CHAPTER 7

POTENTIAL USE OF POLYSACCHARIDES FROM *Nostochopsis* spp. AS PREBIOTIC

7.1. Introduction

At present, cyanobacteria or blue green algae are an interesting source of functional food. These microbes have been reported as rich sources of healthy nutrients such as proteins, carbohydrates, vitamins, minerals, amino acids and fatty acids. *Nostochopsis* (Lon) are filamentous cyanobacteria, normally found in the form of mucilaginous balls. The composition of mucilaginous substances produced by this genus is interesting as a potential source of polysaccharides. Polysaccharides and oligosaccharides from various algae have been found to possess several bioactive activities such as antibacterial activities (Kolankinathan *et al*., 2009), antioxidant potential (Cornish and Garbary, 2010), anti-inflammatory properties (Hong *et al*., 2011), anti-coagulant activity (Rodrigues *et al*., 2011), antimicrobial activity (Kantachumpoo *et al*., 2010), anti-viral activity (Sinha *et al*., 2010) and apoptotic activity (Kwon and Nam, 2007). However, there were few studies on polysaccharide from fresh water algae, especially from *Nostochopsis*. The aim of this study was to compare polysaccharide contents of cyanobacteria in this genus from different areas of northern Thailand and the prebiotic property was also studied.

7.2 Materials and Methods

7.2.1 Polysaccharides extraction

Four samples of fresh *Nostochopsis* were collected during November 2010 to April 2011 from Queen Sirikit Botanical Garden, Mae Rim, Chiang Mai Province (CM), Nan River, Tha Wang Pha, Nan Province (NT), Nam Yao River, Pua, Nan Province (NP), and Khong Muang Canal, Pai District, Mae Hong Son Province (MP). They were washed, cleaned and dried, then extracted with hot water (Huang *et al*., 1998). The extract (1 g dw.100 mL^{-1}) was stirred for 1 h at 90–100 °C. The supernatant was concentrated in an evaporator (Boss Tech) and the obtained gel was precipitated by 95% ethanol. The precipitated gel was separated and dried in hot air oven at 55°C for 48 h.

7.2.2 Size and quantity determination of polysaccharides

Size and quantity of polysaccharides was analyzed by using degree of polymerization value (DP) (FAO/WHO, 1997). The DP was calculated using the ratio between amount of total sugar and amount of reducing sugar. Total sugar was determined by phenol-sulfuric method (Dubois *et al*., 1956) (Appendix A) and reducing sugar was determined by dinitrosalicylic acid method (DNS) (Miller, 1959) (Appendix B). Glucose was used as standard sugar.

$$\textbf{Degree of polymerization = (DP)} \quad \frac{\text{Amount of total sugar}}{\text{Amount of reducing sugar}}$$

7.2.3 Analysis of carbohydrates

Carbohydrates were analyzed by thin layer chomatography (TLC) (Fang and Wang, 2004). Oligosaccharide was prepared by acid hydrolysis and neutralized with $CaCO_3$. Standard sugars were mannose, fructose, xylose, glucose, arabinose, galactose, raffinose, glucuronic acid and rhamnose. They were spotted on TLC silica gel $60F_{254}$ (Merck). The mobile phase was isopropanol: 1-butanol: water (12:6:4) modified from Sa-nguansook (2002). TLC plate was visualized by 5% (v/v) H_2SO_4 in methanol and heating at 150°C for 5 min in hot air oven.

7.2.4 Oligosaccharides from *Thermoascus aurantiacus* SL16W

Lignocellulolytic enzymes from *Thermoascus aurantiacus* SL16W using rice straw as substrate comprising of carboxy methylcellulase (CMCase), filter paper unit (FPU), glucosidase, mannanase, xylanase and xylosidase as 197.57, 2.54, 9.24, 60.26, 54.88 and 15.55 U/g substrate respectively (Sintoon and Khanongnuch 2013) were used to convert polysaccharide from 7.2.1 into oligosaccharides.

7.2.4.1 Effect of CMCase producted by *T. aurantiacus* SL16W

Each 10 ml of 0.2% (w/v) polysaccharide extracted was dissolved in 0.5 M acetate buffer at pH 4.8 and mixed with 0.5, 1, 2.5, 5 and 10 U/g substrate of CMCase. The reaction mixture was incubated in water bath at 50 ºC. The 200 µl of reaction mixture was sampled at 10, 20, 30 and 60 min. Total sugar, reducing sugar and degree of polymerization were calculated as described in 7.2.2.

7.2.4.2 Effect of *Nostochopsis* spp. polysaccharide concentration on CMCase production by *T. aurantiacus* SL16W

Each 5, 10, 20, 30 and 40 mg of polysaccharides was incubated with 1 mL 0.5 M acetate buffer pH 4.8, then hydrolyzed with 2.5 U/g substrate of CMCase for 20 min as described in 7.2.4.1. Total sugar, reducing sugar and degree of polymerization were calculated as described in 7.2.2.

7.2.5 Prebiotic properties

7.2.5.1 *In vitro* study with pure culture

Basal medium was used as a basal growth medium to study the growth ability of *Lactobacillus fermentum* CM33, *Escherichia coli* O157:H7 and *Samonella enteritidis* (Appendix C). Supplemented with different carbon sources such as glucose, oligosaccharide from enzymatic hydrolysis (DP4), extracted polysaccharide from *Nostochopsis* (DP9) and without carbon source. The carbon sources were added to the sterile medium to give a final concentration of 1% (w/v). The 500 µl of those bacteria 12 h of culture at 10^7 CFU.mL^{-1} OD_{650} = 0.68 was used as inoculum. The cultures were incubated at 37 °C for 12, 24 and 36 h and viable cells were examined by spread plate technique. The change of pH in the medium was determined.

7.2.5.2 *In vitro* study with mixed culture

Basal medium was added with 1% extracted polysaccharide (DP9) or oligosaccharide from enzymatic hydrolysis (DP4) as a carbon source for mixed culture of *Lactobacillus fermentum* CM33, *Escherichia coli* O157:H7 and *Samonella*

enteritidis. The samples were compared with glucose and without carbon source supplementation in the medium. Inoculum and viable cell measurement were carried out as described in 7.2.5.1.

7.2.6 Data evaluation

The computer statistical packages, for example SPSS for Windows version 14.0 and Microsoft Excel were used for statistical analysis of variance (ANOVA), correlation and cluster analysis.

7.3 Results and Discussion

The percentage yield of *Nostochopsis* polysaccharide per dry weight was found to be 34-49%. The polysaccharide in NT was higher than MP but did not significantly differ from those of CM and NT (p>0.05) (Table 7.1). The yields of the water-soluble polysaccharide in *Nostochopsis* were similar to many species of cyanobacteria and microalgae and closely related to *Nostoc commune* and *Nostoc sphaeroides* which contained 30%–40% soluble polysaccharide of dry cell (Lee, 1999). Polysaccharide is the major component of *Nostoc* cell wall (Zhanga *et al*., 2010). The highest total sugar was found in NT sample whilst in NP and MP were the lowest when compared with others. Presently, commercial agar and alginate were produced from marine algae, *Porphyra haitanensis*, *Laminaria japonica* and *Enteromorpha linza* which their total sugar contents were comparable with *Nostochopsis* as 78.9, 66.7 and 47.9%, respectively (Painter, 1983). Hence, *Nostochopsis* may have a potential to be developed as a novel polysaccharide source. Reducing sugar in sample from CM was significantly higher than other samples. However, the degrees of polymerization of

polysaccharide (DP) from *Nostochopsis* was in the range of 6-9 (Table 7.1.), which could be classified as a large polymer and it needed to be hydrolyzed for application as beneficial oligosaccharide in various products. In addition, the degree of polymerization of freshwater algae, *Spirogyra* spp. was approximately 14 for fresh sample and 7 for dried sample and they showed potential prebiotics (Phinyo, 2010).

Table 7.1. Polysaccharide content and characterization of all the samples

Samples	%Polysaccharide (yield)	%Total sugar (w/w)	%Reducing sugar (w/w)	Degree of polymerization
CM	42.91 ±0.02ab	41.37±1.81ab	7.16±0.26 b	6
NP	35.60±0.05ab	36.71±4.46 a	5.90±0.64 a	6
NT	49.03±0.08a	49.28±5.08 b	5.82±0.26 a	9
MP	34.05±0.01b	33.52±2.17 a	5.60±0.34 a	6

Data are expressed as mean ± standard deviation (SD) of thee replicates. Different letters represent the statistical comparisons between groups in each column by using ANOVA and post hoc Tukey's b test (p<0.05)

All polysaccharide samples from *Nostochopsis* consisted of monosaccharides such as glucose, fructose, mannose, rhamnose. Glucoronic acid was found only in NT and MP samples Besides, galactose was presented only in CM and MP samples (Figure 7.1). They were similar to those reported by Painter (1983) who found monosaccharide in another cyanobacterium, *Nostoc* consisting of glucose, xylose, glucoronic acid, galactose and rhamnose. Different types of monosaccharide in *Nostochopsis* was found because they were collected from different geographic sources. Composition of cyanobacterial polysaccharides varies according to season,

age, species, and geographic location (Thacher and Paul, 2004; Graham and Wilcox, 2000).

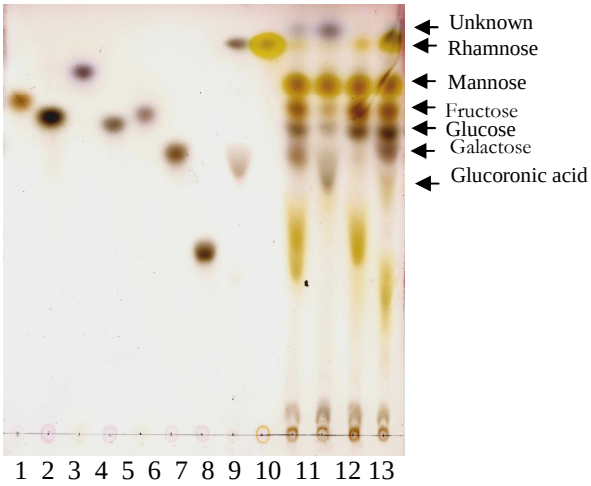

Figure 7.1 Thin layer chromatograms of polysaccharide Lane 1:Mannose,2:Fructose, 3:Xylose, 4:Glucose, 5:Arabinose, 6:Galactose, 7:Raffinose, 8: Glucuronic acid, 9:Rhamnose, 10:Hydrolyzed polysaccharide from *Nostochopsis* (CM), 11:Hydrolyzed polysaccharide from *Nostochopsis* (NT), 12:Hydrolyzed polysaccharide from *Nostochopsis* (NP), 13:Hydrolyzed polysaccharide from *Nostochopsis* (MP).

In this study, the enzyme from *Thermoascus aurantiacus* SL16W was selected because it was produced by using rice straw as substrates which consisted of cellulose and hemicelluloses. It could produce many enzymes such as CMCase, FPU, glucosidase, mannanase, xylanase and xylosidase (Sintoon and Khanongnuch, 2013). Polysaccharide extracted from *Nostochopsis* consisted of mainly monosaccharide *i.g.* rhamnose, mannose, fructose, glucose and galactose which may be hemicellulose polysaccharide. Hemicelluloses are in soluble and insoluble forms and are comprised of a number of branched and linear pentose and hexose containing polysaccharides.

Monosaccharide units include xylose, arabinose, galactose, mannose, glucose, glucuronic acid and galacturonic acid (Lineback, 1999).

Oligosaccharide production is shown in Figure 7.2. All concentrations of CMCase effected DP of polysaccharide. It decreased from 9 to 3-4 and 1-2 for 20 and 30 min respectively. Thus, 2.5 U/g substrate of CMCase was selected because it could hydrolyze polysaccharide to obtain DP4 oligosaccharide (Figure 7.3). Barboza *et al*., (2013) reported that, infant-borne *Bifidobacteria* e.g., *B. breve* and *B. longum* growth can be preferentially stimulated using galacto-oligosaccharide (GOS) that is enriched for DP 4-5 galacto-oligosaccharides.

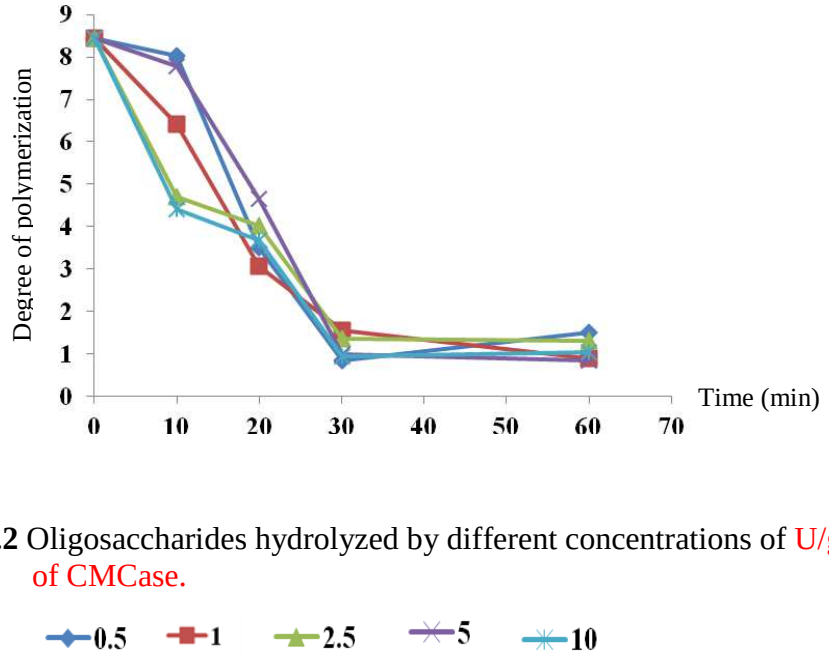

Figure 7.2 Oligosaccharides hydrolyzed by different concentrations of U/g substrate of CMCase.

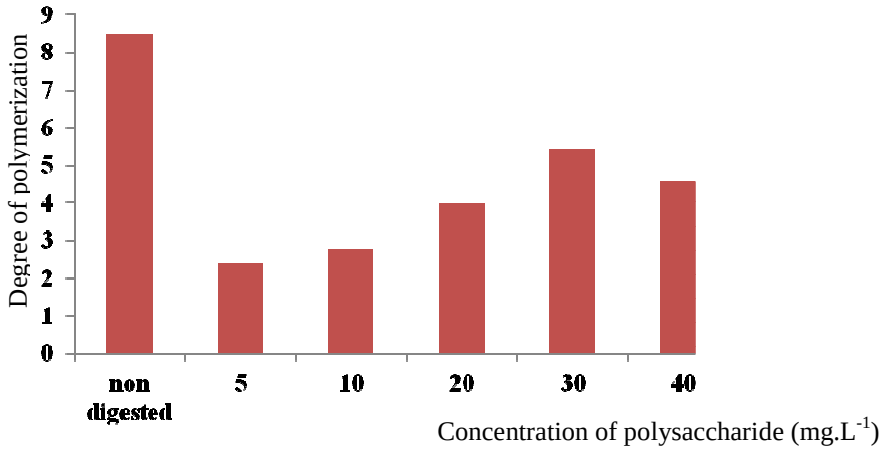

Figure 7.3 Effect of extracted *Nostochopsis* spp. polysaccharide concentration on 2.5 U/g substrate of CMCase.

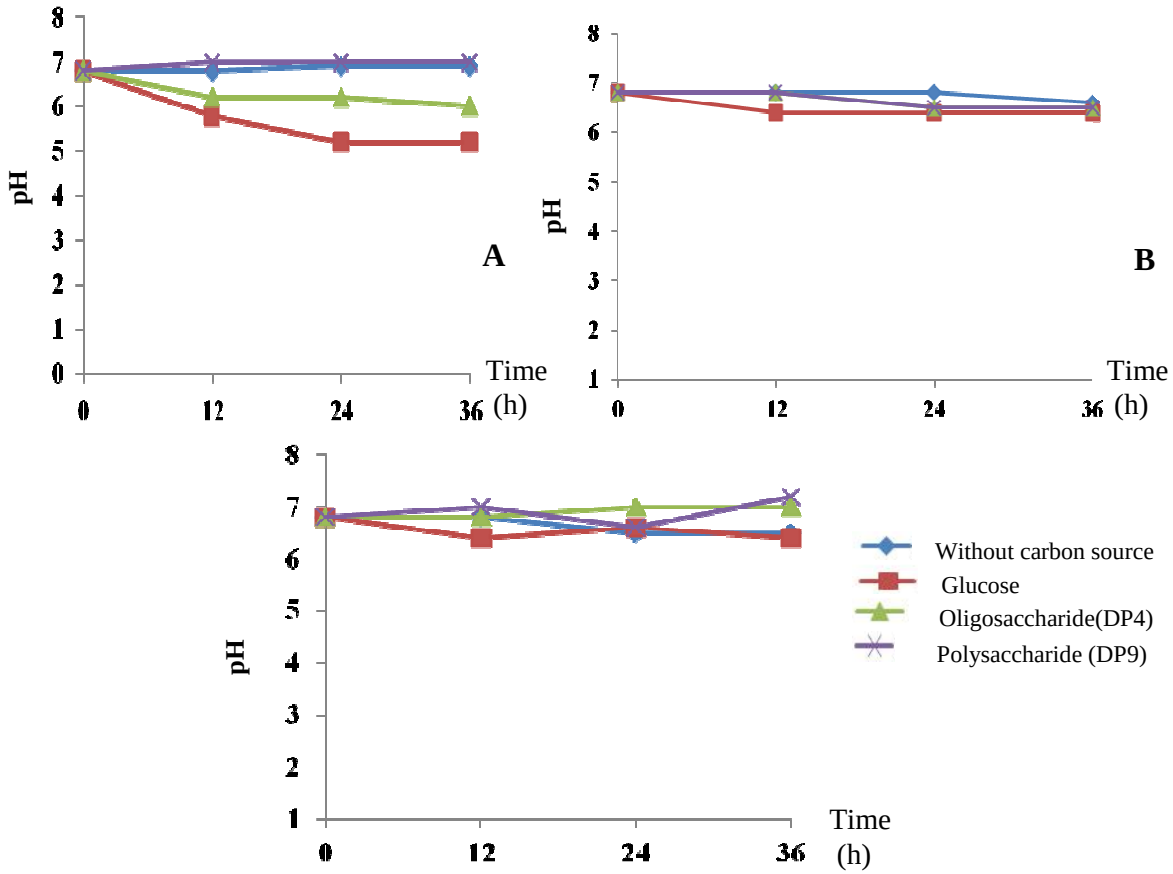

Figure 7.4 pH change in pure cultured
A) *Lactobacillus fermentum* CM33 **B)** *Salmonella enteritidis*
C) *Escherichia coli* O157:H7

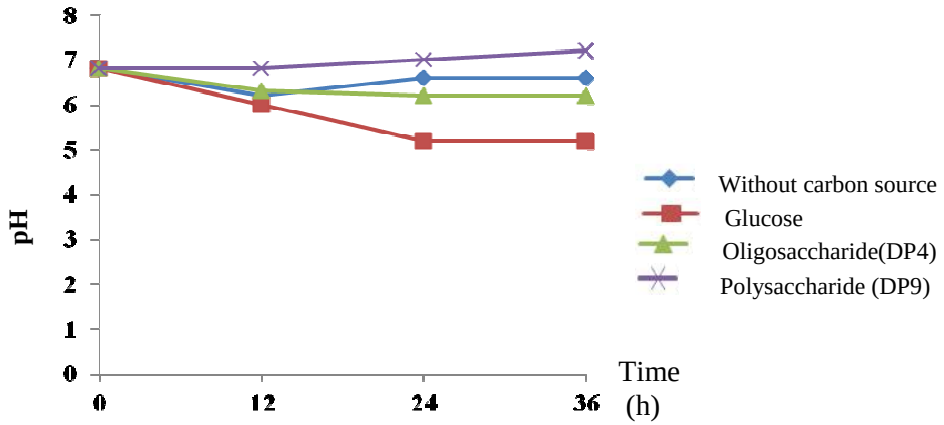

Figure 7.5 pH change in mixed cultured

For prebiotic properties study in pure culture, *Lactobacillus fermentum* CM33 was grown in basal medium either with or without carbon sources. It could also grow in the medium supplemented with polysaccharide (DP9) as carbon source and without carbon source for 12 h. The maximum growth was obtained under cultivation with 1% (w/v) glucose supplementation for 24 h and decreased after 36 h. In addition, the cell growth still increased in the cultivation with oligosaccharide (DP4) after 36 h. The color of the medium changed to yellow and pH dropped to 5 (Figure 7.4). *Salmonella enteritidis* could grow in all media but the growth dropped when glucose was used as carbon source after 36 h. However, *Escherichia coli* O157:H7 could grow in every condition (Figure 7.6). Leyer *et al*. (1995) reported that *E. coli* O157:H7 was adapted to survive in acidic condition by culturing for one to two doublings at pH 5.0. Acid-adapted cells had an increase in resistance to lactic acid.

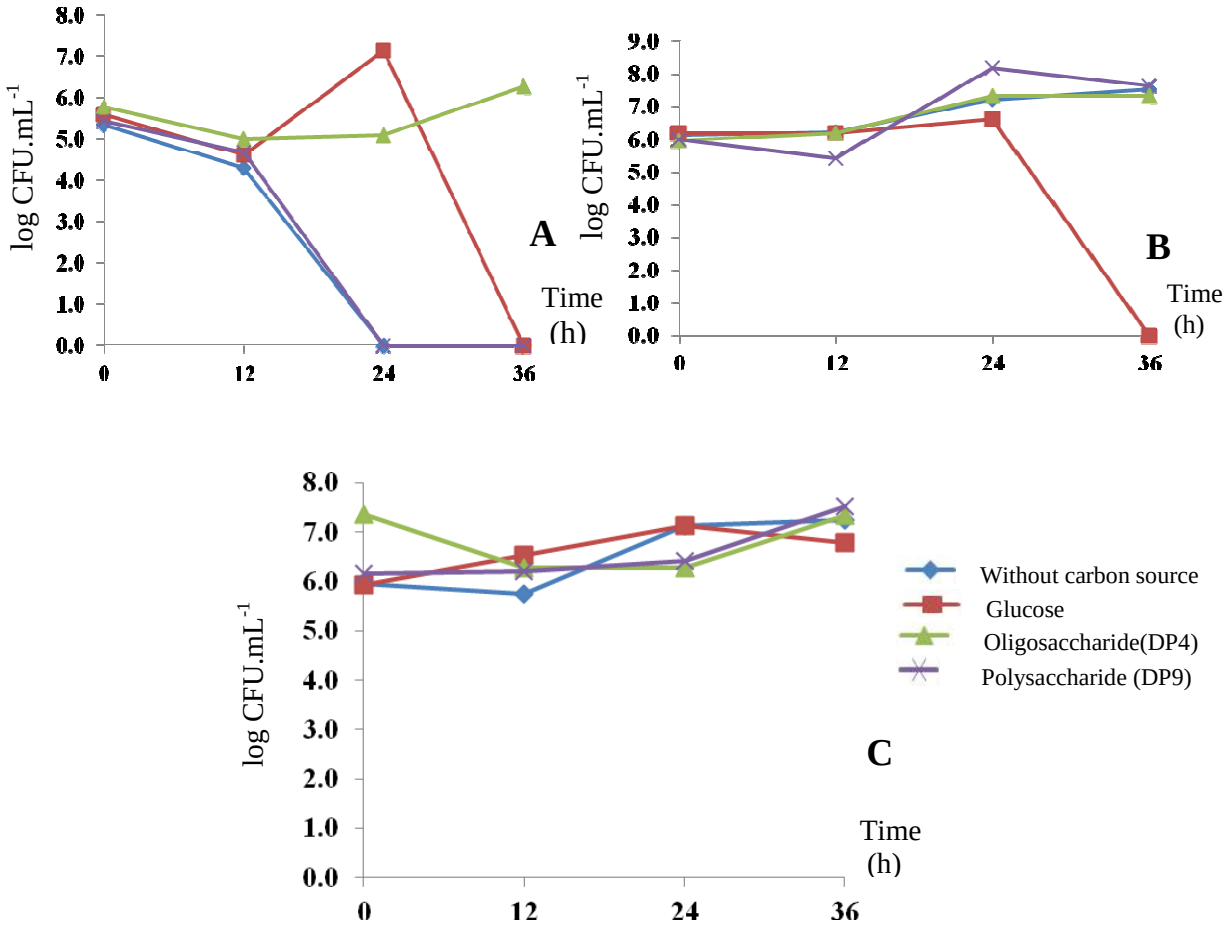

Figure 7.6 Growth of pure culture of bacterial strains in basal medium without carbon sources and with 1% carbon sources
A) *Lactobacillus fermentum* CM33 **B**) *Salmonella enteritidis*
C) *Escherichia coli* O157:H7

For prebiotic properties studied in mixed culture, *E. coli* O157:H7 and *S. enteritidis* could grow in the medium without carbon sources since the basal medium consisted of yeast extract that contained pure amino acid and glucose (Sommer, 1996). *L. fermentum* CM33 could grow slightly after 24 h incubation and the growth

dropped after 36 h. *S. enteritidis* and *L. fermentum* CM33 could not grow in glucose as carbon source after 36 h incubation and pH changed in mixed cultured with glucose as carbon source. It dropped to 5 (Figure 7.5). *L. fermentum* CM33 grew well in oligosaccharide (DP4) than in the polysaccharide (DP9) but it could not inhibit the growth of normal flora and pathogen *i.e. E. coli* O157:H7 and *S. enteritidis*. (Figure 7.7). However, the relationship between the prebiotic effectiveness and DP should be investigated.

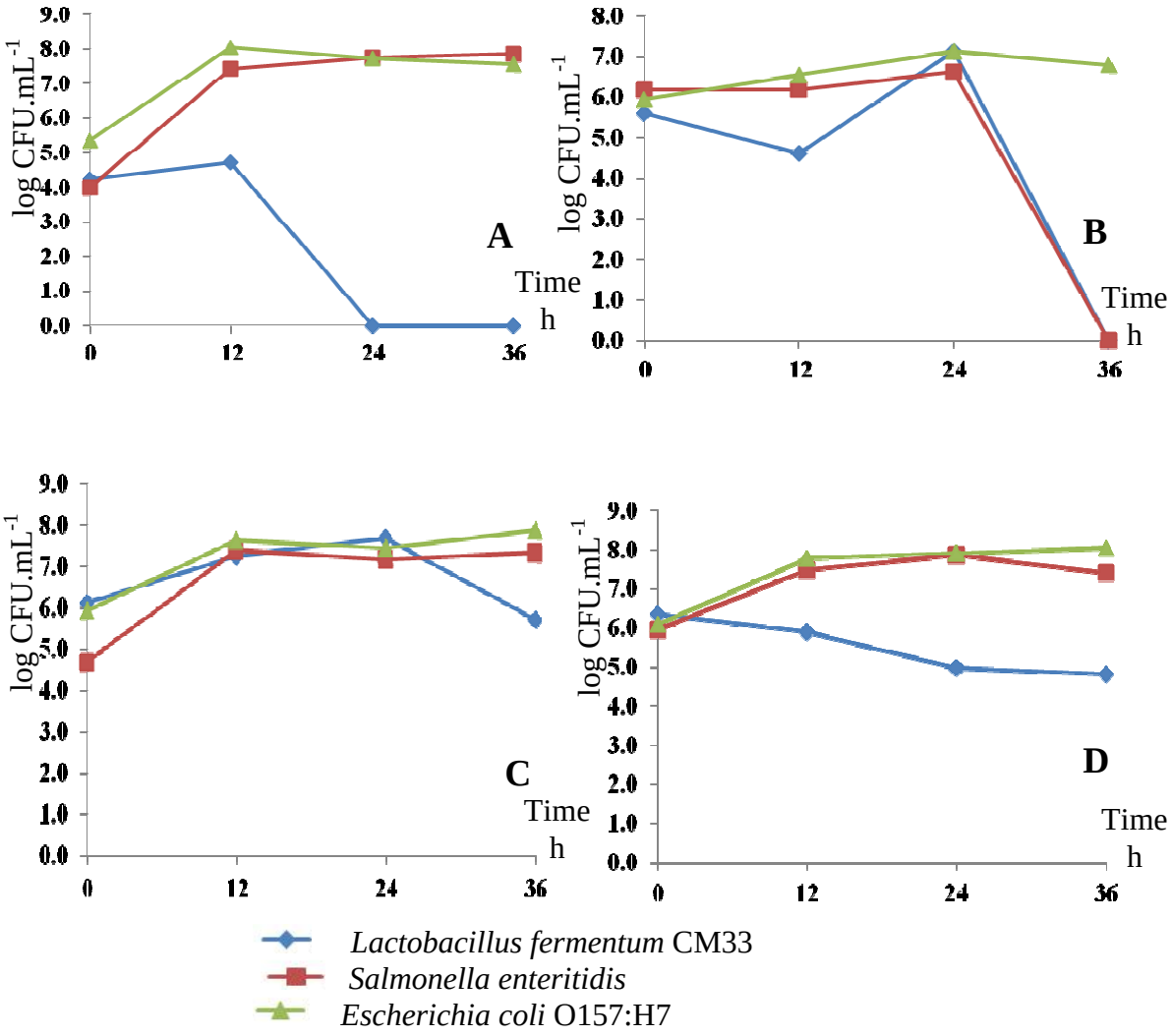

Figure 7.7 Growth of mix culture of bacterial strains in basal medium without carbon sources and with 1% carbon sources **A)** Without glucose **B**) Glucose **C)** Oligosaccharide (DP4) **D)** Polysaccharide (DP9)