Research Report

Effect of encapsulation on *Zymomonas mobilis* survival rate from practical conditions and its levan polysaccharide production ability

By

Teeradate Kongpichitchoke (Ph.D.)
Department of Agro-Industry
Faculty of Biotechnology
Assumption University

September 2017

Research Report

Effect of encapsulation on *Zymomonas mobilis* survival rate from practical conditions and its levan polysaccharide production ability

By

Teeradate Kongpichitchoke (Ph.D.)
Department of Agro-Industry
Faculty of Biotechnology
Assumption University

September 2017

Table of Contents

List of Tables

List of Figures

ACKNOWLEDGEMENT

I would like to express my gratitude to the Institute for Research and Academic Services (IRAS), Assumption University, for financial support. I also would like to mention my thanks to my research assistant, Ms. Apinya Sowatad, for her great contribution in this research. Lastly, I appreciate the deputy chairperson of Agro-Industry department, faculty of biotechnology, Assumption University, Asst. Prof. Dr. Tatsaporn Todhanakasem for her supports and valuable suggestions.

Teeradate Kongpichitchoke (Ph.D.)

Faculty member of Agro-Industry department

Assumption University

September 13, 2017

ABSTRACT

Bioethanol is one of the good sources of liquid energy for automobiles and industries. The bioethanol market has continued to grow rapidly in recent years. Thai government aims to increase ethanol consumption from 1.2 billion liters in 2015 to 3.3 billion liters by 2021 and up to 4.1 billion liters by 2036. *Z. mobilis*, a gram-negative bacterium, is considered as an alternative organism in large-scale fuel ethanol production. The bacterium *Z. mobilis* is a highly potent ethanol producer already used in industrial-scale fermentations which converts sugars to ethanol and carbon dioxide. Levans are natural polymers of the sugar fructose found in many plants and microbial products. They are formed as an undesirable by-product of sugar juice processing. This project aimed to study survivor rate of *Z. mobilis* encapsulated at different concentration of sodium alginate 2%, 2.5% and 3%., from simulated practical conditions including simulated food tract condition (acidic pH at 1.55 and 0.6% bile salt) and high temperature environment (85°). The encapsulated beads were separated into four different sizes according to their average size by sieving process. Results indicated that survival rates of encapsulated cells of *Z. mobilis* were significantly higher ($p < 0.05$) from all simulated conditions compared to free cells. Furthermore, the highest survival rate was obtained from sieve 10 with 3% sodium alginate for both heat and bile salt tests, while the lowest survival rates were performed by 2% alginate beads obtained from sieve 70 with survival rate of 19.52% and 14.72%, and 0.22% and 0.77%, respectively. Levan production was also determined from encapsulated cells providing the highest survivor rate which was beads from sieve 10 of 3% sodium alginate. Results showed they that there was no significant difference ($p > 0.05$) in levan production between free cell and encapsulated cells from high temperature condition test.

1. INTRODUCTION

Thailand is a big producer for many agricultural commodities. For instance, Thai rice sugar and rubber are widely recognized in international markets. Thus, Thai government has implemented long-term strategies to further increase value of the agricultural products by using agro-industry knowledge. Result from this strategy leads to increasing numbers of agricultural-involved processing in which causing higher level of agricultural wastes in consequence. In the last two decades, many researchers have been focusing on utilization of agricultural wastes in various ways. One of a promising ways is to process lignocellulosic materials to produce alternative energy, especially bioethanol, because of energy crisis concerns. Lignocellulosic agricultural wastes can be obtained mainly from rice. They are considered as a good carbon source for growing microbe. However, using of agricultural-based for bioethanol production has an important drawback which is production of toxic compound during fermentation. In this case, *Zymomonas mobilis* is a key player in bioethanol production process because of its ability to produce high ethanol yield and can be toxic compound resistance. After fermentation process, *Z. mobilis* is considered as a byproduct and it is commonly discarded despite of its special benefits. For example, it was reported that this microbe can produce levan polysaccharide which has beneficial bioactive activity. Moreover, it was recently reported that this microbe could produce significant level of cellulase enzyme. Thus, *Z. mobilis* exhibits great potency to be used as probiotic microbe in feed. However, before claiming that this microbe can be used as probiotic, it must be proven that this microbe can survive through feed production process and animal digestive tract. There was a report stating that survivor during the processing, and transit through high acidic conditions in the gastro-intestinal and bile salts in the small intestine is a major challenges (Kailasapathy, 2002). Therefore, this research aims to study survival rate of *Z. mobilis* under high temperature, bile acid and gastric pH

condition in which encapsulation of the cells may improve the cell viability. Furthermore, this study will also ensure that levan production ability of the cells after passing through these harsh conditions and encapsulation will not be terminated.

2. OBJECTIVES

- To determine survival rate of *Zymomonas mobilis*, both free cell and encapsulated form, after treating in simulated feed production process and animal food tract conditions.
- To determine effect of encapsulation, simulated feed production process and animal food tract conditions on production of levan polysaccharide.

3. LITERATURE REVIEWS

3.1 *Zymomonas mobilis*

Zymomonas mobilis is a facultative anaerobic gram negative rod shaped bacteria (as shown in **Figure 1**) which can convert many kinds of carbon sources, such as glucose, fructose, and sucrose, to be ethanol via Enter-Doudoroff pathway. It is a promising organism for bioethanol production as it can efficiently produce ethanol from simple sugars (Dien *et al.*, 2003). Moreover, its dominant advantages, benefitting bioethanol production, such as high ethanol tolerance (12%) and wide pH range (3.5 – 7.5), allows *Z. mobilis* to be recognized as a key producer of bioethanol nowadays (Yang *et al.*, 2010, Panesar *et al.*, 2006, and Rogers *et al.*, 2007). In the last two decades, researchers turned their attention to *Z. mobilis* because it was reported that this microbe has capability of exopolysaccharide 'levan' at large scale (Azerêdo *et al.*, 2010, and Oliveira *et al.*, 2007). After the fermentation, *Z. mobilis* is produced at large scale and therefore is considered as byproduct from bioethanol process. Furthermore, *Z. mobilis* was

also recently reported for its ability to produce extracellular cellulase (Linger *et al.*, 2010).

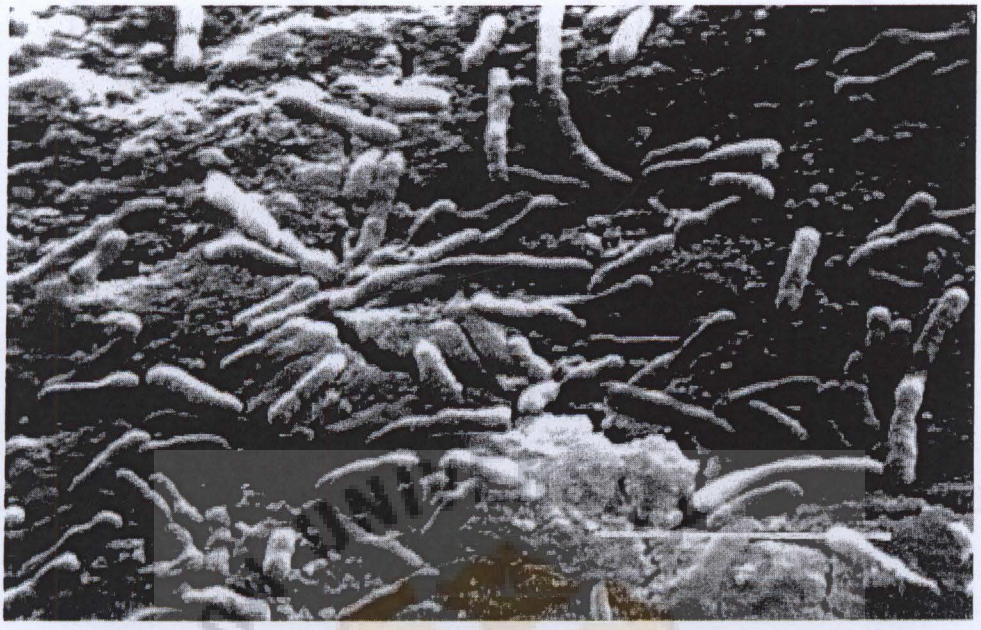

Figure 1. Scanning electron microscope micrograph of *Zymomonas mobilis* (Bekers *et al.*, 2001)

3.1.1 Levan Polysaccharide

Levan polysaccharide also referred as fructans is one of the larger group of polymers, which are used as a source of prebiotic fiber (Franken *et al.*, 2013). Levan is an extra polysaccharide, toxic-free compound, environmental friendly and biologically active which mainly compose of fructose unit. Molecular structure of microbial levan polysaccharide was shown in **Figure 2**. It is being used in food industry, and for animal feed. It also being used in medicine as a hypo-cholesterol, immune system, antitumor, and plasma substitute agent (Liepa *et al.*, 1999). Levan production during sucrose fermentation by *Z. mobilis* is due to extracellular levansucrase which has optimum temperature and pH of 35°C and 5.5, respectively. This enzyme is identified as 2-6-β-fructan 6-β-fructose-transferase containing molecular weight of 48,000 kDa and is produced by several bacteria including *Z. mobilis* (Hestrin *et al.*, 1943).

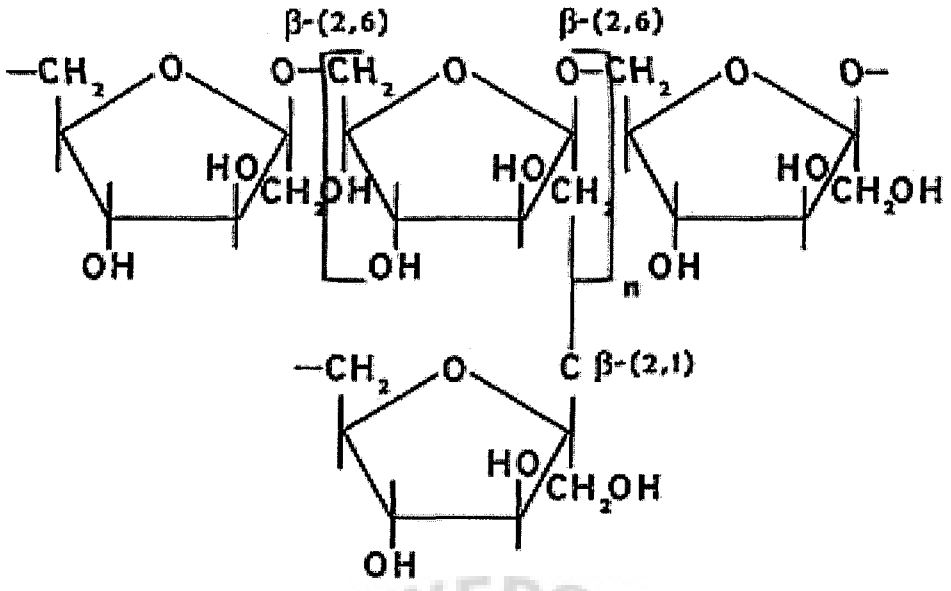

Figure 2. Molecular structure of microbial levan polysaccharide (Srikanth *et al.*, 2015)

3.1.2 Probiotic

Fuller (1989) and FAO/WHO defined meaning of probiotics as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'. Shah (2000) mentioned that probiotics would perform their health benefits when they existed 10^6-10^7 cfu/g of product at the consumption time. Thai FDA issued that a particular product can be classified as probiotic product in case that the product contain probiotic microbes at least 10^6 cfu/g of product. Thus, probiotic bacteria should be active and stable and can survive during their passage through the stomach at level of 10^6–10^7 live microorganisms per gram of product in order to provide the health benefits in intestine (Guarner and Schaafsma, 1998) including growth inhibition of pathogen and contribute to higher growth and feed efficiency, preventing intestinal disorders, and improving utilization of feed by decreasing the amount of feed necessary to be used as animal feed (Wunwisa *et al.*, 2003). Adding of probiotics into food or feed have to consider this minimum requirement because most of food or feed production process contain thermal process which can significantly reduce number of viable

probiotics. In some cases, feed production process temperature, especially pelting process, may reach 90°C (Amerah *et al.*, 2013). Moreover, extreme condition in digestive tracts, such as bile acid and low pH condition, can also greatly damage probiotic cells. For example, *Lactobacillus* sp. cannot survive well in harsh acidity and bile concentration conditions (Mandal *et al.*, 2006).

3.1.3 Characteristics of good probiotic

A good probiotic should have the following requirement (Cock and Castillo, 2013):

a) It should be a strain, which is capable of exerting a beneficial effect on the host animal, for example increased growth or resistance to disease.

b) It should be non-pathogenic and non-toxic.

c) It should be present as viable cells, in large numbers.

d) It should be able live capable of surviving and metabolizing in the gut environment for example, it should be resistant to low pH and organic acids.

e) It should be stable and capable of remaining viable for periods under storage and field conditions.

Z. mobilis is considered to have a generally recognized as safe (GRAS) characteristic (Yang *et al.*, 2010). It also had been reported that it is safe for consumption and exhibit modulating effect on mice immune system (Azerêdo *et al.*, 2010). Therefore, it is interesting to use it as probiotic in food and feed.

3.2 Encapsulation

Microencapsulation is a process in which the cells are retained within an encapsulating membrane to reduce cell injury or cell loss. Encapsulation is used to protect the cells against undesirable environments such as low pH, high temperature, humidity, and heat (Champagne and Kailasapathy, 2008). Furthermore, not only cell survival could be enhanced from encapsulation but also other several benefits, especially in animal feed and play an important role

in the production of dairy product, such as yoghurt, cheese and frozen milk products, as well as for biomass production (Krasaekoopt, *et al.*, 2003). Moreover, encapsulated culture provides high stability of cells and high productivity for metabolite production (Arnaud *et al.*, 1992).

3.2.1 Encapsulant

To protect cells from undesirable environment, encapsulation of cells by various materials are commonly used. Gelatin, vegetable gums, chitosan, carrageenan and sodium alginate were reported to be used as encapsulant in order to protect probiotics from the harsh conditions (Mandal *et al.*, 2006, Rao *et al.*, 1989, Kailasapathy, 2002, and Picot and Lacroix, 2004). Among them, alginate is the most widely used encapsulant for cell encapsulation (Kailasapathy, 2002, Burgain *et al.*, 2011). They are composed of several building blocks (100-3,000 units) liked together in a stiff and partly flexible chain. The relative amounts of the two uronic units and the sequential arrangements of them along the polymer chain vary widely, depending of the origin of the alginate. Sodium alginate is frequently used as a gelling agent, because of its ability to form hydrogels with divalent cations, such as Ca^{2+}, Ba^{2+}, and Sr^{2+} under appropriate conditions. Hydrogel will be formed by mixing block of acid bond with cation. Alginate filament are held together with ionic interaction by a three dimensional network (Simpson *et al.*, 2004). Alginate could increase survival rate of microbes in high bile salt concentration condition at 80-95% compared with free cell (Sheu and Marshall, 1993). Increasing of alginate concentration could also increase survival rate of the entrapped cells by increase microcapsule size (Lee and Heo, 2000).

There are several advantages from using alginate. First, it easily forms gel matrices around bacterial cells. Second, it is safe to the body and requires mild process conditions (such as temperature) for their performance. Third, it is cheap, and can be easily prepared. Fourth, it can be dissolved in intestine and consequently releases entrapped cells. However, some disadvantages are

attributed to alginate beads. For example, alginate microcapsules are susceptible to the acidic environment which is not compatible for the resistance of the beads in the stomach conditions. Other disadvantage of alginate micro particle is that the microbeads obtained are very porous to protect the cells from its environment. Nevertheless, the defects can be compensated by blending of alginate with other polymer compounds, coating the capsules by another compound or structural modification of the alginate by using different additives (Krasaekoopt *et al.*, 2004).

3.2.2 Encapsulation method

The encapsulation techniques, applied to probiotics, are divided into two groups, extrusion and emulsion depending on the method and equipment availability. These two techniques play the same role which is increase the longevity and shelf-life of probiotic bacteria by up to 80–95% (Sheu and Marshall, 1991). Selection of the encapsulation method depends on the physical and chemical properties of the carrier (Burgain *et al.*, 2011).

For encapsulation of probiotics, both extrusion and emulsion techniques can be applied. Extrusion is a relatively simple technique. It usually produces entrapped, rather than encapsulated core material, although encapsulation can be achieved through co-extrusion devices or dropping into a bath of coating material which react at the droplet surface. This method is difficult for large-scale production because of slow formation of beads compared with the emulsion technique. On the other hand, the emulsion technique is relatively new to the food industry and easy to scale up for large-scale production. It provides both encapsulated and entrapped core materials. The size of the beads formed by this method is smaller (25 mm to 2 mm) than that of beads produced by the extrusion method (2–5 mm). The size of beads from the extrusion method depends mainly on the size of the needle used, while the size of beads from the emulsion method depends on the speed of agitation and the type of emulsifier used. Due to the need

for a vegetable oil, the operating cost of the emulsion technique may be higher than that of the extrusion technique (Krasaekoopt *et al.*, 2003). Summarize of advantages and disadvantages of each technique was listed in **Table 1.**

Table 1. Advantages and disadvantages of extrusion and emulsion encapsulation technique

	Extrusion	**Emulsion**
Advantages	Simple and cheap method that uses a gentle operation. No damage to the probiotic cell. Gives high probiotic viability. Does not involve deleterious solvent. Done under aerobic and anaerobic conditions. hydrocolloids (King, 1995)	Large scale productions Higher molecular weight polymer formed at faster rate Large quantity of beads are formed. hydrocolloids
Disadvantages	Difficult to use for large scale production due to slow formation of microbeads. Susceptibility of carbohydrate towards damage and structural defect, a larger size distribution. Limited choice of wall material.	Expensive method Unreacted monomer which are in higher concentration (Arnaud *et al.*, 1990)

3.2.3 Challenges

Challenge that leads to study of this project is that, one of by-products from the production of bioethanol is *Z. mobilis* cells which are usually discarded. Due to their special ability of levan production, leads to efforts of using them as probiotics. However, microbes directly used in animal feed, are mostly neutralized from low acid condition in stomach and bile salt in intestine.

for a vegetable oil, the operating cost of the emulsion technique may be higher than that of the extrusion technique (Krasaekoopt *et al.*, 2003). Summarize of advantages and disadvantages of each technique was listed in **Table 1.**

Table 1. Advantages and disadvantages of extrusion and emulsion encapsulation technique

	Extrusion	**Emulsion**
Advantages	Simple and cheap method that uses a gentle operation. No damage to the probiotic cell. Gives high probiotic viability. Does not involve deleterious solvent. Done under aerobic and anaerobic conditions. hydrocolloids (King, 1995)	Large scale productions Higher molecular weight polymer formed at faster rate Large quantity of beads are formed. hydrocolloids
Disadvantages	Difficult to use for large scale production due to slow formation of microbeads. Susceptibility of carbohydrate towards damage and structural defect, a larger size distribution. Limited choice of wall material.	Expensive method Unreacted monomer which are in higher concentration (Arnaud *et al.*, 1990)

3.2.3 Challenges

Challenge that leads to study of this project is that, one of by-products from the production of bioethanol is *Z. mobilis* cells which are usually discarded. Due to their special ability of levan production, leads to efforts of using them as probiotics. However, microbes directly used in animal feed, are mostly neutralized from low acid condition in stomach and bile salt in intestine.

Nonetheless, high temperature condition in animal feed production process is also another major cause of cell death. In order to deliver live microbes into the host, encapsulation can be applied to protect the cells from extreme conditions. Therefore, it is interesting to study whether encapsulation can increase survival rate of *Z. mobilis* in the harsh conditions. Furthermore, this study will ensure that *Z. mobilis* still has its ability to produce levan polysaccharide when it is administered to the body as probiotic and passed through digestive system.

4. MATERIALS AND METHODS

4.1 Microorganism

Stock cell of *Z. mobilis* was reactivated by transferring one loop of the culture into MRS broth for 24 h and was further scaled-up to 500 mL of MRS broth for 48 h. Cells was harvested by centrifugation at speed 5,000 rpm for 10 min (Kubota High Speed Centrifuge, Osaka, Japan). Cell pellet was collected and washed with 0.85% NaCl. Then, cells were re-suspended with 0.85% NaCl.

4.2 Microencapsulation

Sodium alginate at different concentrations (2%, 2.5% and 3%) in distilled water was prepared for 88 mL and mixed with 22 mL of cells *Z. mobilis* solution obtained from cell harvesting process described in section 3.1. The mixture was dropped one-by-one into 260 mL of vegetable oil containing 0.2% Tween 80, which was spinning by a homogenizer at rotational speed 200 rpm. At the meantime, calcium chloride at concentration 0.2 M was added into the mixture with flow rate 1.9 mL/minutes by using peristaltic pump. Obtained beads were harvested, washed by distilled water with 1% tween 80 and sieved to separate beads according to size.

4.3 Survival rate of free and microencapsulated cells from simulated food tract conditions

Simulated gastric solution of 0.08 M HCL (pH 1.55) containing 0.2% of NaCl was prepared. One gram of cell bead or 1 mL of cell culture was transferred into 9 mL of simulated gastric solution and incubated at 37°C for 0, 30 , 60 and 90 minutes. After the incubation, the beads were removed from simulated gastric solution and transferred into a test tube containing 9 ml simulated intestine juice (0.08 M K_2HPO_4, pH 7.43) with 0.6% bile salt. Incubation was made at 37°C for 150 minutes. After that, the beads were determined for viable cells by using pour plate technique with MRS media.

4.4 Survival rate of encapsulated cells under high temperature condition

Encapsulated cell of 0.5 g or 0.5 mL of cell culture was transferred into 24 mL of sterilized distilled water in a test tube. The tube was incubated at 85°C in a temperature-controlled water bath for 0, 10, 20 and 30 minutes. After that, the beads were determined for viable cells by using pour plate technique with MRS media.

4.5 Levan polysaccharide determination

Z. mobilis either from stock culture or encapsulated cells from high temperature or simulated food tract test was cultured in 500 mL MRS media containing 10% sucrose by incubating at 30°C for 48 h. Levan was precipitated by adding 250 mL of 75% ethanol. KOH at concentration 0.1 M was added to adjust pH to. The precipitant was separated out by using centrifugation at 5,000 rpm for 10 minutes and washed again with 75% ethanol. Obtained solid was hydrolyzed by 0.1 M HCl for one hour at 100°C. Quantification of the levan is done in fructose unit by using DNS method together with fructose standard curve.

4.6 Statistical analysis

Experimental results were used for analysis of variance using Statistical Analysis Software (SAS) computer software. Significant differences between means were determined by Duncan's multiple range test or student t test ($p<0.05$).

5. RESULTS AND DISCUSSIONS

5.1 Survival rate of encapsulated *Z. mobilis* cells from simulated food tract condition.

The survival test of *Z. mobilis* from simulated food tract condition combined both acid and bile salt digestion conditions in animal stomach and intestine, respectively. At first, *Z. mobilis* free cells were tested for their survival level in either simulated food tract or high temperature condition. It was found that the highest survival rate was 5.19% from 30 minutes treatment and the cells could not survive through 90 minutes treatment of simulated food tract condition (**Table 2**). It was obvious that increasing of treatment time caused cell death. It was not surprise because the cells directly exposed to very low pH condition (pH 1.55) and high concentration of bile salt in the simulated food tract condition. Then, encapsulated cells were tested with same condition as free cells. The results were almost every encapsulated cells had higher survival level than free cells (**Figure 3-5**). Higher alginate concentration and bigger size of encapsulated bead provided better protection to the cells in which the highest survival percentage was obtained from 30 minutes treatment time of sieve 10 of 3% alginate concentration with the value of 9.48%. In particular, 3% alginate concentration could significantly ($p < 0.05$) protect the cells better than 2% and 2.5% concentration. Reduction of alginate concentration and bead size, on the other hands, resulted in decreasing of survival rate. The lowest survival value was 0.77% from 90 minutes treatment time of sieve 70 of 2% alginate.

Table 2. Survival rate of *Z. mobilis* free cells in simulated food tract condition or high temperature condition at different treatment time.

	Simulated food tract condition			High temperature condition		
Time (min)	**30**	**60**	**90**	**10**	**20**	**30**
Survival percentage	5.19	0.98	0	15.48	1.28	0

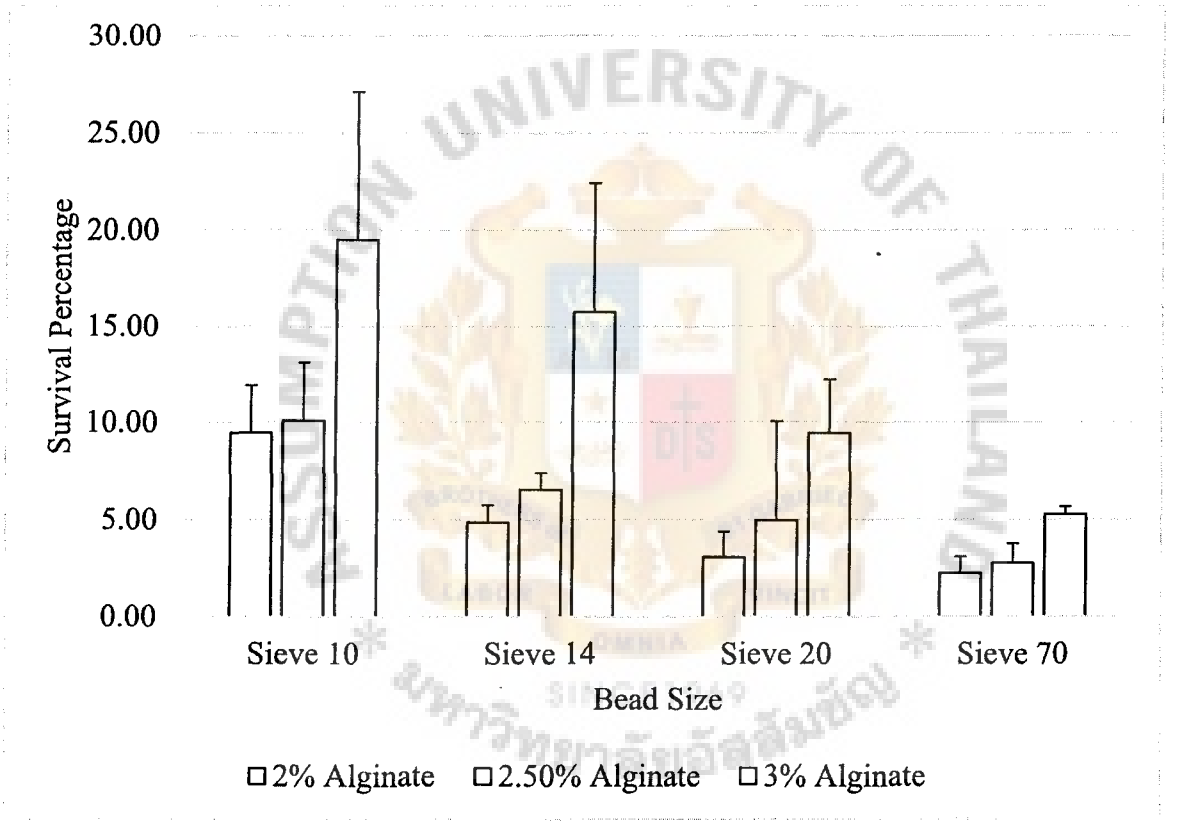

Figure 3. Survival rate of encapsulated *Z. mobilis* at different alginate concentrations from simulated food tract condition for 30 minutes.

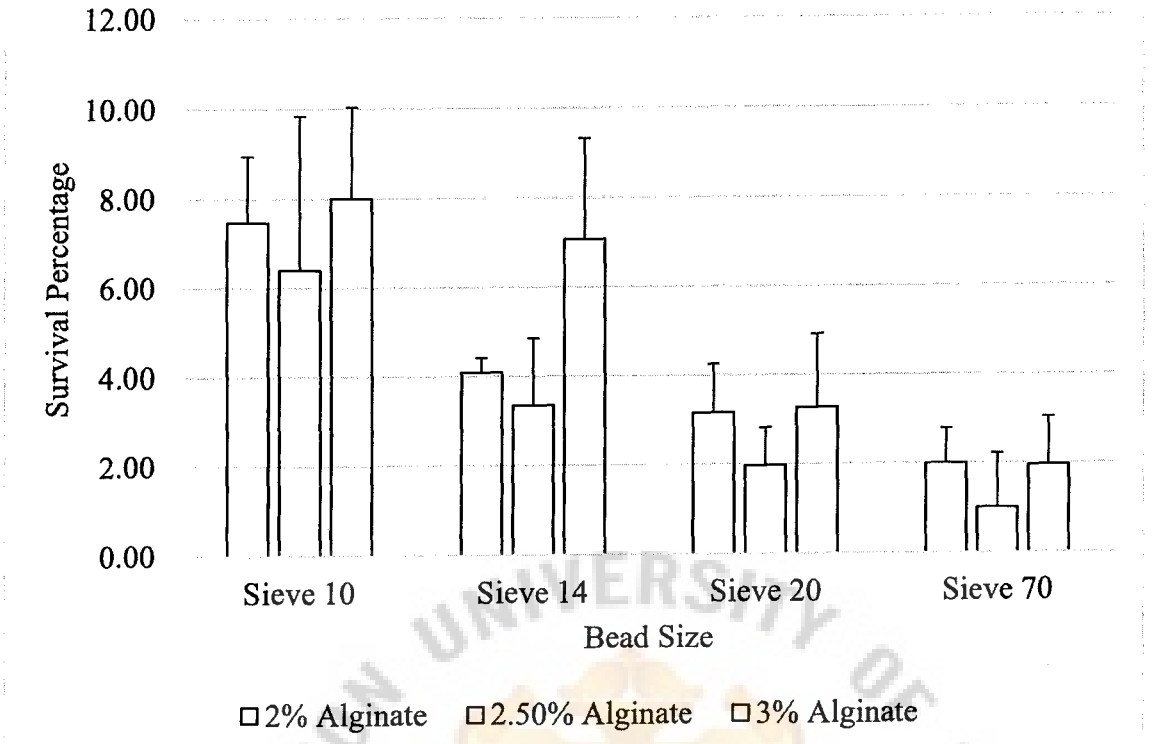

Figure 4. Survival rate of encapsulated *Z. mobilis* at different alginate concentrations from simulated food tract condition for 60 minutes.

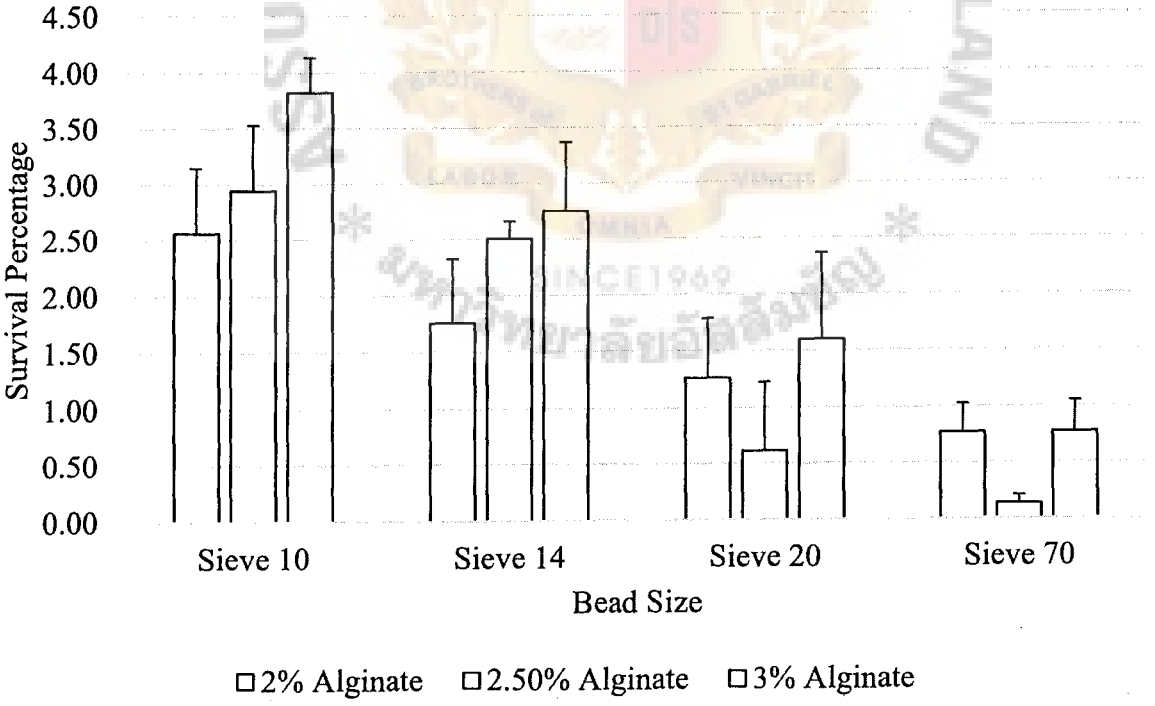

Figure 5. Survival rate of encapsulated *Z. mobilis* at different alginate concentrations from simulated food tract condition for 90 minutes.

5.2 Survival rate of encapsulated *Z. mobilis* cells from high temperature condition.

The high temperature test simulated critical condition to cell viability by feed production process, especially pelleting process where temperature was considered as high. Thus, hot water at 85°C was used in this test to represent the high temperature condition that *Z. mobilis* might face as being used as probiotics in animal feed. Cells without protectant could survive at 15.48% from the heat treatment test for 10 minutes. Longer exposure time dramatically reduced cell viability to 1.28% and 0% for 20 and 30 minutes treatment time, respectively (**Table 2**). Improvement of cell survival rate was found in encapsulated cells. Sieve 10 gel beads obtained from encapsulation of the cells with 3% alginate concentration could provide 17.09% survival rate from 10 minutes high temperature condition compared with untreated gel beads (control). However, smaller bead size (sieve 14, 20 and 70) and lower alginate concentration than 3% provided lower survival rate than free cell group (**Figure 6**). This was because alginate created porous structure gel which might cannot protect heat penetration efficiently. Although our shortest exposure time (10 min) showed insufficient protection to the cells, significant protection was found in longer treatment time 20 and 30 minutes (**Figure 7 and 8**). Gel bead from sieve 10 and 14 for all alginate concentrations gave higher survival rate than free cells in 20 minutes exposure time while sieve 20 bead provided similar cell viability to free cell. The most obvious protection from high temperature condition by encapsulation was found in 30 minutes treatment time. Free cells could not tolerate to this condition, but all encapsulated cells could. In addition, it was confirmed that increasing of encapsulant concentration and bead size resulting in reduction of cell death from extreme conditions.

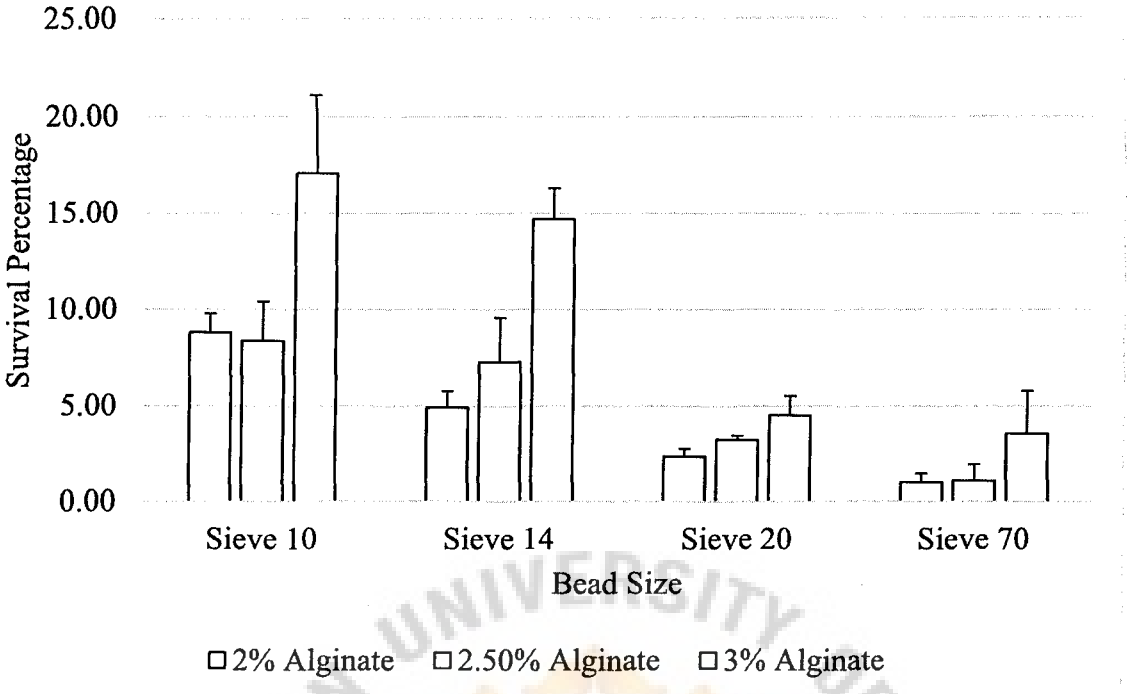

Figure 6. Survival rate of encapsulated *Z. mobilis* at different alginate concentrations from high temperature condition at 85°C for 10 minutes.

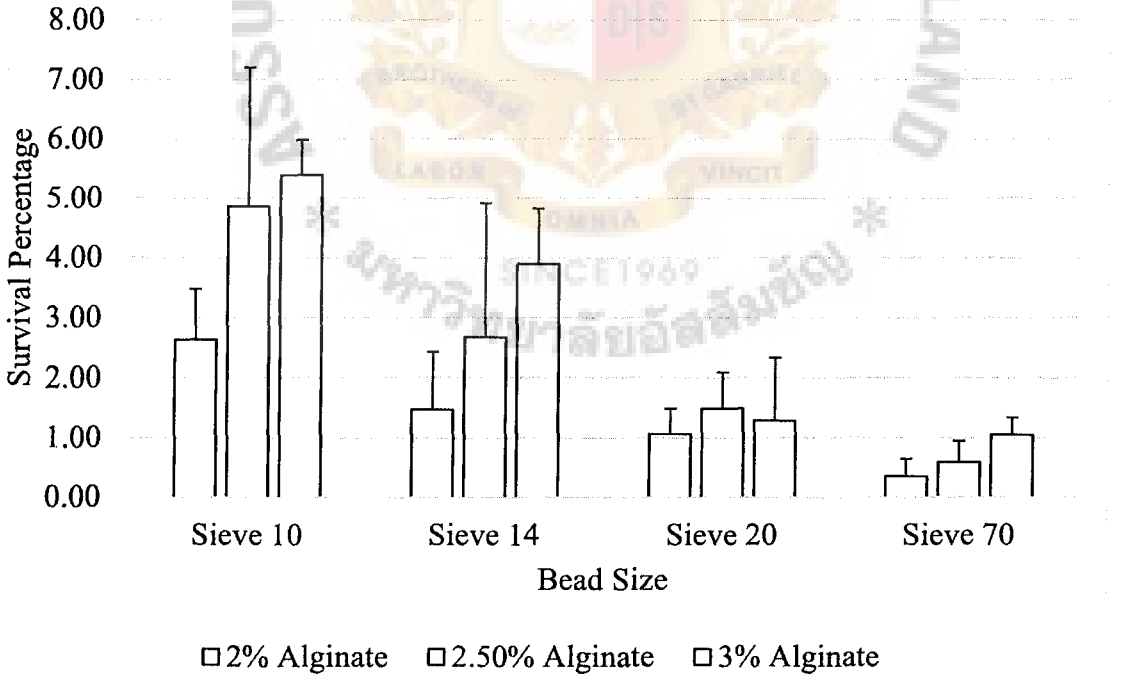

Figure 7. Survival rate of encapsulated *Z. mobilis* at different alginate concentrations from high temperature condition at 85°C for 20 minutes.

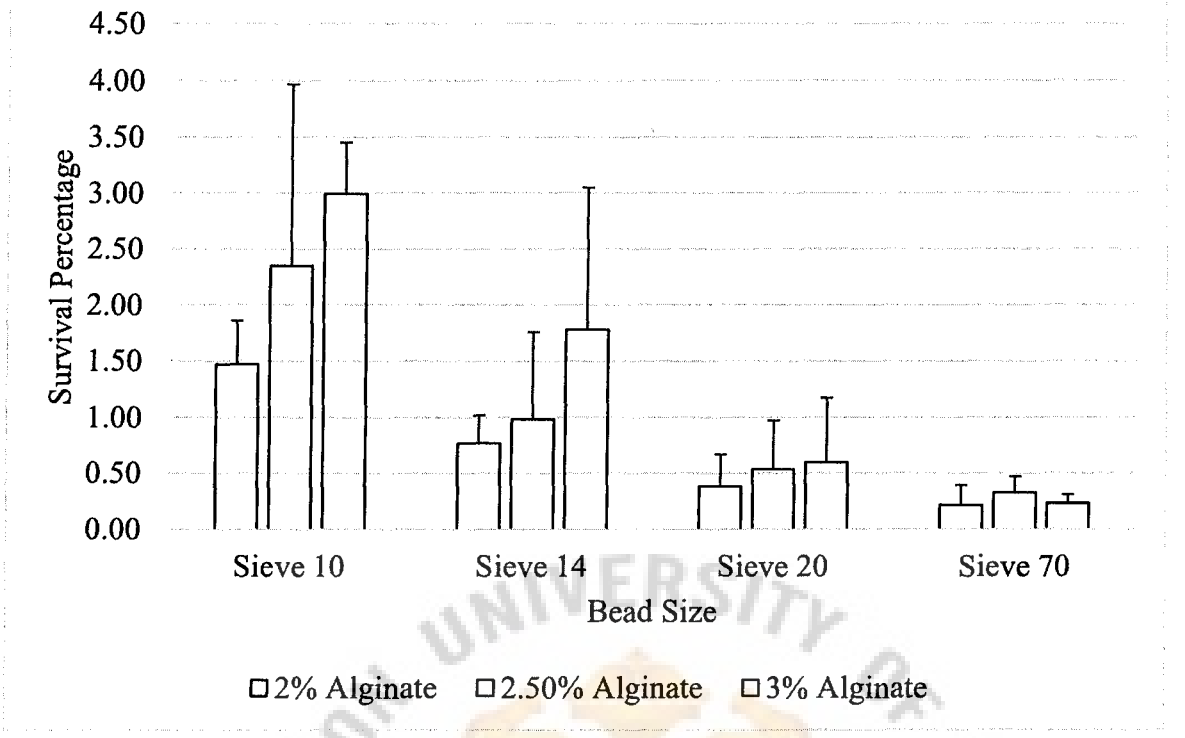

Figure 8. Survival rate of encapsulated *Z. mobilis* at different alginate concentrations from high temperature condition at 85°C for 30 minutes.

5.3 Effect of simulated food tract and high temperature condition on levan polysaccharide production ability

The production of levan polysaccharide was achieved when the bacteria of *Z. mobilis* was cultured and incubated at high concentration of fructose in which the sucrose is considered as a carbon source. In this test, three type of cells were used to study for levan polysaccharide production, including free cells, survived cell released from beads after testing in simulated food tract (SCFT), and high temperature condition (SCHT). Free cell group was a group that used stock cell culture to study for the levan production. Thus, the free cell group played as control group in this study. Survived cell samples were selected from treatment providing the highest survival rate from our study which was from sieve 10 of 3% alginate. Colony was picked up from MRS plate where viable cells, released from beads after passing through simulated food tract or high temperature test, for levan production determination as described in section 4.5. Level of levan

production was determined by following protocol used by Viikari (1984). All polysaccharides, including levan, from culture broth were precipitated and digested by HCl resulting reducing monosaccharide molecules. DNS method together with fructose as standard was used to determine concentration of these monosaccharide. The results (**Table 3**) showed that all cell samples could produce levan polysaccharide and there was no significance difference between among these three cells used for the experiment ($p > 0.05$). Therefore, encapsulation process, simulated food tract condition and high temperature condition did not affect levan polysaccharide ability of *Z. mobilis*.

Table 3. Production of levan polysaccharide by treated *Z. mobilis* from different conditions. (SCFT = Survived cell released from beads after testing in simulated food tract condition, SCHT = Survived cell released from beads after testing in high temperature condition.)

Cell type	**Concentration of produced levan polysaccharide (mg/mL)**
SCFT	0.35[a]
SCHT	0.44[a]
Free cell	0.46[a]

6. CONCLUSIONS

From this study, we found that encapsulation could significantly protect cells from both simulated food tract and high temperature condition over free cells. Encapsulation succeeded its goal and provided a great opportunity for *Z. mobilis* to be used as probiotics in animal feed. In addition, there were two factors; alginate concentration, and bead size, greatly affected cell viability of encapsulated cells. Increasing of alginate concentration and bead size could increase cell survival. Moreover, it seemed that heat was a major cell death cause in this study since viable cell level was lower than the level found in simulated food tract condition at the same exposure time. Lastly, we believed that there are gaps to improve cell protection from unsuitable environments and this would inspire further study to reach the main goal which is to practically use *Z. mobilis* as probiotics.

7. REFERENCES

Amerah, A. M., A. Quiles, P. Medal, J. Sánchez, M. J. Lehtinen, and M. I. Gracia. 2013. Effect of pelleting temperature and probiotic supplementation on growth performance and immune function of broilers fed maize/soy-based diets. *Anim Feed Sci Tech* 180(1-4):55-63.

Arnaud, J. P., C. Lacroix, L. Choplin. 1992. Effect of agitation rate on cell release rate and metabolism during continuous fermentation with entrapped growing. *Biotechnol Tech* 6(3):265-270.

Azerêdo, G. A., T. L. M. Stamford, E. L. Souza, F. F. Veras, E. R. Almeida, and J. M. Araújo. 2010. *In vivo* assessment of possible probiotic properties of *Zymomonas mobilis* in a Wistar rat model. *Electron J Biotechnol* 13(2).

Bekers, M., J. Laukevics, A. Karsakevich, E. Ventina, E. Kaminska, D. Upite, I. Vīna, R. Linde, and R. Scherbaka. 2001. Levan-ethanol biosynthesis using *Zymomonas mobilis* cells immobilized by attachment and entrapment. *Process Biochem* 36:979-986.

Burgain, J., C. Gaiani, M. Linder, and J. Scher. 2011. Encapsulation of probiotic living cells: From laboratory scale to industrial applications. *J Food Eng* 104:467–483.

Champagne, C. P., and K. Kailasapathy. 2008. Encapsulation of probiotics. In: Garti, N. (Ed.), Delivery and Controlled Release of Bioactives in Foods and Nutraceuticals. Woodhead publishing Ltd., Cambridge, UK, pp. 344–369.

Cock, L. S., and V. V. Castillo. 2013. Probiotic encapsulation. *Afr J Microbiol* Res 7(40):4743-4753.

Dien, B. S., M. A. Cotta, and T. W. Jeffries. 2003. Bacteria engineered for fuel ethanol production: current status. *Appl Microbiol Biotechnol* 63(3):258-266.

Franken, J., B. A. Brandt, S. L. Tai, and F. F. Bauer. 2013. Biosynthesis of levan, a bacterial extracellular polysaccharide, in the yeast *Saccharomyces*

cerevisiae. *Plos One* 8(10)

Fuller, R. 1989. Probiotics in man and animals. *J Appl Bacteriol* 66(5):365-378.

Shah, N. P. 2000. Probiotic bacteria: Selective enumeration and survival in dairy foods. *J Dairy Sci* 83:894-910.

Guarner, F., and G. J. Schaafsma. 1998. Probiotics. *Int J Food Microbiol* 39:237-238.

Hestrin, S., S. Avineri-shapiro, and M. Aschner. 1943. The enzymatic production of levan. *Biochem J* 37:450-456.

Kailasapathy, K. 2002. Microencapsulation of probiotic bacteria: technology and potential applications. *Curr Issues Intest Microbiol* 3:39-48.

Krasaekoopt, W., B. Bhandari, H. Deeth. 2003. Evaluation of encapsulation techniques of probiotics for yoghurt. *Int Dairy J* 13(1):3-13.

Krasaekoopt, W., B. Bhandari, H. Deeth. 2004. The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. *Int Dairy J* 14(8):737-743.

Lee, K. Y., and T. R. Heo. 2000. Survival of *Bifidobacterium longum* immobilized in calcium alginate beads in simulated gastric juices and bile salt solution. *Appl Environ Microb* 66:869-873.

Liepa, V., G. Zakenfelds, E. Volpe, Z. Koronova, R. Lapsa, M. Laivenieks, M. Bekeris, and I. Pospishil. 1993. Levan prolongs the life-span of tumor-bearing or irradiated mice, and enhances the antitumor activity. *Oncol Proc Latvian Academy Sci* 47(550):59-64.

Linger, J. G., W. S. Adney, and A. Darzins. 2010. Heterologous expression and extracellular secretion of cellulolytic enzymes by *Zymomonas mobilis*. *Appl Environ Microbiol* 76(19):6360-6369.

Mandal, S., A. K. Puniya, and K. Singh. 2006. Effect of alginate concentrations on survival of microencapsulated *Lactobacillus casei* NCDC-298. *Int Dairy J* 16:1190-1195.

Oliveira, M. R., R. S. S. F. Silva, J. B. Buzato, and M. A. P. C. Celligoi. 2007.

Study of levan production by *Zymomonas mobilis* using regional low-cost carbohydrate sources. *Biochem Eng J* 37:177-183.

Panesar, P. S., S. S. Marwaha, and J. F. Kennedy. 2006. *Zymomonas mobilis*: an alternative ethanol producer. *J Chem Technol Biotechnol* 81(4):623-635.

Picot, A., and C. Lacroix. 2004. Encapsulation of bifidobacteria in whey protein-based microencapsules and survival in simulated gastrointestinal conditions and in yoghurt. *Int Dairy J* 14:505-515.

Rao, A. V., N. Shiwnarain, and I. Mahraj. 1989. Survival of microencapsulated *Bifidobacterium psuedolongum* in simulated gastric and intestinal juices. *Can I Food Sc Tech J* 22:345-349.

Rogers, P. L., Y. J. Jeon, K. J. Lee, and H. G. Lawford. 2007. *Zymomonas mobilis* for fuel ethanol and higher value products. *Biofuels* 108:263-288.

Sheu, T. Y., and R. T. Marshall. 1991. Improving culture viability in frozen dairy desserts by microencapsulation. *J Dairy Sci*, 74(107).

Sheu, T. Y., and R. T. Marshall. 1993. Microencapsulation of lactobacilli in calcium alginate gels. *J Food Sci* 58:557-561.

Simpson, N. E., C. L. Stabler, C. P. Simpson, A. Sambanis, I. Constantinidis. 2004. The role of the $CaCl_2$-guluronic acid interaction on alginate encapsulated βTC3 cells. *Biomaterials* 25(13):2603-2610.

Srikanth, R., C. H. S. S. S. Reddy, G. Siddartha, M. J. Ramaiah, K. B. Uppuluri. 2015. Review on production, characterization and applications of microbial levan. *Carbohydr Polym* 120:102-114.

Viikari, L. 1984. Formation of levan and sorbitol from sucrose by *Zymomonas mobilis*. *Appl Microbiol Biotechnol* 19:252-255.

Yang, S., D. A. Pelletier, T. Y. S. Lu, and S. D. Brown. 2010. The *Zymomonas mobilis* regulator hfq contributes to tolerance against multiple lignocellulosic pretreatment inhibitors. *BMC Microbiol* 10:135.

8. APPENDIX

Composition of media and chemical solutions used in experiments

A. Media preparation (MRS, 500 mL)

- Dextrose sugar 10 grams
- Dihydrogen phosphate 1 gram
- Yeast extract 5 grams
- Agar

B. Phosphate buffer pH 7.0 for cell release

Solution 1: 0.2 M NaH_2PO_4

Solution 2: 0.2 M Na_2HPO_4

Mix 195 mL of solution 1 with 305 mL of solution 2 in a beaker. Then adjust volume to be 1 L by distilled water. One gram of bead is transferred to 9mL of the phosphate buffer. The mixture is shaken at speed 200 rpm at room temperature for 30 minutes.