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PRODUCTION AND PURIFICATION OF BACTERIOCIN-LIKE INHIBITORY SUBSTANCE FROM Pediococcus acidilactici Kp10

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

PRODUCTION AND PURIFICATION OF BACTERIOCIN-LIKE INHIBITORY SUBSTANCE FROM *Pediococcus acidilactici* Kp10

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September 2016

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Recent interest in biopreservatives as natural substitute has grown among consumer for its ability to maintain the organoleptic, nutritional properties and safety of the food with little usage of chemical preservatives and lower heat intensity. Several bacteriocin-like inhibitory substance (BLIS) have been produced from the two principal species of Pediococcus, *Pediococcus acidilactici* and *Pediococcus pentosaceus.*The major problem regarding the application of bacteriocin-producing starter culture and BLIS in food fermentation is to determine the suitable condition that can promote both growth and BLIS production as well as simple and low cost purification method. The characteristics of BLIS also determine the effectiveness of their incorporation into the food products.

In this study, the ability of *P. acidilactici* Kp10 strain in producing bacteriocin-like inhibitory substance (BLIS) in various medium formulations was investigated by shake flask fermentations at condition of 28.5˚C, 120rpm for 24h. M17 (3822.95 AU/mL) was chosen as the best media among MRS, TSB, and NB according to its high bacterial cell growth and BLIS production. A one-way between groups analysis of variance (ANOVA) was used to investigate the ability to produce high BLIS production among those four basal media. The ANOVA was statistically significant ($p<0.05$), indicating that M17 produce highest BLIS production. However, MRS, TSB and NB media did not significantly differ with each other ($p > 0.05$) upon Post-hoc comparison using the Tukey HSD test.

The stability of crude extract of BLIS produced by *P. acidilactici* Kp10 has also been evaluated under different temperatures, pHs and storage periods with different indicator microorganisms (*L. monocytogenes* ATCC 15313, *E. coli* ATCC 35218 and *S. aureus* ATCC 33591).The results represented robust characteristics of BLIS at

wider pH range (pH2-9), temperature (4˚C, -20˚C, -80˚C, 100˚C, and 121˚C) and long period of storage (1, 3, and 6 months) at 4˚C, -20˚C, and -80˚C temperature.

The purification of crude extract of BLIS was conducted using aqueous two-phase flotation system (ATPF) with polyethylene glycol (PEG) and sodium citrate.The optimum purification condition of BLIS by ATPF was achieved at PEG 8,000/sodium citrate comprising TLL of 42.6, V_R of 0.4, C_L of 22% (w/w) at pH 7 with an average F_t of 30 min and F_R at 20 mL/min. BLIS of *P. acidilactici* Kp10was successfully purified using aqueous two-phase flotation system (ATPF) which gave the purification of 5.9-fold with a separation efficiency of 99%.The size of purified BLIS from SDS-PAGE analysis is 12.5 kDa. The findings revealed a great potential for commercial application of BLIS from *P. acidilactici* Kp10 strain.

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PENGHASILAN DAN PURIFIKASI BAHAN PERENCAT SERUPA-BAKTERIOSIN DARIPADA *Pediokokus asidilaktisi* Kp10

Oleh

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Perkembangan terkini terhadap pengawet bergred makanan berasaskan sumber biologi yang selamat telah berkembang di kalangan pengguna kerana ia dapat mengekalkan organoleptik,cirri-ciri nutrisi dan keselamatan makanan dengan penggunaan kurang bahan pengawet kimiadan kurang pendedahan kepada rawatan haba. Beberapa Bahan Perencat Serupa- Bakteriosin (BLIS) telah dihasilkan oleh dua sepsis utama *Pediococcus* iaitu *Pediococcus acidilactici* dan *Pediococcus pentosaceus.* .Namun begitu, masalah utama yang berkaitan dengan penggunaan pemula bacteria penghasil kultur dan BLIS di dalam fermentasi makanan ialah untuk mengenalpasti keadaan yang dapat menggalakkan pertumbuhan dan penghasilan BLIS dan juga penggunaan kaedah purifikasi yang murah dan mudah. Oleh kerana ekosistem makanan itu sendiri adalah kompleks, pH, suhu, atmosfera dan kewujudan pelbagai jenis mikroorganisma akan mempengaruhi pertumbuhan bacteria seterusnya mempengaruhi penghasilan BLIS. Selainitu, ciri-ciri BLIS itu sendiri turut menentukan keberkesanan penggunaannya di dalam produk makanan. Di dalam kajian ini, keupayaan strain *P. acidilactici* Kp10 dalam menghasilkan BLIS dengan menggunakan beberapa formulasi media asas telah dikenalpasti melalui fermentasi menggunakan kelalang goncang pada suhu 28.5°C, 120 kitaran per minit selama 24 jam. Media M17 (acc. to Terzaghi) telah dipilih sebagai media terbaik dengan penghasilan aktiviti BLIS yang paling tinggi sebanyak 3822.95 AU/mL berbanding tiga media lain iaitu deMannRogosa Sharpe (MRS), Tryptic Soy broth (TSB), dan Nutrient broth (NB). Media M17 dipilih berdasarkan keputusan pertumbuhan strain P. acidlactici Kp10 yang tinggi juga penghasilan aktiviti BLIS yang tinggi. Analisis ANOVA satu-hala telah dijalankan bagi melihat keupayaan penghasilan BLIS di dalam keempat-empat media asas yang digunakan. Hasil dapatan ANOVA menunjukkan M17 signifikan berdasarkan statistik ($p<0.05$) dalam penghasilan aktiviti BLIS yang tinggi. Walaubagaimanapun, MRS, TSB, dan NB tidak signifikan secara statistik di antara satu sama lain ($p > 0.05$) berdasarkan ujian perbezaan Post-hoc menggunakan ujianTukey HSD.

Kajian juga telah dijalankan keatas kestabilan ekstrak mentah BLIS yang dihasilkan oleh strain *P. acidilactici* Kp10 berdasarkan suhu, pH, dan tempoh simpanan yang berbeza menggunakan *L. monocytogenes* ATCC 15313, *E. coli* ATCC 35218, dan *S. aureus* ATCC 33591 sebagai mikroorganisma indikator. Keputusan yang diperolehi menunjukkan ciri-ciri BLIS yang memberansangkan untuk tujuan komersial berdasarkan kestabilan pada julat pH yang besar (pH 2- 9), suhu (4°C, -20°C, -80°C dan 100°C) dan juga tempoh penyimpanan yang panjang iaitu 1, 3, dan 6 bulan pada suhu 4°C, -20°C, dan -80°C .

Purifikasi ekstrak mentah BLIS daripada strain *P. acidilactici* Kp10 telah dilakukan dengan menggunakan Sistem Purifikasi Pengapungan Akues dwi-fasa garam-polimer (SDFA) yang terdiri daripada polietilena-glikol (PEG) dan sodium sitrat yang dibentuk untuk perolehan terus BLIS. Di bawah kondisi optimum SDFA, purifikasi BLIS telah diperolehi pada 19% PEG 8,000/14% sodium sitrat merangkumi TLL pada 42.6% (w/w), nisbah isipadu (I*n*) pada 0.4, dan 22% muatan kasar (C_L) pada pH 7 dengan purata masa pengapungan (F_t) pada 30 min dan kadar pengapungan (F_R) pada 20mL/min. Jangkaan saiz BLIS yang telah diperolehi daripada analisis SDS-PAGE ialah 12.5kDa. Hasil daripada kajian ini menunjukkan BLIS daripada strain *P. acidilactici* Kp10 mempunyai potensi besar untuk dikomersialkan sebagai aplikasi dalam industri makanan.

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I certify that a Thesis Examination Committee has met on 21 September 2016 to conduct the final examination of Nurul Lyana binti Md Sidek on her thesis entitled "Production and Purification of Bacteriocin-Like Inhibitory Substance from *Pediococcusacidilactici* Kp10" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
TSB	Tryptic soy broth
NB	Nutrient broth
MRS	De Man Rogosa Sharpe
TLL	Tie line length
ATPF	Aqueous two-phase flotation
ATPS	Aqueous two-phase system
PEG	Polyethylene glycol
V_R	Volume rate
C_L	Crude load
F_R	Flotation rate
Ft	Flotation time
BLIS	Bacteriocin-like inhibitory substances
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

CHAPTER 1

INTRODUCTION

Bacteriocin is defined as proteinaceous compound produces by microorganisms with activity to inhibit the growth of similar or closely related bacterial strains. Bacteriocins are apparently protein in nature and produced by both Gram positive and Gram negative species (Daw and Falkiner, 1996). The production of bacteriocins by lactic acid bacteria (LAB) has been encountered in foods such as dairy products, meats, barley, sourdough, red wine and fermented vegetables. Bacteriocins have wide antibacterial spectrum with increasing interest for feasible application in foods. In addition, the potential of bacteriocins to inhibit Gram-positive pathogens involved in food-borne illnesses such as *Listeria monocytogenes* or *Staphylococcus aureus* has attracted the food industry with increasing demand.For that reason, the potential of LAB as a biopreservative or natural food preservative has been recognized (Cintas *et al*., 2001). A large number of bacteriocins produced by LAB from most common genera such as *Lactococcus, Streptococcus, Pediococcus, Leuconostoc, Lactobacillus,* and *Carnobacterium* have been identified eventhough their potential as biopreservatives is not yet well discovered (Sobrino-López and Martín-Belloso, 2008). In fact, there are few bacteria having the ability to produce more than one bacteriocins and multiply bacteriocinogenic strains which are *Streptococcus salivaris, Streptococcus uberis, and Streptococcus mutans* (Jones *et al*., 2011). Among those bacteriocins encountered, nisin produced by *Lactococcus lactis* and pediocin produced by *Pediococcus acidilactici* have more attractions to the researchers and the industry (Anastasiadou*et al*., 2008).

The progress in bacteriocins research has been supported by the emerging consumer demand towards the applications of natural food preservatives. Biopreservation method using bacteriocin has been chosen since it maintains the organoleptic and nutritional properties even with little usage of chemical preservatives and lower heat intensity (Gálvez *et al*., 2007). Supplementation of food with bacteriocin can be achieved by *ex situ* and *in situ* bacteriocin production. In *ex-situ* method, the produced bacteriocin is added in the form of raw concentrates or applied in the form of immobilized preparations. In *in situ* method, the viable cells of bacteriocin producing strains are incorporated into the food system. However, the actions of bacteriocin in inhibiting growth of pathogens are not easy to predict and describe since the food itself is a complex ecosystem (Balciunas *et al*., 2013).

Suitable media composition and culture conditions shall be used in LAB fermentation not only to enhance the growth but also to enhance the secretion of bacteriocins. In medium formulation, the roles of all components are important to be investigated to find methods that inexpensive, rapid, environmental friendly, high yielding and amenable to large scale production. The key obstacle of bacteriocin applications in the food industry is the food ecosystem itself. Different types of food have different complex ecosystems. In order for the bacteriocin to be incorporated into food system, the physicochemical properties need to be studied first in terms of stability to

temperature, enzymes, pHs, storage duration and other factors that may influence its activity. The method for extraction and purification of bacteriocin from the fermentation broth is also important to be developed for the preparation of active and stable commercial bacteriocin product for application in the food industry. Recently, the use of aqueous two-phase flotation system (ATPF) for protein purification has attracted many reseachers. ATPF system is inexpensive and can be classified as rapid separation techniques. ATPF is a combination of aqueous two-phase extraction system (ATPE) with solvent sublation. The volume of the aqueous phase is much larger than the polyethylene glycol (PEG) phase as compared to conventional ATPE system. Thus, this method can be used to maintain an immiscible two-phase system by manipulating the concentration of salt through salting-out effect (Tan *et al*., 2014).

Throughout this study, the term BLIS (Bacteriocin-like inhibitory substance) will be used as a term of convenience to denote inter-bacterial inhibition that appears likely to be due to the production of bacteriocins, but prior to confirmation of the genetic and molecular identity of the inhibitory agents (Jones *et al*., 2011). The present study was carried out to generate information for subsequence use especially in the food industry in the development of a feasible fermentation for the production of BLIS by the newly isolated strain, *Pediococcus acidilactici* Kp10.

Thus, the objectives of this study were

1. To investigate the most suitable medium that can be used to enhance growth of *P. acidilactici* kP10 and BLIS production.
2. To investigate the stability of BLIS produced by *P. acidilactici* kP10 during storage at different storage conditions.
3. To investigate the possibility of using aqueous two-phase flotation (ATPF) system for the purification of BLIS from fermentation broth of *P. acidilactici* Kp10.

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