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ISOLATION AND CHARACTERIZATION OF BACTERIOCIN PRODUCING LACTIC ACID BACTERIA

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ISOLATION AND CHARACTERIZATION OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA

By

SAHAR ABBASI LIASI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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UPM

Dedicated to my parents

For their endless supports

Abstract of Thesis Presented to the Senate of Universiti Putra Malaysia in Fulfillment of the Requirement for the Degree of Master of Science

ISOLATION AND CHARACTERIZATION OF BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA

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Chairman: Professor Arbakariya Ariff, PhD

Faculty: Biotechnology and Biomolecular Sciences

Lactic acid bacteria (LAB) in fermented foods produce a large variety of compounds which give these products their characteristic flavor and color apart from improving its safety and quality. LAB with potential applications in food industry have been isolated from a local fermented food (budu) and characterized based on their morphological and biochemical characteristics. Gram-staining, catalase and gas production tests were performed for identification while API 50 CHL (BioMérieux) was used for the determination of species. Twelve isolates were identified as genus *Lactobacillus (5 isolates of Lb. paracasei, 2 isolates of Lb. plantarum*, one isolate of *Lb. casei* and 4 isolates of *Lb. sp*). The highest population was *Lb. paracasei* (41.67%). All isolates showed gram-positive, catalase negative and some positive results for homo-fermentative characteristics .

Antibiotic sensitivity test of these twelve isolates of LAB to 24 different types of antibiotics was conducted using the disc diffusion method. Inhibition zone diameter was measured and calculated from the means of five determinations and expressed in

terms of resistance or susceptibility. All LAB isolates from this product were resistant to colestin sulphate, streptomycin, amikacin, norfloxacin, nalidixic acid, mecillinam, sulphanethoxazole/ trimethoprim, and kanamycin but susceptible to erythromycin, ceftriaxone, chlomphenicol, tetracycline, ampicillin and nitrofurantion.

The four isolates (*Lb. casei* LA17, *Lb. plantarum* LA22, *Lb. pracasei* LA07 *and Lb.* sp. LA19) were evaluated for their ability in producing bacteriocins. The inhibitory spectra of the isolates when evaluated against a range of gram-positive and gram-negative test microorganisms showed that antimicrobial substance from these isolates inhibit the growth of indicator microorganisms, such as *Bacillus cereus*, *Lactococcus lactis*, *Staphylococcus aureus*, *Salmonella enterica*, *Listeria monocytogenes* and *Escherichia coli*. Complete inactivation of antimicrobial activity from *Lb. paracasei* LA07 was observed after treatment of cell-free supernatant with proteinase K confirming its proteinaceous nature. Treatment with α-amylase inactivated the antimicrobial activity suggesting that bacteriocin could be glycosylated. Stability of the bacteriocins in the present of catalase enzyme ruled out the possibility of antagonistic activity of bacteriocins due to hydrogen peroxide or organic acids. Lipase caused only a slight reduction of bacteriocin activity, indicating that besides the proteinaceous subunit, some lipid components may also involve in antibacterial activity. The partially purified bacteriocin produced by *Lb. paracasei* LA07 has molecular weight of 10 kDa, based on SDS-PAGE analysis. The antibacterial activity of cell free supernatant was significantly increased by the addition SDS, Triton x-100, Tween 80 and Tween 20. On the other hand, the antibacterial activity was lost with the addition of EDTA.

The influence of pH, temperature, and media composition on growth of *Lb. paracasei* LA07 and bacteriocin production was also conducted in shake flask culture. The highest bacteriocin production was obtained in cultivation using MRS, which was about 51%, 4%, 63 % and 22% higher than those obtained in BHI, M17 NB and TSB, respectively. Production of bacteriocin was also influenced by the cultivation temperature and the highest production was obtained at 30^{o}C. Optimal pH for growth of *Lb. paracasei* LA07 and bacteriocin production was achieved at an initial pH of 8.5. Addition of (5 g/l) yeast extract and meat extract to MRS medium increased further the bacteriocin by about 8% and 5%. Supplementation with glucose at concentrations up to 10 g/l as the sole carbon source, gradually enhanced bacteriocin production, while higher concentrations (20 g/l) did not further increase bacteriocin production. Growth and bacteriocin production were not significantly affected by the agitation rate in either in anaerobic or in microaeration conditions.

From this study, it can be concluded that the locally fermented fish product, budu contained LAB where the highest population was *Lb. paracasei*. The antimicrobial activity produced by the LAB isolated in this research could act as a potential barrier to inhibit the growth of spoilage bacteria and food-borne pathogens.

Abstrak Tesis Untuk Dikemukakan Kepada Senat Universiti Putra Malaysia Sebagai Memenuhi Keperluan Untuk Ijazah Master Sains

PEMENCILAN DAN PENCIRIAN BAKTERIA ASID LAKTIK -PENGHASIL BAKTERIOSIN

Oleh

SAHAR ABBASI LIASI

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Bakteria asid laktik (BAL) dalam makanan tertapai menghasilkan pelbagai sebatian yang memberikan produk tersebut ciri-ciri rasa dan warna selain meningkatkan kualiti dan keselamatannya. BAL yang berpotensi diaplikasikan dalam industri makanan telah dipencilkan dari makanan tertapai tempatan (budu) dan dicirikan berdasarkan morfologi dan biokimianya. Perwarnaan gram, ujian katales dan penghasilan gas telah dijalankan untuk pengenalpastian bakteria tersebut sementara API 50 CHL (BioMerieux) telah digunakan untuk penentuan spesis. Dua belas isolat telah dipencilkan dan dikenalpasti sebagai genus *Lactobacillus* (5 isolat *Lb. paracasei,* 2 isolat *Lb. plantarum*, one isolat *Lb. casei* and 4 isolat *Lb. sp*). Populasi tertinggi yang diperolehi ialah *Lb. paracasei* (41.67%). Kesemua isolat yang dipencil telah menunjukkan ciri-ciri gram-positif, katales-negatif dan hasil yang positif bagi ciri-ciri homofermentatif.

Ujian sensitiviti antibiotik bagi dua belas isolat BAL terhadap 24 jenis antibiotik berbeza telah dijalankan menggunakan kaedah keserapan cakera. Garis pusat zon

perencatan telah diukur dan dikira dari purata lima penentuan dan dinyatakan dalam sebutan rintang atau penerimaan. Semua isolat BAL yang dipencil dari produk ini adalah rintang kepada sulfat kolestin, streptomisin, amikasin, norfloksasin, asid nalidiksik, mesilinam, sulphanetoksazol/trimetroprim dan kanmisin tetapi penerimaan kepada eritromisin, seftriakson, kloramfinikol, tetrasiklin, ampicilin dan nitrofurantion.

Empat isolat tersebut (*Lb. casei* LA17, *Lb. plantarum* LA22, *Lb. pracasei* LA07 dan *Lb.* sp. LA19) telah dinilai keupayaannya dalam menghasilkan bakteriosin. Spektra perencatan yang dinilai terhadap satu julat ujian mikroorganisma gram-positif dan gram-negatif menunjukkan bakteriosin dari isolat tersebut dapat merencat pertumbuhan mikroorganisma penunjuk seperti *Bacillus cereus, Lactococcus lactis, Staphylococcus aureus, Salmonella enterica, Listeria monocytogenes dan Escherichia coli***.** Penyahaktifan lengkap aktiviti antimikrobia *Lb. paracasei* LA07 diperoleh selepas rawatan supernatant bebas-sel dengan proteinase K yang mengesahkan ianya berbentuk protein. Rawatan dengan α-*amilase* tidak menyahaktif aktiviti antimikrobia yang menunjukkan bakteria mungkin terglikosilat. Kestabilah bakteriosin dalam kehadiran enzim katalase menolak kemungkinan aktiviti penentagan bakteriosin disebabkan oleh hydrogen peroksida atau asid organik. Lipase menyebabkan hanya sedikit pengurangan aktiviti bacteriosin yang menunjukkan, di samping subunit berprotein, terdapat sedikit komponen lipid yang mungkin terlibat dalam aktiviti antibakteria. Bakteriosin separa taleh yang dihasilkan oleh *Lb. paracasi* LA07 mempunyai 10 kDa berat molekul, berdasarkan analisis SDS-PAGE. Aktiviti antibateria bagi supernatant bebas sel telah ditingkatkan oleh

penambahen SDS, Triton x-100, Tween 80 dan Tween 20. Selain itu, aktiviti antibakteria hilang dengan penambahan EDTA.

Kajian ke atas pengaruh pH, suhu dan komposisi media kepada pertumbuhan *Lb. paracasei* LA07 dan penghasilan bakteriosin juga telah dijalankan didalam kelalang bergoncang. Penghasilan bakteriosin tertinggi diperolehi daripada pengkulturan menggunakan MRS yang mana lebih kurang 51%, 4%, 63% dan lebih tinggi berbanding BHI, M17 NB dan TSB. Masing-masing. Penghasilan bakteriosin adalah juga dipengaruhi oleh suhu kultur dan penghasilan tertinggi diperolehi pada suhu 30^{o}C. pH optimal untuk pertumbuhan *Lb. paracasei* LA07 dan penghasilan bakteriosin diperolehi pada pH permulaan 8.5. Penambahan extrak yis dan extrak daging (5 g/l) ke dalam medium MRS meningkatkan lagi penghasilan bakteriosin sebanyak 8% dan 5%. Penambahan glukosa pada kepekatan sehingga 10 g/l sebagai sumber karbon tunggal meningkatkan penghasilan bakteriosin; sebaliknya kepekatan yang lebih tinggi (20 g/l) tidak meningkatkan penghasilan bakteriosin seterusnya. Adalah jelas tiada perbezaan yang ketara samada dalam pertumbuhan bakteria atau penghasilan bakteriosin dalam keadaan aerobik atau anaerobik.

Kesimpulan daripada kajian ini menunjukan bahawa budu mengandungi BAL dengan populasi *Lb. paracasei* yang tertinggi. Aktiviti antimikrob bakteriosin yang dihasilkan oleh BAL dalam kajian ini boleh bertindak sebagai penghalang untuk merencat pertumbuhan bakteria perosak dan patogen bawaan makanan.

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I certify that an Examination Committee has met on 3 April 2009 to conduct the final examination of Sahar Abbasiliasi on her thesis entitled "Isolation and Characterization of Bacteriocin-Producing Lactic Acid Bacteria " in accordance with the Universitires 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 fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date: 9 July 2009

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any degree at Universiti Putra Malaysia or at any other institutions.

SAHAR ABBASI LIASI

Date: 17 June 2009

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LIST OF ABBREVIATIONS

Acetyl CoA	Acetyl Coenzyme A
AK	amikacin
AMP	ampicillin
APT	All purpose tween
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
AU	Arbitrary Unit
B	bacitracin
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
^{o}C	Degree centigrade
C	chloramphenicol
CFU	Colony Forming Unit
CH_3CHO	Acetaldehyde
OB	cloxacillin
CIP	ciprofloxacin
CO_2	Carbon dioxide
CN	gentamycin
CRO	ceftriaxone
CT	colestin sulphate
CXM	cefuroxime sodium
Da	Dalton, Identically the atomic mass unit, used mainly for biochemical molecular weights

DNA	Deoxyribonucleic acid
E	erythromycin
E	*Enterococcus* spp.
EDTA	Ethylenediaminetetraacetic acid
EMP pathway	Embden-Meyerhof-Parnas pathway
F	nitrofurantion
g/l	Gram per liter
GAP	Glyceraldehyde phosphate
GRAS	Generally regarded as safe
H	Hour
H_2O_2	Hydrogen peroxide
HCl	Hydrochloride acid
HMM	High-molecular-mass
I	Intermediate
K	kanamycin
kDa	Kilo Dalton
LAB	Lactic Acid Bacteria
L	*Lactococcus* spp.
Lb	*Lactobacillus* spp.
Lc	*Leuconostoc* spp.
LDH	lactate dehydrogenase
LMM	low-molecular-mass
MEL	mecillinam
µg	Microgram, 10^{-6} of a gram
Min	Minutes

Ml	Milliliter, one thousandth (10^{-3}) of a liter
µl	Microliter, one-millionth (10^{-6}) of a liter
Mm	Milimeter, one thousandth (10^{-3}) of a meter
MRS	De Man, Rogosa and Sharpe
MY	lincomycin
N	neomycin
NA	nalidixic acid
NAD (NADH)	Nicotinamide adenine dinucleotide, A coenzyme, $C_{21}H_{27}N_7O_{14}P_2$, occurring in most living cells and utilized alternately with NADH as an oxidizing or reducing agent in various metabolic processes.
NaOH	Sodium Hydroxide
NB	Nutrient Broth
Nm	nanometer, 10^{-9} m
NOR	norfloxacin
NV	novobiocin
OD	Optical Density
P2	penicillin G
PAGE	Poly Acrylamide Gel Electrophoresis
pH	The Power of Hydrogen
pk_a	Acid dissociation constants are also known as the acidity constant or the acid-ionization constant. The term is also used for pK_a, which is equal to minus the decimal logarithm of K_a (cologarithm of K_a)
ppm	The parts of a substance per million parts (by weight) of a solution; equal to 0.0001%
R	Resistant

rRNA	Ribosomal RNA (The RNA that is a permanent structural part of a ribosome, Ribosomal RNA accounts for nearly 80% of the RNA content of the bacterial cell).
RPM	Revolution per Minute
S	Susceptible
S	streptomycin
SDS	Sodium Dodecyl Sulphate
SXT	Sulphanethoxazole/Trimethoprim
TE	tetracycline
TSB	Tryptic Soy Broth
v/v	Volume per volume
VA	vancomycin
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Antagonistic substances are vital factors in microbial ecology. Among the many different substances known to play a role in bacterial interactions, bacteriocins are the most specific and efficient antagonist (Iqbal, 1998). Bacteriocins are antibacterial proteins or protein complexes produced by several gram-positive and gram-negative bacteria.

Although bacteriocins are produced by many gram-positive and gram-negative species, those produced by lactic acid bacteria (LAB) are of particular importance to the food industry (Nettles & Barefoot, 1993). These bacteria have generally been regarded as safe (GRAS status) and as the majority of bacteriocin-producing LAB are natural food isolates, therefore they are ideally suited to food applications. Thus, the production of bacteriocins by LAB is not only advantageous to the bacteria themselves but also to the food industry. When utilised as a tool to control the growth of undesirable bacteria it is directed towards making the food product more acceptable to consumers (Deegan *et al*., 2006).

The demand by consumers for a decrease in the use of chemical additives in food has led to the use of natural antimicrobial substances secreted by food fermentation bacteria to inhibit the growth of undesirable microorganisms (Berry *et al.,* 1991; Schillinger *et al.,* 1991). The use of LAB as starter cultures in fermented food industry has become widespread. Among the beneficial properties of LAB are the production of organic acids, hydrogen peroxide, and bacteriocins which are