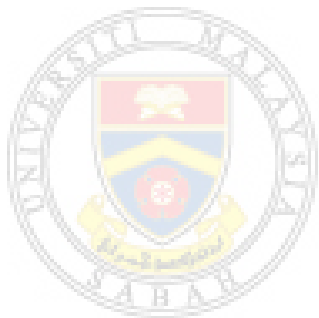


**IMMOBILIZATION OF PROBIOTIC CELLS IN
ALGINATE AND PECTINATE CAPSULES**



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UMS
UNIVERSITI MALAYSIA SABAH

**SCHOOL OF ENGINEERING AND
INFORMATION TECHNOLOGY
UNIVERSITI MALAYSIA SABAH
2011**

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ALGINATE AND PECTINATE CAPSULES**

VOO WAN PING



**THESIS SUBMITTED IN FULFILLMENT FOR
THE DEGREE OF MASTER OF ENGINEERING**

**SCHOOL OF ENGINEERING AND
INFORMATION TECHNOLOGY
UNIVERSITI MALAYSIA SABAH
2011**

DECLARATION

I hereby declare that the materials in this thesis are original except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

3 May 2011

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Voo Wan Ping
3 May 2011

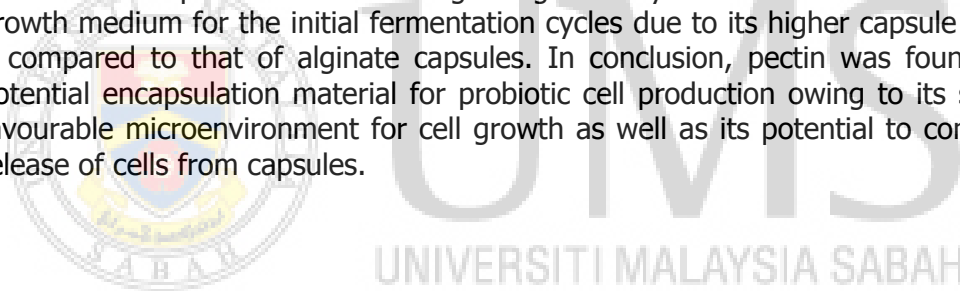


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ABSTRACT

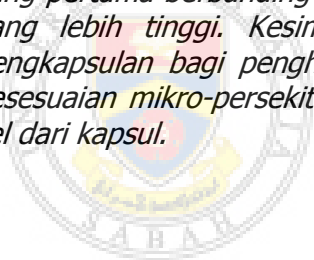
IMMOBILIZATION OF PROBIOTIC CELLS IN ALGINATE AND PECTINATE CAPSULES

A comparative study on the stability and the potential of alginate and pectin based capsules for production of poultry probiotic cells using MRS medium was conducted. The capsule cores, made of three types of materials, i.e. ca-alginate, ca-pectinate and ca-alginate/pectinate, were compared. The mechanical strength of ca-pectinate and ca-alginate/pectinate capsules was 2.5 and 4.3 times, respectively, of that of ca-alginate capsule. The pectin based capsules were found to be more stable than that of the alginate based capsules and their stability was further improved by chitosan coating. However, double layer coating of capsules did not improve the capsule stability due to the competing of polyanion material for chitosan binding, which had weakened the first layer coating. The cell concentration in pectin based capsules was comparable to that of the alginate based capsules. The maximum cell concentration of 1×10^9 CFU/ml was obtained with uncoated ca-alginate/pectinate capsules after two fermentation cycles. Cell concentration in capsules could be influenced by the gel network density since it determines the capsule stability and structural properties. On the other hand, pectin based capsules was found to give significantly lower cell concentration in the growth medium for the initial fermentation cycles due to its higher capsule stability if compared to that of alginate capsules. In conclusion, pectin was found to be potential encapsulation material for probiotic cell production owing to its stability, favourable microenvironment for cell growth as well as its potential to control the release of cells from capsules.



ABSTRAK

Suatu pengkajian atas kestabilan and potensi kapsul alginat dan kapsul yang berasaskan pektin untuk penghasilan sel probiotik ayam dengan menggunakan medium MRS telah dijalankan. Kapsul-kapsul yang mempunyai teras ka-alginat, ka-pektinat dan ka-alginat/pecktinat telah dibandingkan. Kekuatan mekanikal kapsul ka-pektinat dan ka-alginat/pektinat adalah 2.5 dan 4.3 kali ganda kekuatan mekanikal kapsul ka-alginat, masing-masing. Kapsul-kapsul yang berasaskan pektin didapati lebih stabil daripada kapsul alginat dan kestabilannya bertambah apabila dilapiskan dengan chitosan. Akan tetapi, kapsul-kapsul yang dilapiskan dengan dua lapisan tidak menunjukkan peningkatan atas kestabilannya. Ini adalah disebabkan oleh berlakunya perebutan antara polianion dalam proses pengikatan dengan chitosan. Perebutan antara polianion tersebut telah memburukkan kestabilan pengikatan antara chitosan dan polianion dari teras kapsul. Kepekatan sel dalam kapsul yang berasaskan pektin adalah sebanding dengan kepekatan sel dalam kapsul alginat. Kepekatan sel yang tertinggi telah diperolehi dengan menggunakan kapsul ka-alginat/pectinat yang tidak berlapis selepas dua fermentasi proses, iaitu sebanyak 1×10^9 CFU/ml. Kepekatan sel dalam kapsul dipengaruhi oleh kepadatan rangkaian gel kerana ia menentukan kestabilan kapsul dan ciri-ciri yang berkenaan dengan susunan rangkanya. Kapsul yang berasaskan pektin didapati menghasilkan kepekatan sel yang rendah dalam medium pertumbuhan bagi proses fermentasi yang pertama berbanding dengan kapsul alginat disebabkan oleh kestabilan kapsul yang lebih tinggi. Kesimpulannya, pektin didapati berpotensi sebagai bahan pengkapsulan bagi penghasilan sel probiotik disebabkan oleh kestabilannya dan kesesuaian mikro-persekitarannya serta potensinya dalam mengawal pembebasan sel dari kapsul.



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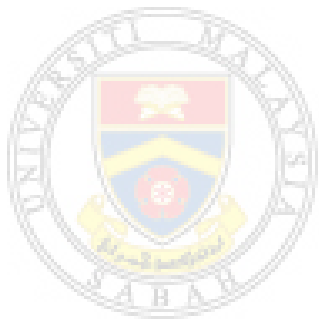
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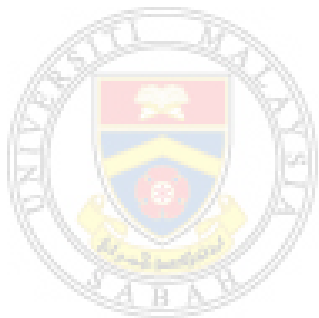
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LIST OF ACRONYMS

CFU	Colony forming per unit
DM	Degree of methoxylation
HM	High methoxylated
ICT	Immobilized cell technology
LAB	Lactic acid bacteria
LM	Low methoxylated
MRS	De Man, Gogosa, Sharpe



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LIST OF SYMBOLS

A	Total external surface area of capsules
C	Concentration of solute in bulk solution
c	Number of colony
C_{eq}	Equilibrium concentration
C_s	Concentration of solute inside capsule close to interphase boundary
D	Capsule diameter
d	Number of dilution
f	Dilution factor
h	Mass transfer coefficient
n	Number of capsule
N_E	Encapsulated cell concentration
N_F	Free cell concentration
τ_d	Doubling time
V_0	Volume of bulk solution
V_c	Total capsule volume accessible for solute

CHAPTER 1

INTRODUCTION

1.1 Research Background

Among the meat commodities, only poultry meat is popularly consumed due to its price and religious acceptability. Malaysia is keen to develop the potential as an international 'halal' food hub since Malaysia is recognized as a truly Islamic country and possesses raw materials, supporting infrastructure, and processing technologies to produce and market 'halal' products. Besides that, there is also worldwide recognition of Malaysia's 'halal' certification due to its stringent criteria and is sought after by other countries.

In recent years, there is a global awareness and concern over the use of antibiotics in poultry production. Concerns over antibiotic usage and residues are primarily related to food safety as intensive use in the feed of livestock would lead to the formation of resistant pathogenic bacteria in humans. In view of the severe restriction or total ban on the use of antibiotics as growth promoters in meat poultry production, probiotic cells, which are beneficial living microbes, have been suggested as an alternative to antibiotics (Khaksefidi and Ghoorchi, 2006). Several studies have shown that the addition of probiotics to the diets of broilers leads to improved performance on the production (Jin *et al.*, 1997; Jin *et al.*, 1998). It is foreseeable in near future that antibiotic-free animal-derived products would be part of the requirement for international trade.

However, preserving the viability of probiotics is challenging as probiotics are very sensitive to many environmental factors such as humidity, heat, gastric acidity and many other chemical and physical stresses. With the advance of technology, such as immobilized cell technology, the probiotics are able to better resist negative factors and improve efficacy. The use of immobilized cell technology has shown to give several desirable effects as well in cell production compare to the conventional method. Eventually, the conventional method of cell production has been replaced

by immobilized cell technology due its expensive separation processes that required for concentrating the cells (Champagne *et al.*, 1994). Immobilized cell technology not only produce concentrated cultures but also separate biomass easily from the medium without centrifugation or filtration because of the large size of the gel matrices thus reducing the overall production cost (Dembczynski *et al.*, 2002).

Cell immobilization has been studied by many researchers and the most commonly used cell immobilization methods is encapsulation in alginate hydrogel matrix. Cell encapsulation is particularly feasible for repeated batch fermentation because of its easy operation. Alginate is known to be biocompatible, non toxic and it can gel at mild condition with the presence of calcium cations. Formation of alginate capsules can be conducted in sterile environment and virtually any ingredient can be encapsulated, whether it is hydrophobic or hydrophilic, sensitive to temperature, a thin liquid or a viscous oil, and solid (Gouin, 2004).

1.2 Research Problem

Of all materials, alginate is the most widely used and investigated biopolymer for cell bioencapsulation. Alginate is biocompatible, and it can gel at mild condition with the presence of calcium cations. Although ca-alginate gel possess no toxicity against cells, it is known to be chemically unstable in the presence of calcium chelators such as phosphate, lactate or citrate and to cations such as sodium, magnesium, which are able to displace calcium. Removal of phosphate from MRS broth or addition of calcium cation into it was found to improve capsule stability (Yoo *et al.*, 1996). However, the change in the composition of growth media may affect cell growth parameters. The use of barium cation as gelling agent was also found to enhance the chemical stability of alginate capsules (Ivanova *et al.*, 2000). However, barium was reported to induce negative effect on cells due to its toxicity to cells (Harel *et la.*, 2000; Wideroe and Danielsen, 2001).

On the other hand, ca-pectinate gel had been reported to be less sensitive to ions and chemical agents if compared to ca-alginate (Kurillová *et al.*, 1992; Berger and Rühlemann, 1988). In addition, the stability constant of ca-pectinate gel was also reported to be one order of magnitude higher than that of ca-alginate gel

(Gemeiner *et al.*, 1996). There are number of studies on pectin based capsules, however, there are very limited reports on probiotic cell production using pectin based capsules especially studies with direct comparison on the use of these materials.

1.3 Research Objectives

The overall objective of this research is to evaluate the stability of pectin based and alginate/pectinate composite based capsules in comparison to alginate based capsules for production of probiotic cells using MRS medium in repeated batch fermentation. Nine types of capsules were used in this study, which include the uncoated capsules, single layer coated capsules and double layer coated capsules.

Non-microbial capsules were used for the characterization of capsule in chemical stability, mechanical strength and mass transfer coefficient. The study of capsule stability is important to ensure that capsules are able to sustain for as many fermentation cycles as possible. The study for mass transfer coefficient of capsules is also important since mass transfer within capsule is necessary for cell production to ensure the required nutrients and cell product to pass through the capsule wall. The experimental set-up and condition for characterizing the capsules were all based on the similar experimental set-up and condition used for the microbial capsules in repeated batch fermentation for better comparison purpose.

The followings are the specific objectives for this study;

- i. To study the mass transfer property of the capsule
- ii. To study the mechanical strength and stability of the capsule
- iii. To determine and compare the cell concentration in fermentation medium and in capsules
- iv. To determine and compare the maximum cell concentration obtained from free suspended cell fermentation and encapsulated cell fermentation
- v. To study the effect of single and double layer coating on capsule stability

1.4 Thesis Layout

This thesis consists of five chapters which discussed the work in details. The contents of each chapter are briefly described in the following paragraphs.

Chapter one presented the introduction of thesis. A brief description on the research background and research problem on probiotic cells and the methods of cell production were discussed. The overall objective and specific objectives for this research were listed in this chapter. In addition, the thesis layout of this research was also presented.

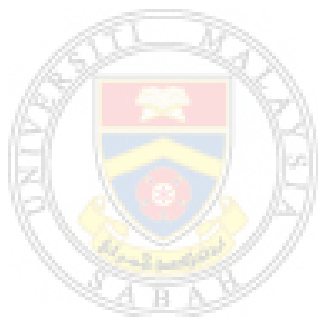
Chapter two mainly focuses on the literature review which began with the substitution of poultry antibiotic by probiotic cells and fermentation methods. The types of biomaterials used for encapsulation were reviewed, including alginate and pectin. In addition, cell growth patterns were also included in this chapter.

Chapter three discussed the materials and methods used in this research. All the equipments used for experiments were mentioned. Cultivation of probiotic cells with different carrier materials in repeated batch fermentation was described in details. In addition, determination of both the free cell and encapsulated cell concentration was presented with the aid of mathematical equations.

Chapter four reported the results on the characterization of capsules and cell growth pattern in capsules. Nine types of capsules were used in this study. Chitosan was used as the first layer coating material whereas the formulation of the core capsule was used as the second layer coating material. A mixed-strain of poultry probiotic cells was encapsulated within the capsules and the capsules were repeatedly used up to six fermentation cycles. The chemical stability, mechanical strength, mass transfer coefficient and cell concentration in the capsule as well as the free cell concentration in the growing medium were all listed in this chapter.

Chapter five discussed the experimental results obtained and compared to the results reported in the literature review. The effect of coating on alginate, pectin and alginate/pectin core capsules was discussed in terms of capsule stability, maximum cell concentration and cell release from the capsules.

Chapter six concludes the thesis based on the findings in this work, which includes the mass transfer of capsules as well as the capsules stability. The effect of different carrier materials used for cell production was also listed in this chapter.



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CHAPTER 2

LITERATURE REVIEW

2.1 Probiotics

Probiotic, which means "for life" in Greek (Gibson and Fuller, 2000), has been defined as a live microbial feed supplements, which beneficially affects the host animal by improving its intestinal balance (Fuller, 1989). Lactic acid bacteria is one of the probiotic groups which make up a large group of microorganism in gastrointestinal tract of all human and animals (Musikasang *et al.*, 2009). Poultry probiotic cells are commonly mixed culture of lactic acid bacteria and they can be grown by using the MRS medium. Recently, emphasis has been placed on the selection, preparation and application of probiotic strains especially lactic acid bacteria. The natural adaptation of lactic acid bacteria to the gut environment and the lactic acid produced by them has provided these organisms with an advantage over other microorganisms to be used as probiotics (Guerra *et al.*, 2007). The basic requirements for an lactic acid bacteria strain which is to be used as probiotic have been described as follows (Lin *et al.*, 2007);

- i. tolerant to acid and bile and be able to adhere to the intestinal epithelium of the hosts
- ii. show an antagonistic activity against pathogenic bacteria, and
- iii. keep their viability during processing and storage.

2.2 Probiotic Cells as Poultry Antibiotic Substitute

The use of antibiotics as growth promoters in animal feeding dates from 1940's, when the addition of subtherapeutic dosages of antibiotics resulted in great benefits for animal rearing, expressed as significant improvements in weight gain, feed conversion and viability (Pelicano *et al.*, 2004). The addition of growth promotion antibiotics became a common practice within five years as a result of improvement in growth (Graham *et al.*, 2007). Antibiotics have been used in poultry production as therapeutic agents to treat bacterial infections that decrease

performance and cause diseases. Many of the antibiotics used in the poultry industry have also been used in human medicine as well (Edens, 2003).

However, the greatest threat to the use of antibiotics is the emergence and spread of resistance in pathogenic bacteria that consequently cannot be treated by previously successful regimens (Mathur and Singh, 2005). Concern over the emergence of antibiotic-resistant pathogens from animals fed antibiotics has resulted in worldwide attempt to reduce antibiotic use in animal production because increased microbial resistance to antibiotics and residues in animal products can be harmful to consumers (Jin *et al.*, 1998). In June of 1999, the European Union had banned the use of some growth promoting antibiotics in poultry feeds due to this problem (Edens, 2003). The ban will ultimately affect most of the poultry exporting countries. Thus, it is foreseeable in near future that antibiotic-free animal-derived products would be part of the requirement for international trade.

As consumers begin to look for minimally processed, organic, and naturally raised products, alternative technologies are required to maintain livestock productivity (Flint and Garner, 2009). One method which is receiving considerable recent attention as a natural alternative to enhancing animal productivity and improving product safety is the feeding of viable microorganisms, which is the probiotics (Brashears *et al.*, 2005; Krehbiel *et al.*, 2003). Past research has shown that administering probiotics can provide the same protection as a naturally developed commensal gastrointestinal tract microflora (Nurmi and Rantala, 1973; Pascual *et al.*, 1999; Kubena *et al.*, 2001; LaRagione *et al.*, 2001). Probiotics that are used in broilers include *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, *Pediococcus*, *Enterococcus* and yeast such as *Saccharomyces cerevisiae* and *Saccharomyces boulardii* (Kabir *et al.*, 2004; Mountzouris *et al.*, 2007).

Probiotics often consist of live microbial cultures that are isolated from the gastrointestinal tract of a healthy adult animal of the same species to which the probiotic product will be administered. The use of probiotics may provide an alternative to the administration of subtherapeutic levels of antibiotics in preventing the colonization of the gastrointestinal tract by unfavourable microorganisms

(O'Dea *et al.*, 2006). Microbial populations within the gastrointestinal tract colonize very quickly after hatching (Guan *et al.*, 2004). Contact with microorganisms on the eggshell (Coates and Fuller, 1977) or in feed (Jones and Richardson, 2004) contribute to microbial colonization of the gastrointestinal tract. It is during this early period, when a stable gut microflora has not yet been established, that the chick is most vulnerable to colonization by pathogens. Hence, establishment of a healthy gastrointestinal tract microflora in newly hatched broiler chicks provides vital protection against these undesirable organisms (O'Dea *et al.*, 2006).

The practice of applying probiotics to animal feedstuffs is more correctly known as direct-fed microbials. The advantages of direct-fed microbials consumption in humans have been recognized for centuries; however, their application and efficacy in livestock operations have only recently been pursued (Flint and Garner, 2009). Several studies have shown that the addition of probiotics to the diets of broilers leads to improved performance (Jin *et al.*, 1997, 1998). Also evidence is accumulating which suggests probiotics exert an essential role in stimulating the immune system in avian (Jin *et al.*, 1997). The mode of action of probiotics in poultry includes (Jin *et al.*, 1997);

- i. maintaining normal intestinal microflora by competitive exclusion and antagonism
- ii. altering metabolism by increasing digestive enzyme activity and decreasing bacterial enzyme activity and ammonia production
- iii. improving feed intake and digestion
- iv. neutralizing enterotoxins and stimulating the immune system

2.3 Conventional Methods for Cell Production

Despite much scientific evidence on the beneficial effects of probiotics on farm animals (Jin *et al.*, 1998; Pascual *et al.*, 1999; Kalavathy *et al.*, 2003; Kosin and Rakshit, 2006), probiotic feed additive is still not well received by local poultry farmers. This is simply because probiotic feed additive costs more than antibiotics due to the production method of the probiotic cells. Probiotic cell cultures are traditionally produced by using the conventional methods of fermentation that uses free cells in continuous and batch processes. The cells are then recovered by