UNIVERSITI PUTRA MALAYSIA

CHANGES IN MICROBIAL AND PHYSICOCHEMICAL PROPERTIES DURING FREE-CELL AND ENCAPSULATED PROBIOTIC FERMENTATION OF RED DRAGON FRUIT JUICE

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By

ONG YIEN YIEN

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Red dragon fruit or red pitaya (*Hylocereus polyrhizus*) has gained its popularity in recent years because of its unique appearance and high nutrient contents (fiber, oligosaccharides, proline, potassium, sodium, and betacyanin). Most of the time, the fruit is consumed freshly but the shelf-life of the fruit is short (5 to 7 days at room temperature). A solution is proposed in this research to extend the shelf-life of the fruit by developing a probiotic beverage from red dragon fruit juice through fermentation. The development of probiotic beverage involves the selection of probiotic strains, the study of fermentation parameters and the improvement of the viability of the probiotic in fermented red dragon fruit juice. The selection of probiotic begins with the isolation and identification of lactic acid bacteria (LAB) from red dragon fruit as probiotics are mostly LAB. Bacteria were isolated on MRS agar and the species of the isolates was identified using biochemical tests, polymerase chain reaction (PCR), randomly amplified polymorphic DNA (RAPD) PCR, 16S rDNA sequencing, and restriction fragment length polymorphism (RFLP). Two species namely *Enterococcus casseliflavus* and *Enterococcus gallinarum* were isolated from red dragon fruit. Although the isolated LAB were found to be non-probiotic strains, the suitability of red dragon fruit juice as medium for probiotic fermentation should not be disregarded. Hence, *Lactobacillus casei* was used as probiotic inoculums for the development of a non-dairy based probiotic beverage using red dragon fruit juice as fermentation medium. Several parameters including the period of fermentation, incubation temperature, size of inoculums and addition of prebiotic (fructooligosaccharides, FOS) were studied in the fermentation. Free cell probiotic femrmentation was carried out successfully in which the amount of probiotic was increased as much as 4.2 log cfu/mL after the juice was fermented for 2 day at 37^{o}C. A minimum of 1% (v/v) probiotic can be inoculated into the juice to product the same amount of cell increment. The cell increment after fermentation can be further improved to 5 log cfu/mL by adding 1% (w/v) FOS into red dragon fruit juice. Viability loss of 1.2 log cfu/mL of probiotic in fermented red dragon fruit juice was observed after 4 week storage at 4^{o}C. The viability loss can be reduced by microencapsulated the probiotic bacteria in alginate. In terms of microencapsulation, parameters such as period of fermentation, addition of second coating in microencapsulation, amount of cell load in alginate beads and concentration of calcium alginate beads were studied. The results of probiotic microencapsulation was promising in which lesser viability loss of probiotic (0.5 log cfu/g) was observed in red pitaya juice fermented with encapsulated *L. casei* after 4 week storage at 4^{o}C when comparing to free cell *L. casei* fermented juice. The viability loss of encapsulated probiotic in red dragon fruit juice can be further improved with 1% (w/v) calcium alginate beads with 10^{7} to 10^{9} cfu/g of probiotic in the beads. Viability loss was reduced to as low as 0.07 log cfu/g in fermented red dragon fruit juice with microencapsulated probiotic and the results were much better than free cell probiotic which reduced 2.2 log cfu/g after stored at 4^{o}C for 4 week. Fermentation of red dragon fruit juice with free cell and microencapsulated probiotic showed no adverse effect on the physico-chemical characteristics especially the antioxidant properties of red dragon fruit juice. The development of probiotic beverage in red dragon fruit juice prolonged the shelf-life of the fruit as well as transforming the fruit juice into a healthy beverage that contains high antioxidant properties (total phenolic content: 29.6 to 31.42 mg/L gallic acid equivalent, ABTS$^{+\bullet}$ inhibition: 12% to 13.1%) and probiotic. In addition, the red dragon fruit juice based probiotic drink is suitable for individuals that are milk allergic and lactose intolerant.

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APPROVAL

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
CAE	Catechin
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DVI	direct to the vat inoculation
FOS	Fructoligosaccharides
GAE	Galic acid equivalent
GRAS	Generally Recognized as Safe
HPLC	High performance liquid chromatography
ITS	Internal transcribed spacer
LAB	Lactic acid bacteria
ME	Microencapsulation
PCR	Polymerase chain reaction
RAPD	Randomly amplified polymorphic DNA
RE	Restriction endonuclease
RFLP	Restriction fragments length polymorphism
RI	Refractive index
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel
SEM	Scanning electron microscope
TEM	Transmission electron microscope
TPC	Total phenolic content
TSC	Total soluble solid content
TTA	Total titratable acidity

CHAPTER I

INTRODUCTION

Fermentation refers to the process of converting raw materials for example carbohydrates, proteins, lipids and others into useful products with the aid of microorganisms and enzymes (Stanbury, 1995). Fermentation aims at producing fermented product such as biomass, microbial metabolites, and microbial enzymes (Stanbury, 1995). In food industry, fermentation is important for food preservations which help to extend the shelf-life of food products apart from improving flavor, texture and the nutritional content (Stanbury, 1995).

In recent years, dragon fruit or pitaya has gained its popularity in all walks of life worldwide due to its unique appearance, taste and nutritional content (e.g. vitamins, minerals, complex carbohydrates, dietary fibres and antioxidant). The fruit is originated from Latin America but it is also available in tropical and sub-tropical countries for example Malaysia. Annual production of dragon fruit has achieved more than 16,000 metric tons in Malaysia (FAMA, 2006). Among the fruits, red dragon fruit (*Hylocereus polyrhizus*) is more preferable to white pitaya (*Hylocereus undatus*) owing to its unique red-colored flesh. The red pigment contained in red dragon fruit was found to be betacyanin, which is also associated with antioxidant properties of red dragon fruit (Khalili *et al.*, 2012). Red dragon fruit brings a number of health benefits when it is consumed fresh such as prevention of colon cancer and diabetes, neutralization of toxic substances, reducing the levels of cholesterol and high blood pressure (Jaafar *et al.*, 2009). The matured red dragon fruit can be stored at ambient temperature for 3-4 days and 1-2 weeks at temperature 20^{o}C or 14^{o}C (Le Bellec *et al.*, 2006). The shelf-life of red dragon fruit is relatively short at room temperature (5-7 days) and medium (1-2 weeks) at cool temperature compared to other fruits. Therefore, the development of a new food product from red dragon fruit is proposed to overcome the problem of shelf-life. Existing food products derived from red dragon fruit include juices, jams, jellies, candies and yogurts (Jamilah *et al.*, 2011). Owing to the significant prebiotic content in red dragon fruit (Wichienchot *et al.*, 2010), a potential probiotic beverage could be developed from this fruit as prebiotic are known to promote the growth and activities of probiotic (Saminathan *et al.*, 2011; Soccol *et al.*, 2010; Nazzaro *et al.*, 2008; Kaplan and Hutkins, 2000; Shin *et al.*, 2000).

Before starting the probiotic fermentation, it is crucial for us to know whether there is any probiotic that is derived from red dragon fruit. Screening of probiotic is important because strains isolated from natural sources tend to be more stable and suitable for food utilization (Adnan and Tan, 2007). In addition, red dragon fruit has been studied in various aspects such as physico-chemical characteristics, nutritional contents, crop plantation and so on but not the microbiology content. Thus, the isolation and identification of microorganisms (especially lactic acid bacteria, LAB) from fresh red dragon fruit has to be done to figure out the suitability of the isolated strains to be applied in probiotic fermentation.

Probiotic is a group of microorganisms that exerts positive health effects when sufficient amount of the active microorganisms reach the intestine (de Vrese and Schrezenmeir, 2008). Common probiotics are derived from the genera *Lactobacillus* and *Bifidobacterium*, however there are also other genera such as *Enterococcus* spp. *Lactococcus* spp., *Streptococcus* spp. and yeast that fall into the group of probiotic (de Vrese and Schrezenmeir, 2008). Probiotics are able to modulate the intestinal microflora of human intestine so that a balanced microflora is achieved (de Vrese and Schrezenmeir, 2008). They are capable to prevent and reduce symptoms of diseases such as diarrhea and inflammatory bowel disease (de Vrese and Schrezenmeir, 2008). The health benefits brought by probiotics prompt the incorporation of probiotic in various food products such as fermented milks, ice cream, cheese, milk powder, buttermilk and yogurts (Kechagia *et al.*, 2013). Among these food products, fermented milks and yogurts are found to be unsuitable for lactose intolerance and milk allergic individuals. Considering these groups of individual, non-lactate based drink such as red dragon fruit juice is a suitable choice to be developed into a non-dairy probiotic drink.

Selection of suitable probiotic strain for fermentation is important and the viable cell number of probiotics should reach a desirable population for the drink to be beneficial. Some researchers proposed the amount of probiotic to be above 10^6 cfu/g or ml and minimum ingestion of probiotic of 10^8 to 10^9cfu is required to compensate possible reduction of cell concentration when the probiotic passes through intestinal passage (Prado *et al.*, 2008; Shah, 2007; 2000; Vinderola and Reinheimer, 2000; Dave and Shah, 1997b). The amount stipulated by researchers also ensures the induction of changes in microflora for gut health such as balancing microflora in human intestine and inhibiting the colonization of pathogenic microbes (de Vrese and Schrezenmeir, 2008).

During the process of fermentation, probiotic strains may produce various metabolites and end products for example lactic acid that would decrease the pH and affecting the survivability of probiotic especially after 4 weeks storage. Amount of living probiotics in the product is important for exerting the health benefits when probiotics enter into human guts. Studies showed a decrement of probiotic for more than 2 log cycles while some even lost their viability totally after 2 weeks of cold storage due to the low pH and high acidity of the medium after fermentation (Rivera-Espinoza and Gallardo-Navarro, 2010; Charernjiratrakul *et al.*, 2007; Yoon *et al.*, 2006). Therefore, it is recommended that the probiotic strains should be protected to extend their shelf-life. Microencapsulation technique has been reported to improve the survival rate of probiotic strains against adverse environmental conditions thus it is used in current study to ensure the viability of probiotic in red dragon fruit juice (Chen and Chen, 2007).

Red dragon fruit contains substrates that are suitable for the utilization of probiotics, therefore a probiotic drink is can be developed from red dragon fruit juice through fermentation. Other than that, the viability loss of *L. casei* in fermented red dragon fruit juice during the storage period can be minimized by incorporating microencapsulation technique during fermentation.

1.1 Objectives

Red dragon fruit possesses a short shelf-life at room temperature and the problem could be overcome by transforming the fruit into probiotic beverage. Owing to the nutrient contents and significant amount of prebiotic, red dragon fruit juice could serve as a potential medium for probiotic fermentation. In order to develop a fermented red dragon fruit juice containing probiotic, the following objectives were formulated.

1. To isolate and identify lactic acid bacteria (LAB) from fresh red dragon fruit.
2. To study the effect of probiotic fermentation on the properties of microbial and physico-chemical characteristics of red dragon fruit juice and its prolong storage.
3. Study of the effects of microencapsulation on the survivability of probiotic strain during storage of 4 weeks at cold temperature (4^{o}C).

1.2 Organization of the Thesis

Chapter I includes the general introduction on current study about probiotic fermentation using red dragon fruit juice. Objectives and scope of the study are stated clearly in this chapter and the organization of the whole thesis is illustrated.

Chapter II provides information regarding red dragon fruits, fermentation, probiotic, and microencapsulation techniques. This chapter also reports some recent researches that investigate the mentioned subjects which are related to the current study.

Chapter III describes the isolation and identification of LAB from red dragon fruit and its naturally fermented product using biochemical tests and molecular approaches. Detailed methodology is included in this chapter followed by results and discussion. The results are summarized in conclusion section and LAB strain isolated from red dragon fruit and its naturally fermented product will be revealed. Suitability of the isolated LAB strain for the application of fermentation is also discussed in this chapter.

Chapter IV begins with the screening of probiotic strains for the probiotic fermentation in red pitaya juice. Materials and methods used in probiotic fermentation are described in this chapter. The results of each probiotic strain in terms of their increment of viable cell count after fermentation and viability loss after 4 week storage are reported in results and discussion part and the most suitable strain was chosen to proceed with the study of the fermentation of probiotic in red dragon fruit juice. The fermentation is studied for a few parameters such as fermentation time, fermentation temperature, inoculum sizes, and fermentation with addition of prebiotic. The detailed methodologies are reported in this chapter and the results are discussed to obtain the best fermentation condition that has the greatest increase in viable cell count. Changes of physico-chemical characteristics of fermented red dragon fruit are also analyzed in this chapter. Lastly, the shelf-life of the probiotic fermented red pitaya juice is studied over a period of 4 weeks to determine the viability loss of the fermented juice.

Chapter V describes the incorporation of microencapsulation techniques into probiotic fermentation of red dragon fruit juice. Several parameters (fermentation time, addition of prebiotic as second coating, concentration of inoculums in calcium alginate beads, and concentration of calcium alginate) of microencapsulation techniques are demonstrated and studied in the red pitaya juice fermentation. Detailed methods of studying different parameters are reported in this chapter. Physico-chemical characteristics, growth of probiotic during fermentation and viability loss of probiotic after 4 week storage are studied and discussed to examine the ability of calcium alginate beads in protecting the living probiotic.

Chapter VII summarizes and concludes the significances and findings of current research. Limitations of the current research are illustrated and recommendations for further research are also included in this chapter.

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