharaide from depolimerisation enzimatically process with polimerisation degree at 2-8 which use as prebiotics, do not inhibit the prebiotic growth. It has been predicted that *L. bulgaricus* FNCC – 0041can not efficiently utilize the nutrient of chitooligosaccharide from H_2O_2 polimerisation for growth and cell metabolism. On the other hand, the *S. thermophillus* FNCC – 0040 culture was managed to increase the growth significantly due to its ability to utilize chitooligosaccharide as nutrition to stimulate in the same manner.

Sangwan *et al*. (2014) confirmed that *S. thermophillus* produce high β-galactocide enzyme. It is postulated these probiotic bacteria produced this enzyme in the media and this enzyme supported the utilization chitosan and oligosaccharide as nutreints for their growth.

CONCLUSION

Chitosan and Chitooligosaccharide added at 0.05; 0.1; dan 0.2 mg/ml in MRS broth for *Streptococcus thermophillus* FNCC – 0040 had the significantly effect ($p<0,05$) on growth and have a good potency as prebiotics. Chitosan and Chitooligosaccharide added at 0.05; 0.1; and 0.2 mg/ml in MRS broth for *Lactobacillus bulgaricus* FNCC – 0041 had a non significantly effect ($p>0,05$) on growth. The application of chitosan and chitooligochitosan at the correct concentration was able to support the probiotic bacteria growth without any antibacterial activity. In the future, our self-production chitosan promising a good potency to explore and develop in further aspects.

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