Isolation and selection of antifungal lactic acid bacteria to use as inoculants for improving quality of fermented plant beverages

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ชื่อวิทยานิพนธ์ การแยกและการคัดเลือกแบคทีเรียแลกติกที่ผลิตสารยับยั้งยีสต์และรา เพื่อเป็นกล้าเชื้อปรับปรุงคุณภาพของน้ำหมักชีวภาพ
ผู้เขียน นายภากร ปราชญากิจ
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บทคัดย่อ

ผลิตภัณฑ์เครื่องดื่มน้ำหมักชีวภาพจากพืชมักพบการปนเปื้อนด้วยยีสต์อยู่เสมดังนั้น เพื่อให้ทราบถึงสายพันธุ์ของยีสต์ที่ปนเปื้อน จึงได้มีการตรวจสอบน้ำหมักชีวภาพจากแหล่งต่างๆจำนวน 40 ตัวอย่าง และสามารถแยกยีสต์ได้ 59 ไอโซเลท เมื่อนำยีสต์ทุกไอโซเลทไปทำการเทียบเคียงด้วยวิธีการดั้งเดิมพบว่าสามารถจัดได้เป็น 7 กลุ่มใหญ่ เมื่อนำตัวแทนของแต่ละกลุ่มไปเทียบเคียงสายพันธุ์ ด้วยวิธี 26S rRNA gene sequence พบว่ายีสต์ที่มีการปนเปื้อนมากที่สุดคือ *Rhodotorula mucilaginosa* (14 ไอโซเลท, 23.7%) รองลงมาคือ *Pichia membranifaciens* (11 ไอโซเลท, 18.6%) ส่วนกลุ่มย่อยที่เหลือ ได้แก่ *Pichia anomala* (9 ไอโซเลท, 15.3 %) *Saccharomyces cerevisiae* (6 ไอโซเลท,10.2 %) *Issatchenkia occidentalis* (5 ไอโซเลท, 8.5 %) และ *Candida tropicalis* (4 ไอโซเลท, 6.8 %) ตามลำดับ และที่เหลือไม่สามารถเทียบเคียงได้ (10 ไอโซเลท, 16.9%) โดยเกือบทั้งหมดของยีสต์ที่เทียบเคียงได้เป็นกลุ่มที่สามารถเจริญที่ผิว (film yeast) ยกเว้น *S. cerevisiae*

เพื่อที่จะควบคุมยีสต์ที่ปนเปื้อนในน้ำหมักชีวภาพโดยการใช้แบคทีเรียแลกติกเป็นกล้าเชื้อจึงแยกแบคทีเรียดังกล่าวจากน้ำหมักชีวภาพจากพืช และอาหารหมักชนิดต่างๆ จำนวน 500 ไอโซเลท พบว่ามี 261 ไอโซเลทภายใต้สภาวะที่ไม่จำกัดการสร้างกรด และไฮโดรเจนเพอออกไซด์ที่มีความสามารถยับยั้งยีสต์ *Rhodotorula mucilaginosa* DKA ได้ดี แต่เมื่อสารออกฤทธิ์ทั้งสองถูกจำกัดให้สร้างน้อยลงพบว่ามีเพียง 6 ไอโซเลทที่ยับยั้งยีสต์ดังกล่าวได้ในระดับดี และเมื่อทดสอบขอบเขตการยับยั้งยีสต์ต่าง ๆ 14 ชนิด พบว่ามีเพียงไอโซเลท DW3 ที่สามารถยับยั้งยีสต์ทดสอบได้ถึง 9 ชนิด และเมื่อนำไอโซเลท DW3 ไปเทียบเคียงโดยใช้ชุดทดสอบ API 50CH และยืนยันผลด้วย 16S rDNA sequence พบว่าคือ *Lactobacillus plantarum*

เมื่อนำน้ำเลี้ยงเชื้อปราศจากเซลล์ที่มีความเข้มข้น 10 เท่ามาทำให้เป็นกลางหมดสภาวะความเป็นกรดไม่พบฤทธิ์การยับยั้งยีสต์และรา แต่ยังคงยับยั้งแบคทีเรีย *Staphylococcus aureus* PSSCMI 0004 *Vibrio parahaemolyticus* PSSCMI 0064 *Shigella sonneii* PSSCMI 0032 และ*Escherichia coli* PSSCMI 0001 ได้ดี สารดังกล่าวออกฤทธิ์ได้ดีในการควบคุม

S. aureus PSSCMI 0004 และ *Escherichia coli* PSSCMI 0001 ในสภาวะเป็นกรดดีกว่าเป็นกลาง และน้อยลงในสภาวะเป็นด่าง ที่อุณหภูมิน้อยกว่า 60°C นาน 30 นาทียังออกฤทธิ์ได้ดี และเอนไซม์ Catalase Amylase และ Lipase ไม่มีผลต่อสารต้านจุลินทรีย์ในการควบคุมเชื้อทั้งสอง แต่ Trypsin Pepsin α-Chymotrypsin และ Pronase E มีผลทำให้ฤทธิ์ต้านจุลินทรีย์หมดไป จากคุณสมบัติที่กล่าวมาแสดงว่าสารต้านจุลินทรีย์ที่สร้างโดย *L. plantarum* DW3 จัดอยู่ในกลุ่มแบคเทอริโอซินที่ไวต่อความร้อน (Class III: large heat-labile protein) และเรียกว่าแบคเทอริโอซิน DW3 ซึ่งมีฤทธิ์ยับยั้ง *S. aureus* PSSCMI 0004 และ *E. coli* PSSCMI 0001 ในระดับ 80 และ 60 AU/ml และการเก็บไว้เป็นเวลา 30 วันในรูปแช่แข็งเข้มข้นมีความเสถียรดีกว่าการเก็บในรูปสารละลายเข้มข้น นอกจากนี้ *L. plantarum* DW3 ยังสามารถสร้างสารที่ควบคุมยีสต์ *R. mucilaginosa* DKA ได้ดี และผลจากการศึกษาด้วยเทคนิค Gas chromatography-Mass spectrometer (GC-MS) พบว่าสารดังกล่าวคือ Phenyllactic acid (PLA) ซึ่งมีฤทธิ์ยับยั้งแบคทีเรีย ยีสต์ และราบางชนิดได้ดี โดยเชื้อผลิต PLA ได้ 31 mg/L ในอาหาร MRS และค่า MIC_{90} ของ PLA ต่อเชื้อยีสต์ดังกล่าวคือ 5 mg/ml

ผลการเติมกล้าเชื้อ *L. plantarum* DW3 7.5 x 10^6 CFU/ml ในการหมักสาหร่ายผมนางมีบทบาทสำคัญมากในการควบคุมปริมาณยีสต์ที่ปนเปื้อนมากับวัตถุดิบ โดยสามารถควบคุมยีสต์ให้ต่ำกว่า 100 CFU/ml เมื่อสิ้นสุดการหมัก 60 วัน สำหรับชุด No partial sterilization-Starter: N-S และสำหรับชุด Partial sterilization-Starter: P-S ไม่พบยีสต์เลยเมื่อหมักไปได้ 45 วัน ขณะที่การไม่เติมกล้าเชื้อ (No partial sterilization-No starter: N-N มียีสต์มากกว่า 100 CFU/ml แต่ชุดที่ผ่านการฆ่าเชื้อบางส่วน (Partial sterilization-No starter: P-N) ด้วย 0.5% potassium metabisulfite (KMS) พบยีสต์ต่ำกว่า 100 CFU/ml เช่นกัน และเมื่อสิ้นสุดการหมักพบว่าชุดการทดลองที่เติมกล้าเชื้อมีปริมาณ LAB เหลืออยู่มากกว่าชุดไม่เติมกล้าเชื้อ โดยชุด N-S และ P-S มี LAB อยู่ 4.7 และ 4.0 log CFU/ml ขณะที่ชุด N-N และ P-N มี LAB อยู่ 2.4 และ 2.3 log CFU/ml ตามลำดับ

เมื่อสิ้นสุดการหมักพบว่าการเติมกล้าเชื้อ LAB ลงไปทำให้ค่าความเป็นกรดมากกว่าโดย ที่ชุด P-S และ N-S ต่างก็มีปริมาณกรดสูงสุดพอๆกันคือ 0.95% โดยต่างก็มีค่าพีเอชเท่ากันคือ 2.9 และมีน้ำตาลเหลืออยู่ 4% และพบว่าน้ำหมักมีค่าการนำไฟฟ้า 1.0 mS/cm เป็นไปในรูปแบบเดียวกับปริมาณกรดที่เกิดขึ้น และแร่ธาตุต่างๆ (ทองแดง: Cu เหล็ก: Fe โปแตสเซียม: K โซเดียม: Na และสังกะสี: Zn) มีปริมาณสูงขึ้นตามอายุการหมักซึ่งสอดคล้องกับปริมาณกรดและค่าการนำไฟฟ้า โดยที่ปริมาณของธาตุเหล่านั้นอยู่ในเกณฑ์ที่ปลอดภัยสำหรับการดื่ม นอกจากนี้จากผลการทดลองตรวจไม่พบธาตุที่เป็นพิษ Pb (ตะกั่ว) และ สารหนู (As) ในทุกชุดการทดลอง และพบว่าน้ำหมักสาหร่ายผมนางเป็นแหล่งที่ดีของ Fe (ค่าสูงสุด 17 mg/L ในชุด N-N และ P-S)

ปริมาณเมทานอลตรวจไม่พบตลอดการหมัก และปริมาณของกรดอะซีติกและกรดแลกติกที่พบแบ่งเป็น 2 กลุ่มคือชุดการทดลองที่ผ่านการฆ่าเชื้อบางส่วน (P-N และ P-S) มีปริมาณต่ำกว่าชุดที่ไม่ผ่านการฆ่าเชื้อบางส่วน (N-N และ N-S) อย่างมีนัยสำคัญทางสถิติ ($P = 0.05$) และเมื่อสิ้นสุดการหมักปริมาณกรดอะซีติกของทุกชุดอยู่ระหว่าง 1.8-2.5 g/L ขณะที่กรดแลกติกมีปริมาณระหว่าง 3.2-7.0 g/L สำหรับฤทธิ์ยับยั้งเชื้อก่อโรคพบว่าชุดการทดลองที่เติมกล้าเชื้อทำให้น้ำหมักมีฤทธิ์ยับยั้งเชื้อก่อโรคทุกชนิดที่ทดสอบ (*S. aureus* PSSCMI 0004 *E. coli* PSSCMI 0001 *Salmonella* sp. *V. parahaemolyticus* PSSCMI 0064 และ *Bacillus cereus* ATCC 11778) ได้ดีกว่าไม่เติม และ *V. parahaemolyticus* PSSCMI 0064 เป็นเชื้อที่มีความไวต่อน้ำหมักมากที่สุด

ผลการทดสอบทางประสาทสัมผัสพบว่าการเติมกล้าเชื้อทำให้แต่ละปัจจัยที่ทดสอบได้รับคะแนนสูงกว่าการไม่เติมกล้าเชื้อทั้งที่อายุการหมัก 30 และ 60 วัน โดยชุด N-S ได้รับคะแนนสูงสุดในทุกปัจจัยแม้ว่าไม่มีความแตกต่างทางสถิติ ($P > 0.05$) และพบว่าการฆ่าเชื้อบางส่วนด้วย 0.5% KMS ส่งผลให้การยอมรับในแต่ละปัจจัยต่ำกว่าที่ไม่ผ่านการฆ่าเชื้อบางส่วน และเมื่อเก็บน้ำหมักไว้ 3 เดือน (อายุน้ำหมัก 150 วัน) พบว่าในทุกชุดการทดลองระยะเวลาไม่มีผลต่อการเปลี่ยนแปลงการยอมรับรวมของผู้ทดสอบ โดยแต่ละชุดการทดลองได้รับคะแนนเฉลี่ยตลอดอายุการเก็บมากกว่า 3.0 ซึ่งจัดอยู่ในเกณฑ์ยอมรับได้ในระดับปานกลาง และในทุกชุดการทดลองตรวจไม่พบ total coliforms และ *E. coli* (แบคทีเรียบ่งชี้การปนเปื้อนด้วยอุจจาระ) รวมถึงเชื้อก่อโรค *S. aureus Clostridium perfringens* และ *Salmonell*a spp. เมื่อสิ้นสุดการหมัก และเก็บน้ำหมักไว้ 3 เดือน นอกจากนี้การเก็บน้ำหมักไว้ 3 เดือนทำให้น้ำหมักอยู่ในสภาพปราศจากเชื้อ โดยที่ฤทธิ์การยับยั้งแบคทีเรียก่อโรคที่ทดสอบยังคงอยู่แต่ลดไปบ้าง และชุดที่เติมกล้าเชื้อ (N-S และ P-S) ให้ผลการยับยั้งดีกว่าไม่เติมอย่างมีนัยสำคัญทางสถิติ ($P = 0.05$)

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Abstract

Yeast contamination invariably occurs with fermented plant beverages (FPBs). To understand what strains of yeast were, the most prevalent contaminants, 40 FPB samples collected from various sources were investigated. Fifty-nine contaminating yeast strains were isolated and identified into seven groups by conventional methods. A representative yeast strain from each group was then selected for identification using 26S rRNA gene sequences. The results of DNA sequence analysis indicated that the main group of contaminating yeasts was *Rhodotorula mucilaginosa* (14 isolates, 23.7%) followed by *Pichia membranifaciens* (11 isolates, 18.6%). Other minor groups were *Pichia anomala* (9 isolates, 15.3 %), *Saccharomyces cerevisiae* (6 isolates, 10.2 %), *Issatchenkia occidentalis* (5 isolates, 8.5 %) and *Candida tropicalis* (4 isolates, 6.8 %) and the remaining 10 isolates, (16.9%) could not be identified. All isolates except for *S. cerevisiae* were film yeasts.

In order to control yeasts that might be contaminated, FPBs selected lactic acid bacteria (LAB), isolated from FPBs and fermented foods, were tested for their ability to inhibit growth of yeasts when used to assist with the FPB process. 500 isolates of LAB were investigated. 261 LAB isolates produced inhibitory effects against an isolate of *Rhodotorula mucilaginosa* DKA and previously isolated as an FPB contaminant, when production by the LAB of acid or hydrogen peroxide was not limited. However, only 6 of the LAB isolates showed good yeast inhibitory activity as the productions of both compounds were limited. Based on the spectrum of yeasts inhibited, isolate DW3 was selected for further studies due to its ability to inhibit 9 of the 14 contaminating yeast species tested, and it was identified as *Lactobacillus plantarum* by a commercial test kit API 50CH and confirmed with 16S rDNA sequence.

A 10X concentrated neutralized culture filtrate (CNF or freeze dried supernatant: FDS) from isolate DW3 did not inhibit the molds and yeasts tested. However, the FDS gave good inhibition against *Staphylococus aureus* PSSCMI 0004, *Vibrio parahaemolyticus* PSSCMI 0064, *Shigella sonneii* PSSCMI 0032 and *Escherichia coli* PSSCMI 0001. The FDS was active against tested bacteria (*S. aureus* PSSCMI 0004 and *E. coli* PSSCMI 0001) in an order of acidity > neutralization > alkalinity and in the temperature range of less than 60ºC, for 30 min. The enzymes catalase, amylase and lipase had no effect on the FDS antimicrobial activity. However, trypsin, pepsin, α-chymotrypsin and pronase E, completely inhibited its antimicrobial activity. Based on these results - a class III bacteriocin - (large heat-labile protein), we suggest that the antimicrobial activity of *L. plantarum* DW3 was named bacteriocin DW3. The bacteriocin DW3 gave higher inhibitory effect against *S. aureus* PSSCMI 0004 (80 AU/ml) than that of *E. coli* PSSCMI 0001 (60 AU/ml). Besides, the bacteriocin DW3 was kept for 30 days in a form of FDS which had higher stability to control both organisms than that found in a concentrated solution form. In addition, the isolate DW3 also produced the antifungal substance that inhibited variety of microbes, particularly *R. mucilaginosa* DKA. The substance was isolated and identified by Gas chromatography-Mass spectrometer (GC-MS) and it was phenyllactic acid (PLA). The isolate DW3 produced 31 mg/L PLA in MRS medium and its MIC_{90} against the target yeast was 5 mg/ml.

In order to identify whether the use of *L. plantarum* DW3 could control the amount of yeast in a fermented seaweed beverage (FSB), it was introduced as a starter culture at approximately 7.5 x 10^6 CFU/ml into partial sterilization (0.5% KMS: potassium metabisulfite) or no partial sterilization FSB substrates, together with appropriate controls (No Partial sterilization-No starter: N-N and Partial sterilization-No starter: P-N). At the end of the fermentation (60 days) the amount of the yeast in the FSB with DW3 as starter cultures was less than 100 CFU/ml (N-S) and no yeast was found at day 45 in a set of P-S. The natural fermentation (N-N) did not pass a standard guideline for yeast count; however, partial sterilization without inoculation of DW3 (P-N) could pass the standard level. The amounts of LAB at day 60 in the N-S and P-S sets were 4.7 and 4.0 log CFU/ml, whereas those in N-N and P-N sets were 2.4 and 2.3 log CFU/ml, respectively.

At the end of the FSB fermentation with starter, there was more acidity as both sets of inoculated cultures obtained 0.95% acidity with 2.9 pH and remaining sugar at 4%. The FSB had similar changes of acidity, electrical conductivity (1.0 mS/cm) and elements (Na, K, Cu, Fe and Zn). In addition, all these values increased during the fermentation time and were below the recommended safety levels for drinking. Toxic elements, As and Pb, were not detected in any sets of the FSB. Moreover, the FSB was a good source of Fe with as much as 17 mg/L in the N-N and P-S sets.

In the FSB, no methanol was detected throughout the fermentation and the organic acid levels in the partial sterilization treatment sets were significantly lower than those in the no partial sterilization sets ($p = 0.05$). Acetic acid levels at day 60 in the FSB were in the range of 1.8 to 2.5 g/L while lactic acid was between 3.2 to 7.0 g/L. The inhibitory effect of culture fluids from all inoculated sets on all target organisms (*Staphylococcus aureus* PSSCMI 0004, *Escherichia coli* PSSCMI 0001, *Salmonela* sp., *Vibrio parahaemolyticus* PSSCMI 0064 and *Bacillus cereus* ATCC 11778) was much higher than that of the uninoculated sets ($P = 0.05$) and *V. parahaemolyticus* PSSCMI 0064 was the most sensitive organism to the beverage.

Results of the sensory tests for the FSB at days 30 and 60 were similar in both inoculated sets with higher scores from the factor tests (most acceptable) than those from the uninoculated sets although no significant difference was found ($P>0.05$). The maximum score in each sensory test was found in the N-S set. Moreover, sets partial sterilization using 0.5% KMS obtained lower scores than no partial sterilization treatment sets. A three month-storage time produced no effects on the sensory tests for the beverage because there is no change in the acceptable level. Each treatment set of the beverage obtained an average score of more than 3.00 which is moderately acceptable.

The finished product of FSB at the end of fermentation and after storing for 3 months passed microbiological quality tests because there were no detectable coliforms and *E. coli* (fecal indicators). Moreover, none of sets found *S. aureus, Clostridium perfringens* and *Salmonella* spp. The storage time of the beverage provided sterile conditions. The inhibitory effects of the beverage against pathogenic bacteria were retained with only minor reductions. The inoculated treatment sets had a higher inhibiting activity than that in the uninoculated sets ($P = 0.05$).

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Pakorn Prachyakij

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LIST OF ABBREVIATIONS AND SYMBOLS

AFLAB	=	antifungal lactic acid bacteria
BSA	=	N, O-bis (trimethylsilyl)-acetamide
CFU	=	colony forming unit
^{o}C	=	degree Celsius
cm	=	centimeter
DNA	=	deoxyribonucleic acid
EMB	=	Eosin Methylene Blue medium
EtOAc	=	ethyl acetate
EtOH	=	ethanol
FPB	=	fermented plant beverage
FPBs	=	fermented plant beverages
FDS	=	freeze dried supernatant
g	=	gram
GC	=	gas chromatography
h	=	hour
HCl	=	hydrochloric acid
HPLC	=	high performance liquid chromatography
ICP-AES	=	inductively coupled plasma-atomic emission spectroscopy
kDa	=	kilodalton
kg	=	kilogram
KMS	=	potassium metabisulfite
LAB	=	lactic acid bacteria
L	=	liter
M	=	molarity
ME	=	malt extract
MEA	=	malt extract agar
MEB	=	malt extract broth
MIC	=	minimum inhibition concentration
MID	=	minimum inhibition dose

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

MeOH	=	methanol
MRS	=	de Man Rogasa Sharp medium
mg	=	milligram
min	=	minute
ml	=	mililiter
mm	=	milimeter
mM	=	milimolar
MS	=	mass spectometer
N	=	normality
NaOH	=	sodium hydroxide
NaCl	=	sodium chloride
nm	=	nanometer
OD	=	optical density
PCA	=	plate count agar
pH	=	-log hydrogen ion concentration
PLA	=	phenyllactic acid
PSU	=	Prince of Songkla University
RNA	=	ribonucleic acid
rpm	=	revolution per minute
SD	=	standard deviation
TBC	=	total bacterial count
TSA	=	Tryptic soy agar
TSB	=	Tryptic soy broth
U	=	unite
v/v	=	volume per volume
w/v	=	weight per volume
w/w	=	weight per weight
µg	=	microgram
µl	=	microliter
%	=	percent

CHAPTER 1

INTRODUCTION

Fermented plant beverage (FPB or FPBs) is a kind of non alcoholic beverage, which produced from varieties of plants (fruits, cereals, vegetables and herbs) and fermented by the natural microorganisms, particularly lactic acid bacteria (LAB) (Kantachote *et al.,* 2005). There is no record of how long Thai people have consumed FPBs but the health promoting effect of FPBs have long been believed by Thai consumer (Kantachote *et al*., 2005; Kantachote *et al*., 2008) Some kinds of FPBs such as Noni juice from Polynesia, EM-X from Japan and Vita Biosa from Denmark have been developed to the commercial products. Moreover their scientific information and health promoting effect have been reported (Chui *et al.*, 2006; Deiana *et al.*, 2002; McClatchey, 2002)

Unfortunately, Thai FPBs have not been accepted as a commercial product by Thai Food and Drug Administration (Thai FDA) due to the remaining of yeast in finished products (Kantachote *et al*., 2005). To prevent this organism by using chemical preservatives such as potassium metabisulfite (KMS) is not accepted by health care consumers although this chemical has long been widespread used in wine production. Moreover, heat treatment like pasteurization and sterilization is also unacceptable in some groups of consumer on the ground of their believe that the nutritional values of FPBs are destroyed during the processes (Kantachote and Charernjiratrakul, 2008b). As regards, the natural way found that covering the surface of FPBs in a vessel with a water contained plastic bag before closed with its lid was able to control film yeast in FPBs namely Noni fermented beverage and Seaweed fermented beverage (Kantachote and Charernjiratrakul, 2008a).

It has long been recognized that lactic acid bacteria play an important role on food fermentation and many researchers have intensively studied about their antibacterial activity and their antibacterial compounds such as bacteriocins, hydrogen peroxide, diacetyl, organics acid namely lactic acid and acetic acid (Beasley and Saris, 2004; Bromberg *et al.*, 2004b; Choi *et al.*, 2000; Corsetti *et al.*, 2004; Danova *et al.*, 2005; De Muyncka *et al.*, 2004; Ivanova *et al.*, 2000; Leroy and De Vuyst, 2004a;

Nardi *et al.*, 2005; Okkers *et al.*, 1999; Yang, 2000). Successful application of LAB was reported in many fermented foods such as pickles (Jay, 2000), yoghurt (Schwenninger and Meile, 2004), bread (Lavermicocca *et al*., 2000) and FPBs (Kantachote and Charernjiratrakul, 2008a). Nevertheless, a few researches have focused on antifungal lactic acid bacteria, particularly antiyeast (Lavermicocca *et al*., 2000; Magnusson and Schnurer, 2001 ; Magnusson *et al*., 2003; Okkers *et al*., 1999; Roy *et al*., 1996; Schnürer and Magnusson, 2005; Strom *et al*., 2005; Strom *et al*., 2002).

At recent years, the spoilage yeast of foods and drinks has increased importance for economic losses. The main yeast species present in foods and beverages was reported (Deak and Beuchat, 1996; Pitt and Hocking, 1997; Rose and Harrison, 1993; Tudor and Board, 1993). In addition, the emergent of foodborne yeast has gained significant (Loureiro and Querol, 1999).

In Thailand, red seaweed such as *Gracilaria fisheri* has long been used as an edible plant and also a source of producing agar. Generally, the red seaweed contains useful phycocolloids, vitamins and some contain antibiotic principals (Chopin *et al.*, 1999; Werawanich *et al.*, 2000; Zandi *et al.*, 2007a). Recently, antibiotic substances i.e. antibacterial, antifungal and antiviral of this red marine alga have been investigated (Kantachote and Charernjiratrakul, 2008a; Zandi *et al.*, 2007a). Therefore, it is interesting to use this red seaweed as a raw material for producing fermented beverage. It is not only to increase value of seaweed by using an appropriate technology but also to increase alternative way for people who consume healthy beverages.

To achieve a natural way for controlling yeast in FPBs, antifungal lactic acid bacteria (AFLAB) was isolated and selected from a variety of fermented foods and FPBs to use as inoculants for a fermented seaweed beverage. In addition, antifungal compound produced by a selected strain was also studied.

Overall, the outcomes from this research provide information on how to overcome yeast contamination in FPBs and how to use the appropriate technology for controlling the quality of FPBs.

OBJECTIVES

1. To isolate and identify contaminated yeast strains in fermented plant beverages
2. To isolate, select and identify antifungal lactic acid bacterium from fermented food and FPBs
3. To investigate antimicrobial substances produced by a selected lactic acid bacterium
4. To identify the antifungal substance produced by a selected lactic acid bacterium and partially characterize its activity
5. To apply a selected lactic acid bacterium as inoculants on the quality of a fermented seaweed beverage

CHAPTER 2

LITERATURE REVIEWS

1. Fermented plant beverages

I. Fermented plant beverages

A fermented plant beverage (FPB or FPBs) is a lactofermentative beverage, produced from different kinds of plants. FPB is composed of 3 main compositions as follows: plant, potable water and sugar. FPBs have been believed by the consumers that they have health promoting effect and could cure some diseases (Chaiyasut, 2007; Kantachote and Charernjiratrakul, 2008a; Kantachote *et al.*, 2005). At present, foods which can prevent delay of onset diseases such as cancer, diabetes mellitus are receiving more attention. Functional food or medicinal food is the fresh or processed food claimed to have a health-promoting and/or disease-preventing property beyond the basic nutritional function of supplying nutrient (Whang, 2007). According to the meaning of functional foods, FPBs attracted health care people.

Noni juice is the most well known FPB. It has been used for centuries by the ancient in Polynesia for centuries as a general tonic which treat imbalances of the digestive, respiratory nervous system. There are many evidences about it's benefit on animal and human such as anticancer, anti-inflammatory and immune enhancing effects (Hirazumi and Furusawa, 1999; McClatchey, 2002). The other FPB is EM-X Rice Bran Beverage (EM-X), product of the Tropical Plant Resources Research, Japan. EM-X is an extract beverage made of an effective microorganism (EM), organic brown rice, organic rice bran, papaya and kelp. Health promoting effect of this product has been studied by many researchers in over 50 countries. There are some reports stated that it has anticancer, anti-inflammatory and antioxidant effects (Chui *et al.*, 2006; Deiana *et al.*, 2002).

Another FPB is Vita Biosa, produced from Biosa Denmark ApS. It is a mixture of water, organic molasses from sugarcane, organic fructose, organic

dextrose, organic herbs and fermentation cultures such as *Lactobacillus acidophillus*, *Bifidobacterium animalis* subsp. *lactis*, *Streptococcus thermophilus*, *Lactobacillus casei*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *lactis* biov. *diacetyllactis*, *Leuconostoc pseudomesenteroides*. Vita Biosa gives a positive effect on the digestion system. However, both EM-X and Vita Biosa producers have not mentioned about an acceptance from any food or drug organization as Vita Biosa product is only valid date tested by the Danish Ministry of Food, Agriculture and Fisheries (www.biosa.dk). Products of noni juice, EM-X and Vita Biosa are showed in Figure1.

A B C

Figure1. Commercial fermented plant beverages. A: Noni juice from FrenchPolynesia; B: EM-X® Rice Bran Beverage Manufactured by Tropical Plants Resources Research Japan and C: Vita Biosa Manufactured by Biosa Danmark ApS, Denmark (www. Noni-leader.com 4 April; www.emamerica.com; www.biosamidwest.us/Founders.html4 April)

There is no record regarding how long that Thai people have consumed plant fermented beverage (FPB). At the present, public concern about healthy food and functional food has dramatically increased. Noni juice and other FPBs have been imported to Thailand for many years as they have impacted on Thai health care consumer. Nevertheless, these products seem to be expensive for Thai people.

In recent years, there are many communities in Thailand which have produced FPBs by themselves. Among of those communities, Sunti-Asoke

community network and Organic agricultural network are the highest FPBs production communities (Chaiyasut *et al.*, 2004a). Many recipes of FPBs have been produced depending on areas and kind of seasonal fruits. However, FPBs produced from Thai Noni (*Morinda*, Yor) is still the most requisite (Figure 2) and has become dramatically widespread in the country due to the fact that Thai noni juice is much cheaper than the imported products.

However, the safety and quality of FPBs are needed to concern. Yeasts are the most remaining microorganism in FPBs while mold and bacteria are also detected in finished product of FPBs (Kantachote *et al.*, 2005). The remaining yeasts in final products are a barrier to produce FPBs in a commercial scale. Moreover, high level of alcohol and some heavy metals were also detected in some FPB samples (Chaiyasut, 2007).

Therefore to develop Thai FPBs to become a commercial product, scientific data and controlling yeast technique are urgently required.

A B C

Figure 2. Thai Noni fermented beverage products; A: product from Tumbon Nongaein, Mukdaharn province; B: product from khaokho tale phu Co., Ltd., Phetchabun province; C: product from Tumbon Chang noi, Ayutthaya province (http://www.thaitambon.com May 1)

II. Cases study of Thai Fermented Plant Beverage

1. Investigation of the safety and some characteristics of the products available in Thai market

Chaiyasut *et al.,* (2004a) surveyed 26 FPBs producers. There were 11 producers from the central region, 5 from north east, 4 from north, 1 from eastern and 1 from south of Thailand. It was found that 61.5% of FPB products produced from *Morinda* and 57.7% of producers produced the FPBs by the mixture of fruit, sugar and water is 3:1:10, respectively. Most of FPB products were fermented not less than 3 months. Only 30.8% of the producers informed the expiry date on the product label while 76.9% did not provide any information.

Siriloon *et al.,* (2004) reported the biological characteristic and the safety of 70 FPB products available in Thailand including FPBs were produced in the laboratory. Based on microbiological quality, 28 samples contaminated with some microorganisms; however, pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* spp. and *Clostridium perfringens* were not found. Yeast was found in 4 samples in range of $2.2 \times 10^2 - 9.1 \times 10^3$ CFU/ml, and identified as non pathogenic strain *Candida* sp. Mold was found in 2 samples (190 and 430 CFU/ml) and identified as *Aspergillus niger*. Bacteria were found in range of $1.4 \times 10^2 - 5.8 \times 10^4$ CFU/ml, and also nonpathogenic strains such as *Flavobacterium* sp and *Bacillus* sp. Lactic acid bacteria were also found in the range between $1.7 \times 10^3 - 6.4 \times 10^5$ CFU/ml. In addition, pH of the samples was between 2.78-3.89, except Kefer (6.49) and EM-X (5.72). According to the standard of the Ministry of Public Health for beverage product in closed container, it could be concluded that mostly of FPB products available in Thailand are microbiological safe beverages.

Kantachote *et al*., (2005) reported the characteristics of 19 FPBs which collected from southern of Thailand had varied odor, color and clarity and the presence of Cu, Zn, K, and Na depended on the types of plant used and the additive of sugar or honey. The samples had pH between 2.63-3.72 while sugar and ethanol were found in ranges of 0.21-4.20% and 0.03-3.32%, respectively. The amount of methanol was between 0.019-0.084% and methanol production was dependent on both the

fermentation process and the plant used. Microbiological quality differed depending on the fermentation time (3 months - 3 years) and also the level of sanitation of the production process.

Chaiyasut, (2004) investigated nitrogen, phosphorus compound (P_2O_5) and the contaminative metals in 68 Thai FPBs. Nitrogen compound was found in all samples in a range of 0.11-0.41%. Phosphorus was detected only in 4 samples (0.01-0.1%). Calcium and magnesium were found 31.3-912 mg/kg and 0.3-23.3 mg/kg, while zinc, copper and cadmium were not found. Iron was found between 0.05-84.75 mg/kg in 29 samples and 10 samples were found higher content than the limitation for the beverage product in closed container (15 mg/kg). Selenium was found 0.0043-0.49060 mg/kg in 61 samples, and 14 samples showed higher amount of selenium than the standard of Australian food products as Se in beverage products is not more than 0.2 mg/kg. Limitation of Thai RDI (Thai Recommended Daily Intakes) from the Ministry of Public Health was showed in Table 1. Chaiyasut *et al*.,(2007) reported that sodium and potassium were found in 68 Thai FPB samples in ranges of 0.01-1.46 mg/ml and 0.19-2.21mg/ml, respectively; however, both elements content did not exceed guideline of Thai RDI (Table 2).

Table 1. Thai Recommended Daily Intakes (Thai RDI) for elements (Chaiyasut, 2007)

People	Age	Ca (mg)	P (mg)	Mg (mg)	Fe (mg)	Zn (mg)	Cu (mg)	Se (mg)
Infant	3-5 months	360	240	50	6	3	0.5-0.7	0.01-0.04
	6-8 months	420	280	70	7	5	0.7-1.0	0.02-0.04
	9-11months	480	320	70	8	5	0.7-1.0	0.02-0.05
Child	1-3 years	800	800	150	10	10	1.0-1.5	0.02-0.08
	4-6 years	800	800	200	10	10	1.5-2.0	0.03-0.12
	7-9 years	800	800	250	10	10	2.0-2.5	0.05-0.20
Boy	10-15 years	1200	1200	350	12	15	2-3	0.05-0.20
	16-19 years	1200	1200	400	10	15	2-3	0.05-0.20
Girl	10-15 years	1200	1200	350	15	15	2-3	0.05-0.20
	16-19 years	1200	1200	400	10	15	2-3	0.05-0.20
Adult Male	20+ years	800	800	350	15	15	2-3	0.05-0.20
Female	20+ years	800	800	300	10	15	2-3	0.05-0.20
Pregnant women	-	+400	+401	+150	+30	25	2-3	0.05-0.20

Table 2. Thai Recommended Daily Intakes (Thai RDI) for Na and K (Chaiyasut, 2007)

People	Age	Na (mg)	K(mg)
Infant	3-5 months	115-350	350-925
	6-8 months	250-750	425-1,275
	9-11months	250-750	425-1,275
Child	1-3 years	325-975	550-1,650
	4-6 years	450-1,350	775-2,325
	7-9 years	600-1,800	1,000-3,000
Boy	10-15 years	900-2,700	1,525-4,575
	16-19 years	900-2,700	1,525-4,575
Girl	10-15 years	900-2,700	1,525-4,575
	16-19 years	900-2,700	1,525-4,575
Adult Male	20+ years	1,100-3,300	1,875-5,625
Female	20+ years	1,100-3,300	1,875-5,625
Pregnant women	-	1,100-3,300	1,875-5,625

Chaiyasut *et al*., (2004d) studied the type and the quantity of organic acids in 68 FPBs produced in Thailand by HPLC techniques. They found tartarlic, citric, and lactic acids in ranges of 0.018-0.241 mg/ml, 0.024-0.276 mg/ml and 0.028-1.244 mg/ml, respectively. Malic, acetic, sorbic and fumaric acids were also found in ranges of 0.015-0.389 mg/ml, 0.052-0.55 mg/ml, 0.049-0.373 mg/ml and 0.025-0.980 mg/ml, respectively while ascorbic acid was found in only 2 samples and less than 0.080 mg/ml. Only 1 sample was found propionic acid (0.310 mg/ml) while shikimic acid was not found in any samples.

In FPBs, organic acids such as lactic and acetic acids were produced by glucose metabolic pathway of LAB. The first period of fermentation, obligate heterofermentative LAB such as *Leuconostoc* sp. were predominant LAB, and they metabolize glucose via Phosphoketolase pathway, organic acids (lactic and acetic) and carbon dioxide are obtained. An increase of acidity, *Lactobacillus* sp. i.e. *L. brevis*, *L. fermentum* and *L. plantarum* become dominant LAB after a few days of fermentation. *L. brevis* and *L. fermentum* are obligate heterofermentative LAB while *L. plantarum* is facultative heterofermentative LAB which utilize glucose via ether Embden-Meyerhof-Parnas pathway or Phosphoketolase pathway (Figure 3) (Kantachote and Charernjiratrakul, 2008a).

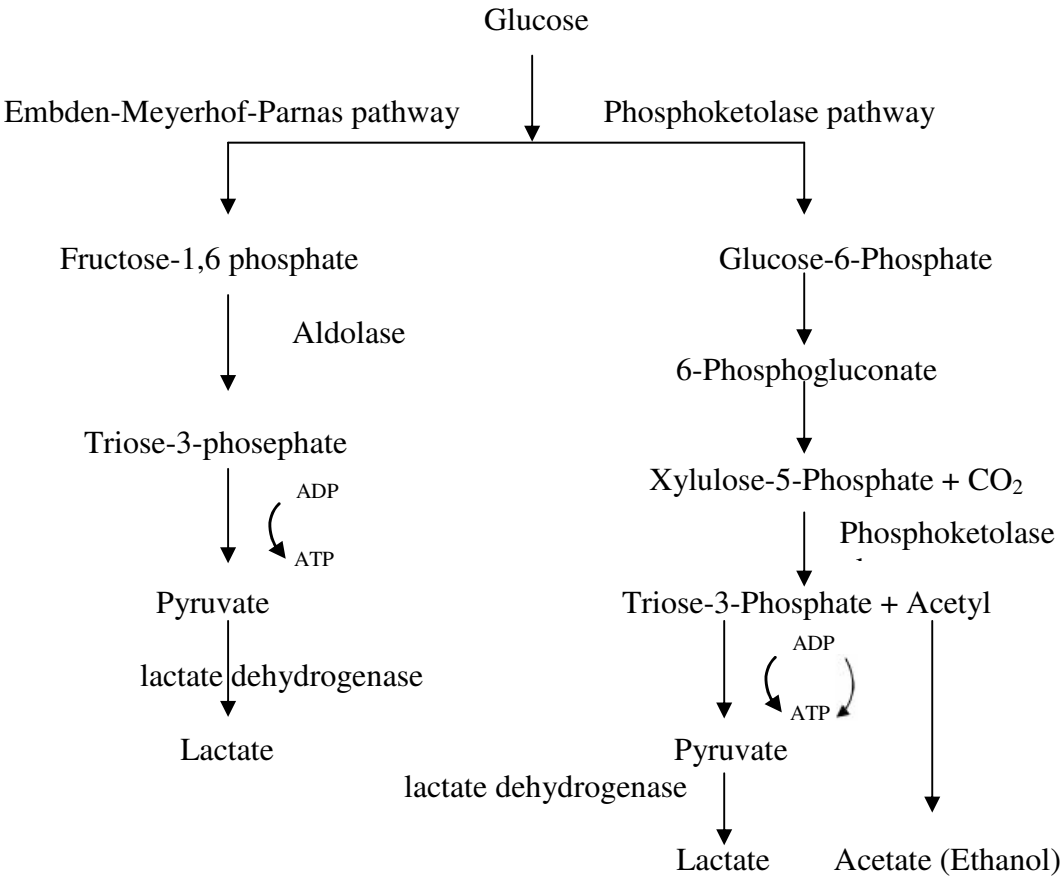


Figure 3. Glucose metabolic pathways of Lactic acid bacteria (Adopt from Wood and Holzapfel, 1995)

As Figure 3, Embden-Meyerhof-Parnas pathway, glucose is metabolized to pyruvate and then pyruvate is further reduced to lactic acid via the enzyme lactate dehydrogenase. The pathway that converts glucose to lactic acid is called lactic acid fermentation. While Phosphoketolase pathway involves the initial splitting of CO_2 from the glucose molecule, followed by a further splitting of the resulting pentose (xylulose -5-phosphate) into two-carbon and three-carbon fragments in a phosphoroclastic reaction catalyzed by phosphoketolase, yielding glyceraldehydes-3P and acetyl phosphate, respectively. The three carbon fragment is eventually reduced to lactate in the same way as homolactics, and the two-carbon fragment is reduced to ethanol or acetic acid. In the presence of oxygen or other oxidants increased amounts of acetate may be produced at the expense of lactate or ethanol, whereby one additional mole of ATP is obtained via the acetate kinase reaction. Thus products other than lactate are generated, and the pathway is therefore called the heterofermentative pathway (Wood and Holzapfel, 1995).

Levels of ethanol and methanol in 70 FPBs were reported by Chaiyasut *et al*., (2004b) and Chaiyasut *et al*., (2004c). More than 0.5% ethanol was found in 55 samples (78.5%), less than 0.5% was found in 11 samples and no ethanol was found at all in 4 samples. Level of methanol was between 0.0010-0.0297% in 47 samples while it was not found in 23 samples. According to the limitation of Public Health Ministry for the standard of beverage in closed container, total alcohol occurred by the natural process which is more than 0.5% is not permitted. Results indicated that among 70 FPB samples, only 11 FPB samples had total alcohol of less than 0.5 %.

Methanol is produced from pectin (polymethylgaracturonate) degradation by pectin methylesterese (Whittaker, 1990) (Figure 4). Pectin is converted to pectic acid (polygalacturonate) and methanol by pectin methylesterese. This enzyme is normally found in fruits such as banana, orange, and tomato. In addition, it is produced by *Erwinia carotovora, E. chrysanthemi* and some species of yeast namely, *Rhodotorula glutinis* and *R. rubra* (Kantachote *et al.,* 2005). Therefore, occurrence of methanol in FPBs might be pectin and pectin methylesterese of each kind of fruit or plant including microorganisms and fermented condition (Chaiyasut, 2007).

Ethanol is produced by contaminated yeast in raw materials of FPB. Yeast metabolizes carbohydrate (glucose) via Embden-Meyerhof Pathway (EMP), which is

the same pathway as some strain of LAB used. By means of this pathway, pyruvate, intermediate substance is produced and changed into acetaldehyde and carbon dioxide by pyruvate decarboxylase, then acetaldehyde is further reduced to ethanol by alcohol dehydrogenase. For some LAB like *Streptococcus*, *Lactococcus* and *Lactobacillus*, pyruvate is change into lactate by lactate dehydrogenase (Figure 5) (Alcamo, 1991).

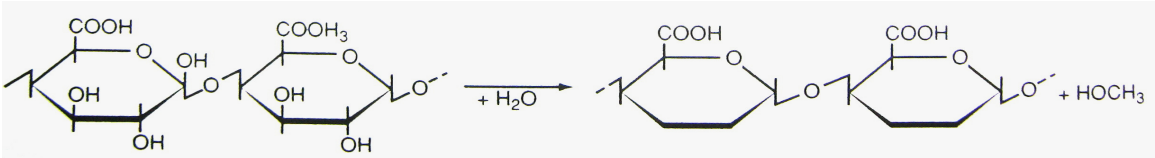


Figure 4. Methylation of pectin by pectin methylesterase (Chaiyasut, 2007; Uhlig, 1998)

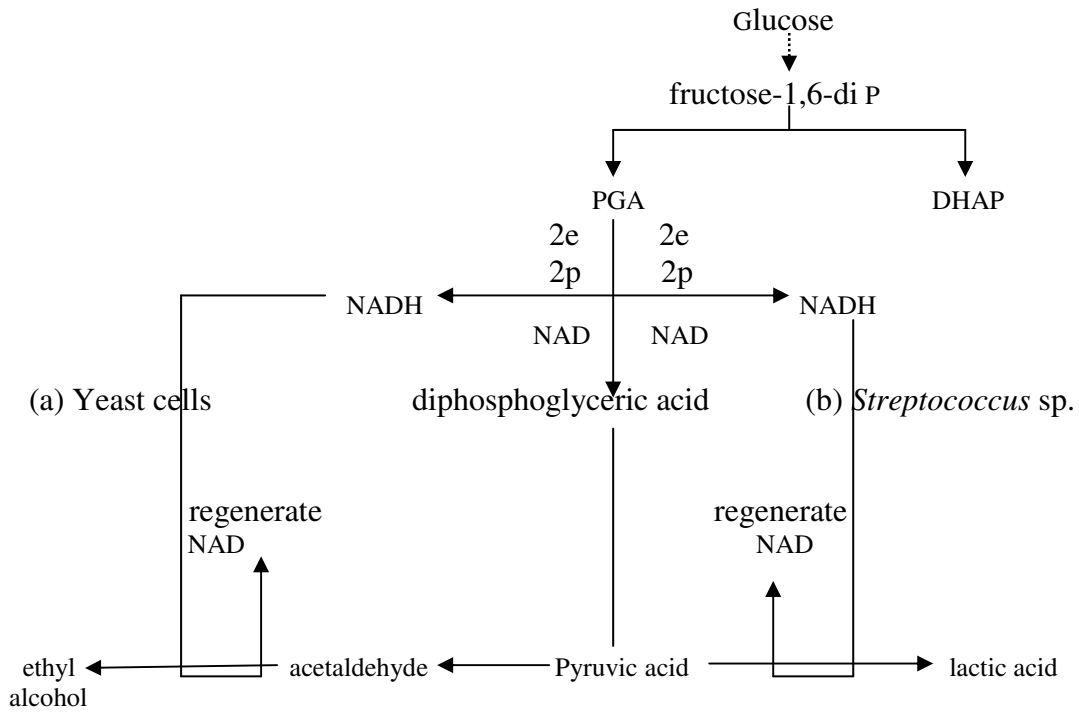


Figure5. Glycolysis in yeast cells and *Streptococcus* sp.(Adopted fro Alcamo,(1991))

2. Antioxidative of the FPBs

Chaiyasut *et al*., (2004f) studied the oxidative activity of 28 Thai FPB selected from 8 provinces by ABTS method (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical)) compared with vitamin C, vitamin E, Trolox and Quercetin. The antioxidative level of these samples could be divided into 3 groups. The first group (14 samples) was low antioxidative activity and the antioxidative equivalent in forms of VCEAC, VEEAC, TEAC, and QEAC were 4.372-24.440, 3.715-73.741, 9.914-41.055, and 0.338-11.447 mg/100ml, respectively. It was noticed that most of these samples contained *Morinda* (Noni) as the main composition. The second group (12 samples) was intermediate antioxidative activity and these samples were comprised of many kinds of plant i.e. *Galingale*, *Morinda*, *Phyllanthus emblica* and so on. The antioxidative equivalent in forms of VCEAC, VEEAC, TEAC, and QEAC were 34.612-90.192, 126.565-337.283, 50.779-134.423 and 19.674-53.239 mg/100ml, respectively. The last group (2 samples) was the high oxidative level, which *Terminalia chebula* Retz., *Terminalia bellirica* (Gaertn.) Roxb., and *Terminalia* sp. were the main component. The antioxidative equivalent in forms of VCEAC, VEEAC, TEAC, and QEAC were 1042.275-1947.755, 3406.255-6379.087, 1762.741-3650.359 and 535.216-990.656 mg/ 100 ml, respectively.

Antioxidative level of FPB is depended on many factors such as kind of plant, material preparation, fermentation time and fermentation process. Chaiyasut *et al*., (2004e) detected the oxidative activity of 7 formulas of FPBs produced from noni (*Morinda citrifolia* Linn) as follows:

1) Noni: water: cane sugar and starter Myrobalan Wood
2) Noni: water: honey and starter Beleric Myrobalan
3) Noni: water: cane sugar
4) Noni: water: honey
5) Noni: water: cane sugar (solid state fermentation for 15 days before adding water)
6) Noni: water: cane sugar (washed material by 200 mg/L KMS before adding starter)
7) Noni: water: cane sugar (pasteurization by heat before adding starter)

The antioxidative activity was investigated since from day 0 until day 180 by the ABTS method and also by reduction of power on ferric assay. Both methods gave a similar result and the activity increased following the fermentation time with the highest at day 15 and days 90-150. It was noticed that using cane sugar provided the higher oxidative activity than honey. In addition, pasteurization by heat clearly affected the antioxidative activity, while starter (*Lactobacillus* sp.) had no effect on antioxidative activity of FPBs (Chaiyasut *et al.*, 2004f).

3. Antimicrobial activity of the FPBs

Siriloon *et al.,* (2004) studied antimicrobial activity of 70 FPBs available in Thailand. They reported that 57 samples had an antimicrobial activity as 48 samples inhibited *Staphylococcus aureus* ATCC 25923, 43 samples inhibited *Escherichia coli* ATCC 25922. In contrast, *Pseudomonas aeruginosa* ATCC 27853 and *Candida albicans* 25922 were inhibited by 33 and 36 samples. Taking into consideration about the inhibitory spectrum, it was found that 23 samples inhibited all of 4 pathogens, 11 samples inhibited 3 pathogens while 10 and 13 samples inhibited 2 and 1 pathogens, respectively. Thirteen samples showed no antimicrobial activity that included 2 import products, Kefir and EM-X.

Kantachote *et al*., (2008) investigated the antipathogenic bacterial activity of 19 FPB samples collected from southern Thailand. Among 19 samples, FPBs produced from banana (*Musa sapientum* L.) and wild forest Noni (*Morinda coreia* Ham) provided the highest degree of bacterial inhibition. Both FPBs significantly inhibited all Gram positive bacteria (*S. aureus*, *Bacillus cereus*). Among Gram negative bacteria *E. coli* was the most resistant bacterium followed by *Salmonella* sp. while *P. aeruginosa*, *Shigella* sp. and *V. parahaemolyticus* were more sensitive. They concluded that antibacterial activities of FPBs were mainly attributed to their total acidity and some bioactive compounds derived from the plants used.

III. Thai FPB production

According to the study of Praditsrikul and Sittigool, (2004) and Patanakanok *et al.,* (2002), Thai FPB production is consisted of three steps.

1. Materials and Equipments preparation

Fruits are selected and washed. Whole, cut or sliced fruits are prepared depending on recipes. Brown sugar or white sugar or honey is commonly used as sweetener and substrate for fermentation. The container is a narrow or wide top diameter, or an earthen jar. According to the principle of fermentation, a narrow top diameter container is recommended because it prevents air from the outside. Hence the growth of contaminate aerobic microorganisms such as mold and yeast is limited while lactic acid bacteria, anaerobic or microaerophilic bacteria are encouraged.

2. Fermentation step

Normally, the ratio of ingredients; sugar, fruit and water is 1:3:10 (w/w/v), respectively. If the starter is used, the mixture of the starter, sugar, fruit and water is usually 1:1:3:8, respectively. The starter is obtained from the backslopping (previous successful FPB). Two types of fermentation process are normally used.

2.1. Wet fermentation (Submerge fermentation), water is added along with fruit, sugar and starter at the beginning of the process.

2.2. Dry fermentation (Solid state fermentation), fruit is well mixed with sugar in a container and the top of container is tightly covered. After 15 days, water is added and then leave it for three months or until the bubbles are not observed.

To prevent the growth of mold and yeast on the surface of fruit, run over the surface by sugar before cover the top of container is recommended. Kantachote and Charernjiratrakul, (2008a) revealed that covering the surface of FPB with a water contained plastic bag before close the container by its lid was a successful technique. In addition, gently stir the container to move the air contact side of the fruit which result in the growth of mold and yeast are prohibited.

CO_2 from the fermentation process affects the swelling of container. Some producers release this gas by using the special container by the lid contain with a small tube to release some air (Kantachote and Charernjiratrakul, (2008a).

2.3. Favoring and packaging

After complete fermentation process, the liquid from the container is filtrated by cheesecloth and/or favored by adding honey before packaging to obtain the concentrate FPB from this step. For ready to drink formula, the liquid is diluted (about 8 times by clean water) and favored before packaging.

IV. The standard for community fermented plant beverage (มผช 481/2547)

Although there is no standard of Thai FPB from Thai FDA, recently there has a standard of a community product which is able to use as a temporary guidance for producers. Details of this standard are as following provided:

1. The definition of FPB is the beverage derived from fermentation of one or several kind of plants.
2. Fermentation process means fermentation or liquid extraction from plants by microorganisms. Generally, the main microorganism is lactic producing bacteria namely *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, *Bifidobacterium* and *L. acidophilus*. Moreover, some other plant fermentable microorganism is allowed to use in the process.

The required characteristic of FPBs

1. General appearance

FPBs have to be liquid form and the sediment or a few of scrap of plant materials are allowed in the finished product.

2. Color, odor and flavor

Varied color, odor, and flavor occur according to the kinds of plant and fermentative process. All of these characteristics including the general appearance of a FPB product are determined by the sensory analysis following the method as described in the standard.

3. Unusual particle

Unusual particles i.e. hair, soil, sand, pebble, and fraction or wastes from animals are not allowed in the product.

4. Food additive (some cases)

The types and amounts of food additive used in FPB must follow the regulations of Thai FDA.

5. Ethyl alcohol ≤ 3%

6. Methyl alcohol ≤ 240 mg/L

7. pH ≤ 4.3

8. Microorganisms

Salmonella spp. must not be detected in 50 ml of the sample.

Staphylococcus aureus must not be detected in 1ml of the sample.

Clostridium perfringens must not be detected in 0.1 ml of the sample.

Escherichia coli by MPN method ≤ 2.2 per 100 ml of the sample

Yeast and Mold ≤ 100 colonies per 1 ml of the sample

The information about hygiene, packaging, trademark and sample testing are described in the community standard for FPBs (http://www.tisi.go.th/otop/standard/standards.htm (มผช 481/2547) April 1)

V. Yeast contamination; problem of Thai FPB

Contamination of FPBs with undesirable yeasts is a serious problem in the FPBs production and consumer acceptability. Although, there are several FPBs produced from many communities in Thailand, but they have not yet been authorized by Thai FDA (Food and Drug Administration) since the excess amount of yeasts remaining in FPBs (Kantachote *et al.*, 2005). Yeasts and molds can grow even at low pH value, existing in plant fermented products. Contaminating fungi in raw materials and post contamination from the poor manufacturing process might be the main cause of the problem. The FPBs contaminated with yeasts are shown in Figure 6.

Kantachote *et al.,* (2005) revealed that yeasts are the most remaining microorganism in the FPB samples, 9 of 19 samples, while molds and bacteria were found in samples 6 and 5 of 19, respectively. Due to the fact that a great number of yeasts are usually detected in FPBs and exceeded the guideline of Thai FDA for the

enclosed beverage (yeasts must not be exceed 100 CFU/ml in product), some researchers have tried to control yeasts in the product. Kantachote and Charernjiratrakul, (2008a) investigated 3 types of fermented patterns of 2 FPBs ; (*Morinda coreia* Ham) and (*Gracilaria fisheri*). The first pattern called M pattern, a fermented condition that initial air was removed by covering the empty area of fermented vessels with water contained in a plastic bag and also covered above with its lid. The second called N pattern which is similar to the first type but it has a ventilating tube jut out from the vessel. The last one is a traditional method (T) used as a control set, fermentation by a normal vessel covering with its lid. It was found that film yeasts grew covering the surface of both N and T patterns, but it did not appear in the M pattern. Nevertheless, numbers of yeast in the both FPBs were not different among 3 patterns over 90 days of fermentation as one produced from *Gracilaria fisheri* had yeast approximately 2 log CFU/ml as well as another one produced from *Morinda coreia* Ham had 3 log CFU/ml of yeast.

Figure 6. Contamination of film yeasts in fermented plant beverages (FPBs)

2. Lactic acid bacteria (LAB)

Lactic acid bacteria are found in many nutrient rich environments and occur naturally in various food products such as dairy, meat and vegetables. Besides, LAB are added as pure cultures or mixed cultures to various fermented food products. LAB are recognized to be GRAS (generally recognized as safe) and have been established as a natural consumer and environmental friendly way to preserve foods and feeds

(Armaforte *et al.*, 2006; Magnusson, 2003). LAB are a group of bacteria that have some similarities in term of morphological, physiological, metabolic and phylogenetic attributes. In general characteristics of LAB are Gram positive, rods or cocci shape, non spore forming, catalase-negative and devoid of cytochromes, nonaerobic habit but some strains are aerotolerant, fastidious and acid-tolerant. In addition, lactic acid is the main end product of carbohydrate metabolism (Stiles and Holzapfel, 1997). At the present, LAB are divided following recent taxonomic techniques into many genera such as *Lactobacillus, Leuconostoc, Pediococcus, Streptococcus Aerococcus, Alloiococcus Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactococcus, Oenococcus, Tetragenococcus, Vagococcus*, and *Weissella*. Lactobacilli, carnobacteria and some *Weissella* are rods while the remaining genera are cocci (Axelsson, 1998).

Carbohydrate metabolism

Based on carbohydrate metabolism, LAB can be divided into two groups (Vuyst and Vandamme, 1994). The first is Homofermentative LAB which metabolizes glucose via Embden-Meyerhof-Parnass pathway (EMP) or named Fructose Biphosphate Aldolase pathway (Figure 3). Lactic acid is the end product of this pathway. Another group is Heterofermentative LAB metabolizes glucose via 6-Phosphogluconate pathway/ Phosphoketolase pathway (Figure 3) or Bifidus pathway (Figure 7). Besides lactic acid, acetic acid, ethanol and carbon dioxide are the end product of these pathway (Moat and Foster, 1995; Vuyst and Vandamme, 1994; Wood and Holzapfel, 1995). The difference of homo and heterofermentative is summarized by following equations.

Homofermentation: 1 Hexose + 2 ADP + 2 Pi $\longrightarrow$ 2 Lactic + 2 ATP

Heterofermentation:1 Hexose +1 ADP + Pi $\longrightarrow$ Lactic + EtOH + CO_2/Acetic+1ATP

Generally, glucose is the first substrate for heterotrophic bacteria, however LAB are able to use other substrates such as lactose, fructose and sucrose as a carbon source (Kantachote, 2003; Vuyst and Vandamme, 1994). In homofermentative LAB,

lactose is taken up from the medium via 2 systems depend on strain of LAB, The first is ATP-dependent permease system (most lactobacilli and *Streptococcus thermophilus*). Lactose is transferred to cells by this system and then β-galactosidase converts lactose to glucose and galactose. The second is Phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS). Normally lactococci use this system for lactose transportation into the cells, and lactose is degraded to glucose and galactose-6-phosphate by phospho-β-galactosidase. Galactose is phosphorylated and further metabolized to glucose-6-phosphate and finally to lactate via glycolytic pathway. Whilst galactose-6-phosphate is utilized through the tagalose-6-phosphate pathway resulting in the production of additional lactic acid (Vuyst and Vandamme, 1994). Carbohydrate metabolism in homofermentative lactic acid bacteria is showed in Figure 8.

For heterofermentative LAB, lactose catabolism involving oxidation of glucose-6-phosphate to 6-phosphogluconate, followed by decarboxylation of hexose resulting in pentose moiety (xylulose-5-phosphate), which further split by means of phosphoketolase into a triose phosphate (glyceraldehyde-3-phosphate) and acetyl phosphate. The acetyl phosphate is either metabolized to acetic acid, or it is reduced by dehydrogenase to acetaldehyde and finally to ethanol. While triose phosphate is further metabolized via glycolytic pathway and excrete as lactic acid (Vuyst and Vandamme, 1994). Carbohydrate metabolism in heterofermentative lactic acid bacteria is showed in Figure 9.

For fructose, LAB contain fructokinase and phosphoglucoisomerase to phosphorylate fructose to fructose-6-P and then isomerizes to glucose 6-P. In the homofermentative pathway, glucose 6-P is further metabolized to pyruvate via glycolysis. The pyruvate is then reduced to lactate via lactate dehydrogenase. In contrast to homolactics, heterolactics contain mannitol dehydrogenase, which catalyzes the reduction of fructose to mannitol and oxidizes NADH under anaerobic conditions. In this reaction a small amount of fructose is used as an electron acceptor with the remaining fructose is converted to lactate, ethanol, acetate, and CO_2 (Li, 2004).

Similar to lactose, LAB are able to hydrolyze sucrose with β-glucosidase and β-fructosidase possessing sucrase. Sucrose transport across the cell membrane is

due to a specific sucrose phosphotransferase system (PTS) which simultaneously phosphorylates the glucose moiety of sucrose. Extracellular sucrose is hydrolyzed by sucrase and the resulting monosaccharides are transported into the cells and phosphorylated by the fructose and glucose PTSs. Both products, glucose and fructose, can be metabolized via the pathways previously mentioned (Li, 2004).

In fact, homo or heterofermentative pathway is glycolytic cycle. There is only one part of carbohydrate metabolism of bacteria. To obtained total energy from carbohydrate, Krebs cycle (tricarboxylic acid cycle: TCA or Citric Acid Cycle) and oxidative phosphorylation are taken part in the carbohydrate metabolism. In brief, pyruvate produced from glycolytic pathway transfers into Krebs cycle and change to other substance such as citric acid, succinic acid, fumaric acid, malic acid and oxaloacetic acid. Many intermediate substances produced via Krebs cycle are precursor of amino acid synthesis, purine and pyrimidine synthesis as well. For oxydative phosphorylation involving energy synthetic via electron transport system, combination of PO_4^{3-} and ADP is related to electron transport order, and resulted in ATP synthesis. To sum up, one molecule of glucose can be created 38 ATP via glycolysis, Krebs cycle and oxidative phosphorylation (Alcamo, 1991).

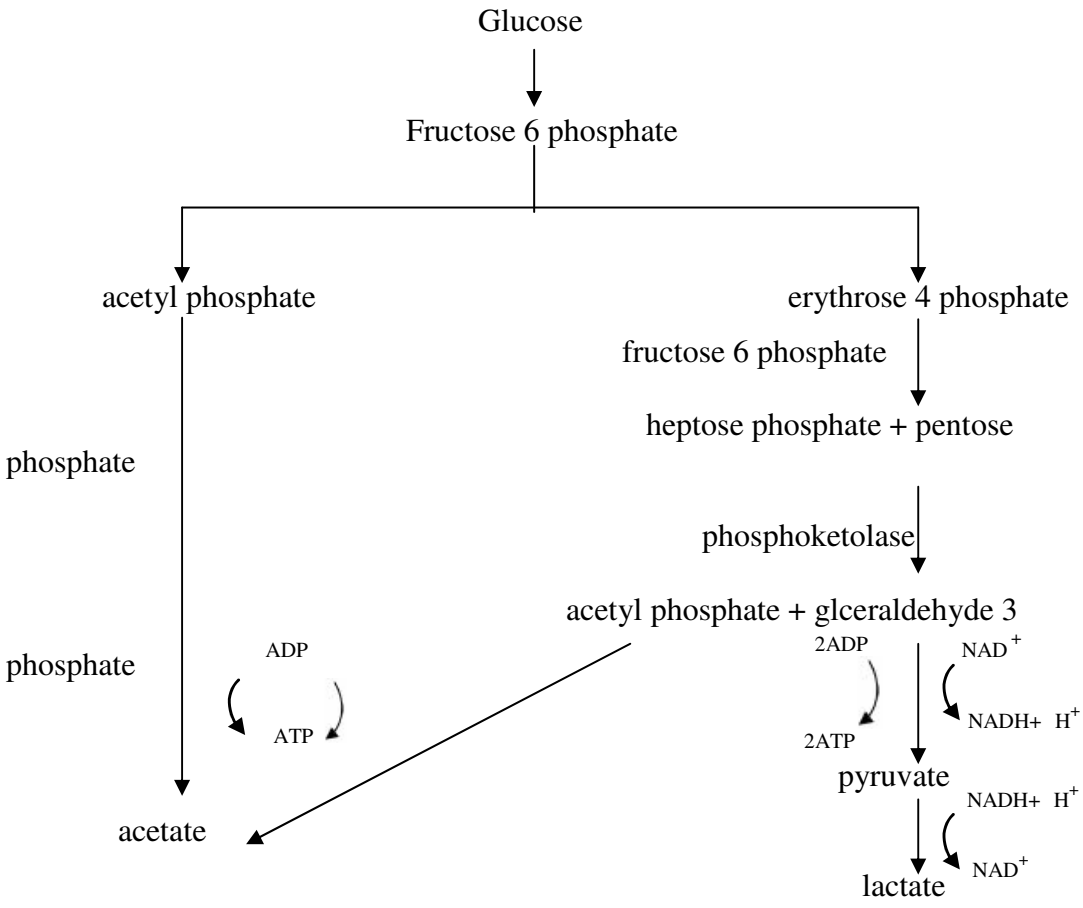


Figure 7. Carbohydrate metabolism in bifidobacteria (Vuyst and Vandamme, 1994)

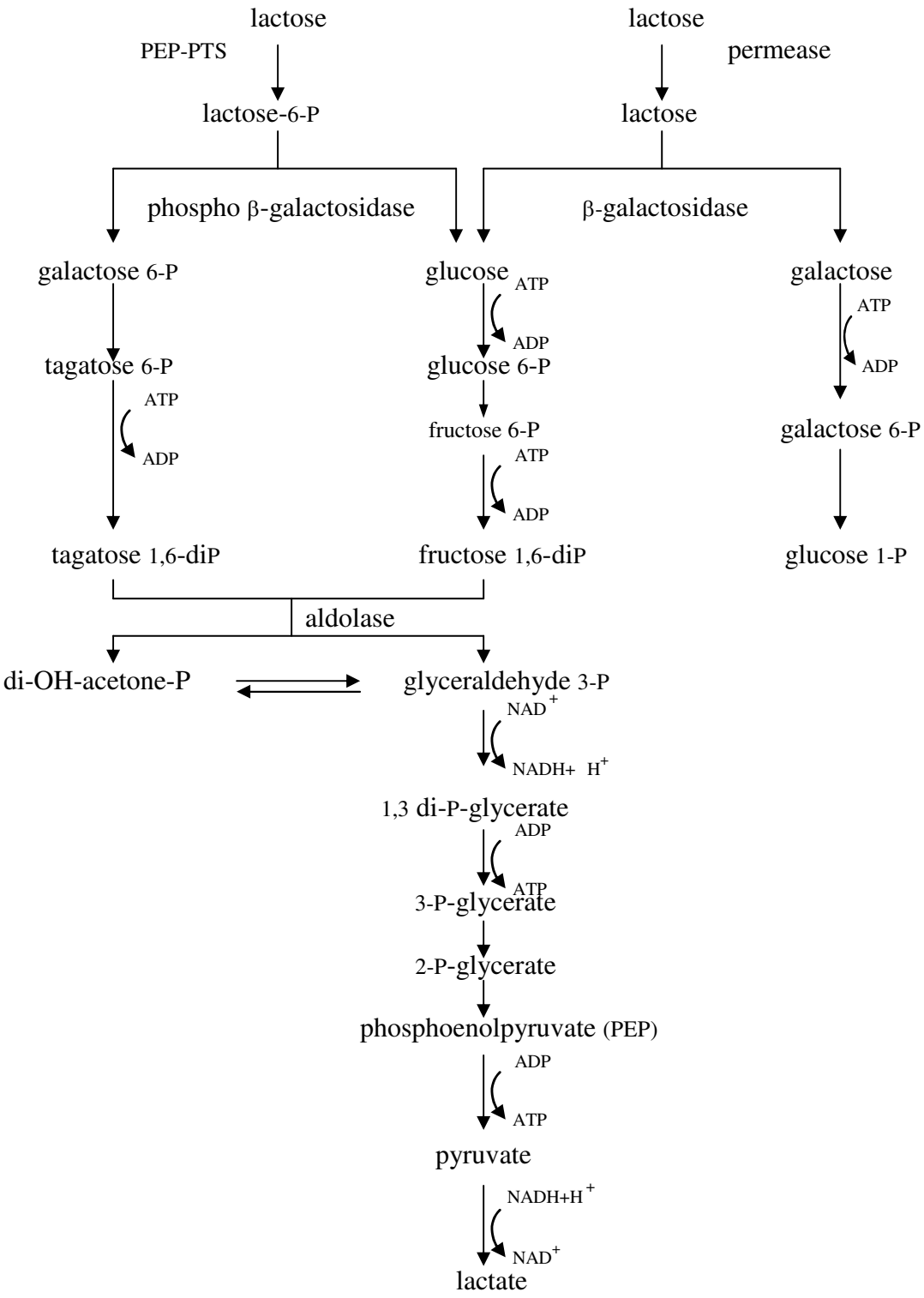


Figure 8. Carbohydrate metabolism in homofermentative lactic acid bacteria (Adopted from Vuyst and Vandamme, (1994))

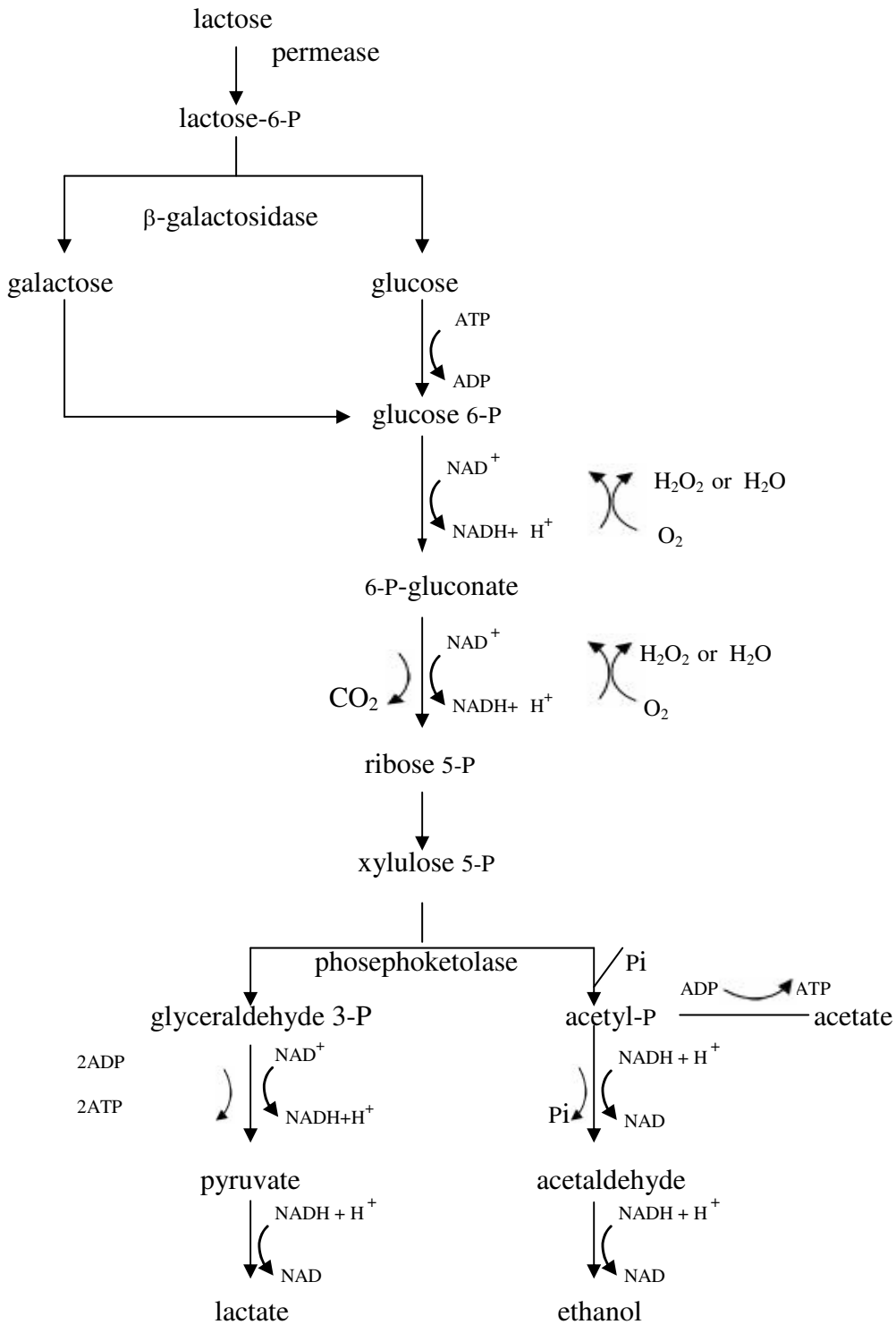


Figure 9. Carbohydrate metabolism in heterofermentative lactic acid bacteria (Adopted from Vuyst and Vandamme, (1994))

Lactobacilli

Amongst LAB, lactobacilli are frequently detected in FPBs (Kantachote *et al*., 2005; Kantachote and Charernjiratrakul 2008) and they can be classified into 3 groups as follows (Hammes and Vogel, 1995).

1. Obligately homofermentative lactobacilli

The EMP is used to convert hexoses to more than 85% lactic acid. Members of this group possess fructose 1, 6-bisphosphate aldolase but lack of phosphoketolase. Therefore, neither gluconate nor pentoses are fermented.

2. Facultatively heterofermentative lactobacilli.

The organisms have both aldolase and phosphoketolase. Hence, not only ferment hexoses but also pentoses (and often gluconate). The enzymes of the phosphogluconate are repressed n the presence of glucose.

3. Obligately heterofermentative lactobacilli

The phosphogluconate pathway or phosphoketolase is used to degrade hexoses and the end products are lactic acid, ethanol (acetic acid) and CO_2. Pentoses enter this pathway and they may be fermented.

Starter cultures for fermentation

Biopreservation, the use of microorganisms to preserve food and feed, has become well known during recent years due to consumers demand to reduced the use of chemical preservatives. Moreover, physical methods such as pasteurization in dairy products results in loss of the flavors and give the end products that they are not satisfy by the consumer (Schnurer and Magnusson, 2005). The preserving ability of lactic acid bacteria (LAB) has been used since ancient times in food and animal feed, such as sauerkraut and silage. The antimicrobial effect of the LAB is mainly related to production of lactic acid and acetic acid (Strom *et al.*, 2005), but some strains, synthesis other antimicrobial compounds; i.e., phenyllactic acid, caproic acid, carbon dioxide, hydrogen peroxide, diacetyl, ethanol, bacteriocins, reuterin, reutericyclin and some organic acids such as propionic acid (Leroy and De Vuyst, 2004b). Due to the "generally regarded as safe: GRAS" status of LAB, the interest in using them for biopreservation has increased during recent years.

Traditional food fermentation is depended on natural LAB which presents in raw materials; therefore, inconsistency either amount or species of LAB affect directly to the variable of final product qualities. Backslopping, inoculation of the raw material with a small quantity of the previously performed successful fermentation which is adapted from the former fermentation by using backslopping resulted into more shorten fermentation period and less fermentation failure than the original process due to the dominant strains of LAB remaining in previous fermented product work as a selected starter culture (Kantachote and Charernjiratrakul, 2008c; Schnurer and Magnusson, 2005). Despite more advantage, precise ratio of mix starters in Backslopping is variable, and some characteristic may be lost during propagation (Schnurer and Magnusson, 2005). In another way, pure culture of LAB isolated from complex ecosystems of traditionally fermented food exhibits a diversity of metabolic activities. It is more advantage in term of competing other microbial growth rate, quality attribute such flavors and aroma, and antimicrobial properties (Schnurer and Magnusson, 2005).

Driehuis *et al*., (1999) found that after *L. buchneri* had been inoculated in maize silage the amount of yeast was decreased and enhance of aerobic stability occurred. Ranjit *et al*., (2002) also evaluated the effect of *L. buchneri 40788* on the silage fermentation, aerobic stability of silage was increased while yeasts in the silage decreased. Similarly to laboratory silos, on-farm, treatment of maize silage with *L. buchneri* 40788 resulted in fermentation and aerobic stability. Laitila *et al*., (2002) revealed that great anti-fusarium activity of *L. plantarum* VTTE-78076(E76) both in vitro and in malting barley was detected. Nevertheless, contaminated level and the fungal strain are directly effect on antifungal potential of *L. plantarum* VTTE-78076(E76). Schwenninger and Meile, (2004) studied the effects of *L. paracacei* subsp. *paracasei* and *Propionibacterium jensenii* on following spoilage yeasts; *Candida pulcherrima*, *C. magnoliae*, *C. parapsilosis* and *Zygosaccharomyces balii*. Results showed that antiyeasts activity in dairy product such yoghurt or cheese had occurred while quality properties of the products had not changed. Optimal amounts of *L. paracacei* subsp. *paracasei* and *P. jensenii* were 1 x 10^8 cells/g and 5x10^7 cells/g, respectively. *L. plantarum* 20B, phenyllactic and 4-hydroxy-phenyllactic acids producing strain was selected as antifungal starter culture in bread. It was found that

A. niger FTDC3227 grew after 2 days in breads that was started with *Saccharomyces cerevisiae* 141 alone or with *S. cerevisiae* and *L. brevis* 1D, an unselected but acidifying lactic acid bacterium, while fungal growth was delayed for 7 days in bread started with *S. cerevisiae* and *L. plantarum* 21B (Lavermicocca *et al.*, 2000). Co-cultivation of antifungal *L. plantarum* MiLAB393 and *Aspergillus nidulans* was conducted and results showed that not only the morphology of fungal was changed and biomass was reduced, but the increasing of several *A. nidulans* proteins was also detected (Ström *et al.,* (2005). Furthermore, they also studied the effect of *L. corynifomis* MiLAB 123 on morphological changing, biomass and protein expression of *A. nidulans* as well. However, effectiveness of *L. corynifomis* MiLAB 123 was less than *L. plantarum* MiLAB393.

Although, many researchers reported about antifungal properties of LAB but conducting antifungal LAB as inoculants in FPBs is relatively low. Kantachote and Charernjiratrakul (2008b) studied the effect of using two types of starter, 5% of *L. plantarum* DW44 (LAB set) and 5% of previously successful FPB (FPB set) compared with no using starter fermentation (control set) on the microbial quality of FPBs. It was found that using the both type of starter (LAB and FPB sets) could reduce the total bacterial and yeast counts. FPB produced from *Gracilaria fisheri* met a standard for yeast number set by the Medical Science Department of no more than 100 CFU/ml. On the contrary, the yeast number of the control set was over this standard. Yeasts were not found in another FPB produced from *Morinda coreia* Ham of the both starter sets, while in the control set, yeast count did not pass the standard.

According to the reviews above, we have an assumption that using a selected promising antifungal LAB strain from fermented foods, particularly from FPBs as a starter culture could inhibit contaminated yeasts in FPBs. Furthermore, we are also interested in the identification of the antifungal substances produced by a selected antifungal LAB strain.

3. Antifungal substances produced by lactic acid bacteria

Antifungal substances of LAB have been recognized and varieties types of bioactive active compounds have been studied such as organic acids, short chain fatty

acids, cyclic dipeptides, phenyllactic acid, reuterin and proteinaceous compounds. (De Muynck *et al.*, 2004; Gourama, 1997; Kantachote and Charernjiratrakul, 2008b; Laitila *et al.*, 2002; Lavermicocca *et al.*, 2000; Magnusson, 2003; Magnusson and Schnurer, 2001; Nakanishi *et al.*, 2002; Okkers *et al.*, 1999; Roy *et al.*, 1996; Sjogren *et al.*, 2003; Talarico and Dobrogosz, 1989).

3.1. Organic acids

Lactic acid, a major organic acid produced by LAB can inhibit many microorganisms by pH reduction. The antimicrobial mechanism was explained by Gould, (1991). Firstly, undissociated molecule of the acid across through the cell membrane and then it dissociate due to a higher pH of cytoplasm, protons releasing and accumulation of anion of the acid resulting in decreasing of intracellular pH. Besides of lactic acid, acetic acid and trace amount of propionic acid are produced from heterofermentative LAB as well (Magnusson *et al.*, 2003; Schnurer and Magnusson, 2005; Wessels *et al.*, 2004). Acetic acid and propionic acid interact with cell membranes, and cause intracellular acidification and protein denaturation. They are more antimicrobially effective than lactic acid due to their higher pKa values (lactic acid 3.08, acetic acid 4.75, and propionic acid 4.87), therefore higher percent of their undissociated acids than lactic acid at a given pH (Earnshaw, 1992a; Earnshaw, 1992b). In yeast, a lowering of intracellular pH affects the carbohydrate metabolism (glycolysis), therefore intracellular acidification directly inhibits the growth of yeast cells (Krebs *et al.*, 1983; Strom *et al.*, 2005). In addition, inhibitory mechanism via destruct of cell membrane have been reported by Piper *et al.*, (2001) and also inhibition of amino acids uptake (Bauer *et al.*, 2003). It was found that *S. cerevisiae* is inhibited by sorbate via intracellular pH decreasing and dysfunction of cell membrane (Plumridge *et al.*, 2003). Nevertheless, Piper *et al*., (1998) found that *S. cerevisiae* has a mechanism for defense organic acids by induction of the plasma membrane proteins Pdr12 and Hsp30. *Zygosaccharomyces baiili* defenses the across of acids by changing the cell envelope moreover it has an ability to degrade the acids (Piper *et al.*, 2001). In filamentous fungi, organic acids are not much inhibitory effect. Anyhow there has an evidence that sorbic acid causes intracellular acidification and

inhibition of conidial germination and mycelial growth in *Aspergillus niger* (Plumridge *et al.*, 2003). Corsetti *et al*. (2004) discovered that a *Lactobacillus sanfrancisco* isolate produced a mixture of organic acids with antimold activity.

Phenyllactic acid (PLA)

It is well recognized that PLA is produced by some strains of LAB i.e. *L. plantarum* 21B (Lavermicocca *et al.*, 2000), *L. plantarum* MiLAB 393.(Strom *et al.*, 2002), *L. corynifomis* Si3, *Pediococcus pentosaceus* and *L. sake* (Magnusson *et al.*, 2003), *L. plantarum* TMW1.468 and *L. sanfranciscensis* DSM20451T (Valerio *et al.*, 2004), and *Lactobacillus* sp. SK007 (Li *et al.*, 2007). The structure of PLA and its configuration are shown in Figure 10.

L-phenyllactic acid D-phenyllactic acid

Figure 10. Structural form of L and D- phenyllactic acid (Magnusson, 2003)

PLA showed fungicidal activity against 13 of the 14 species fungal tested, and these fungi were isolated from bakery products, which included potential toxigenic strains, such as *Aspergillus ochraceus*, *A. flavus*, *Penicillium roqueforti*, *P. verrucosum*, and *P. citrinum*. Less than 7.5 mg/ml of PLA showed 90% growth inhibition of various fungi. Levels of growth inhibition of 50 to 92.4% were observed for all fungal strains after incubation for 3 days in the presence of 7.5 mg/ml of PLA in buffered medium at pH 4, which is similar in real food systems. While less than 10 mg/ml of PLA showed fungicidal activity (Lavermicocca *et al.*, 2003).

Due to broad antifungal spectrum and fungicidal activity, application of PLA to control contaminated fungi in food systems is more effective than commonly used preservatives now in bakery products, such as propionic acid and its salts, which acts as a fungistatic mechanism (Lavermicocca *et al.*, 2003). Similar to the activities of

other weak acid preservatives (propionic acid, benzoic acid, sorbic acid, etc.) and organic acid acidulants (lactic acid, malic acid, citric acid, acetic acid, etc.), the activity of PLA (pKa 3.46) was shown to be pH dependent, indicating that its mode of action is somewhat related to the lipophilic properties which enable the undissociated form to cross microbial membranes (Gould, 1991; Lavermicocca *et al.*, 2003).

Apart from bacteria, PLA is produced by yeasts, *Geotrichum candidum*, which is like member of the natural milk flora that is used as a maturing agent for soft and hard cheeses. Moreover, the anti-*Listeria monocytogenese* activity of PLA was also found. Lactic acid and PLA were compared with their anti-listeria activities. It was found that inhibitory properties of lactic acid are due to its acid nature, not to the molecule itself. lactic acid (120 mM) at pH 5.6 had no action against *L. monocytogenese* in the agar well test, while 120 mM PLA at the same pH gave an inhibitory zone of 37 $\pm$ 07 mm in diameter (Dieuleveux *et al.*, 1998). Besides, *L. monocytogenese*, Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcuss faecalis*, and Gram-negative bacteria such as *Providencia stuartii* and *Klebsiella oxytoca* were also inhibited by PLA. Evidence from the scanning electron microscope indicated PLA affects on bacterial cell wall. Firstly, the bacteria formed aggregation and secreted polysaccharides; their cell walls lost their rigidity, causing the cells to swell. Finally the bacteria broke down completely and the cells disintegrate (Dieuleveux *et al.*, 1998).

It has been shown that PLA is a product of phenylalanine metabolism. The schematic of phenylalanine degradation is shown in Figure 11. Phenylpyruvate is generated after transamination. Phenylpyruvate can then be reduced by hydroxyl acid dehydrogenases, which results in the production of PLA. When phenylpyruvate is decarboxylated, then phenylacetaldehyde is formed, which in turn can be converted to phenylethanol or phenylacetate. When phenylpuryvate is not enzymatically converted, it may undergo a chemical oxidation to benzaldehyde (Valerio *et al.*, 2004). Valerio *et al.*,(2004) studied the influence of amino acids on PLA production. They found that PLA is enhanced when the concentration of phenylalanine increased and the amount of tyrosine decreased. They explained that tyrosine involves in PLA production through the competition with phenylalanine for enzymes is associated with PLA formation. Vermeulen *et al.*,(2006) found that supplying peptides to the culture

medium of *L. plantarum* TMW1468 and *L. sanfranciscensis* DSM20451T resulted in distinguish increasing of PLA production, while PLA formation which transports single amino acids is not efficient (only 1% of the offered phenylalanine was converted to PLA). Li *et al.*, (2007) studied the influence of phenylpyruvic acid (PPA), an intermediate substrate and single amino acid (Phe) on PLA production by *Lactobacillus* sp. SK007. It was obvious that using PPA (6.1 mM) as a substrate resulted in increasing PLA 14-fold compared to that using Phe at the same concentration.

In short, PLA is a novel antimicrobial compound active against both Gram-positive and Gram-negative bacteria (Dieuleveux *et al.*, 1998). Moreover, PLA has a broad inhibitory activity against a wide range of fungi including some mycotoxigenic species (Lavermicocca *et al*. 2003). Phenyllactic acid has also been found as a metabolite involved in formation of cheese flavor produced by LAB strains through degradation of phenylalanine and tyrosine, respectively. In addition, application PLA producing LAB as starter in food and feed was successful. For many reasons mentioned above, PLA is an effective marker of antifungal LAB and LAB starter for fermented foods, (Li *et al.*, 2007; Valerio *et al.*, 2004).

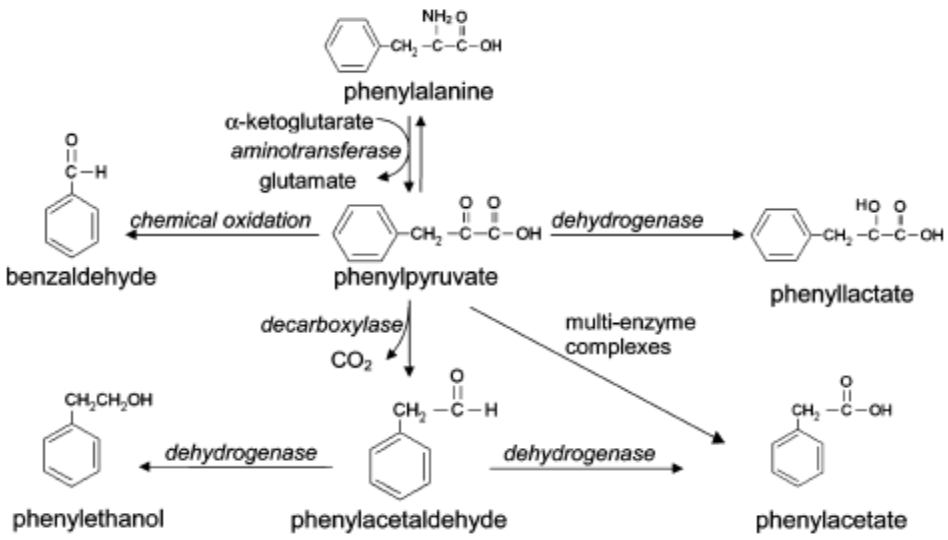


Figure 11. Pathways involved in phenylalanine metabolism in lactic acid bacteria (Valerio *et al.*, 2004)

3.2. Reuterin

It has long been known that reuterin is produced by *L. reuteri*, a heterofermentative LAB normally found in gastrointestinal tract of human and animals (Axelsson *et al*. 1987). It is formed during the anaerobic growth of this strain by the action of glycerol dehydratase which catalyzes the conversion of glycerol into reuterin (Talarico and Dobrogosz, 1989). Reuterin has been chemically identified to be 3-hydroxypropanal (®-hydroxypropionaldehyde), a highly soluble pH-neutral compound which is in equilibrium with its hydrated monomeric and cyclic dimeric forms (Axelsson *et al*. 1989, Talarico and Dobrogosz 1989). The biosynthesis pathway from glycerol to the three forms of reuterin is shown in Figure 12.

The active compound reuterin is in fact an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-HPA (Talarico and Dobrogosz, 1989). Reuterin exhibits a broad spectrum of antimicrobial activity against certain Gram-positive and Gram-negative bacteria, yeast, fungi and protozoa. Spoilage organisms are sensitive to reuterin include genera of *Salmonella, Shigella, Clostridium, Staphylococcus, Listeria, Candida*, and *Trypanosoma* (Axelsson *et al*. 1989 sited by Yang, 2000). The antifungal effect of the addition of glycerol to *L. coryniformis* strains has been investigated by Magnusson and Schnurer (2001), they found that accidental addition of glycerol to the overlay assay led to a dramatic increase of the inhibitory effect against several filamentous fungi and yeast.

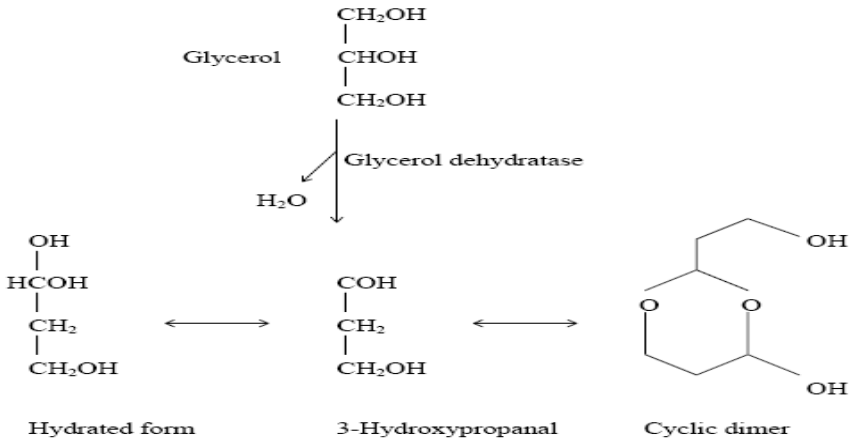


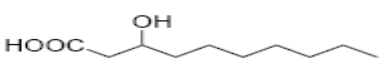
Figure 12. The biosynthesis pathway from glycerol to the three forms of reuterin (Yang, 2000)

3.3. Short chain fatty acids

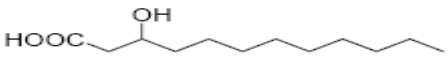
Sjögren *et al*., (2003) discovered four types of 3-hydroxylate fatty acid, 3-hydroxydecanoic acid (myrmicacin, 3-HDA), 3-hydroxydocecanoic acid, 3 hydroxytetradecanoic acid and 3-hydroxy-5-cisdodecenoic acid (Figure 13), which produced from *L. plantarum* MiLAB14. These fatty acids are able to inhibit many yeasts and molds. However, yeasts seem to be more sensitive to 3-hydroxylate fatty acid than to molds. The minimum inhibitory concentration (MIC) of 3-hydroxydecanoic acid was between 10 and 100 μg ml^{-1}, of 3-hydroxydodecanoic acid between 10 and 50 μg/ml, and of 3 hydroxytetradecanoic acid between 10 and >100 μg/ml against molds and yeasts . This could be compared with standard antifungal drugs, *e.g.* amphoteracin B that inhibits fungal growth at concentrations in the μg/ml range (Strom *et al.*, 2005).

The metabolic role of fatty acids and their antifungal mechanism are unknown but the antimicrobial action of fatty acid might be due to the undissociated molecule. At lower pH, their antifungal activity is more effective (Gould, 1991). Moreover, antifungal activity of fatty acids is also depended on chain length (Baird-Parker, 1980; Kabra, 1983; Woolford, 1975). Bergsson *et al* (2001) reported that yeast cells treated with 10 mM of the fatty acids, it was only capric (C10) and lauric (C12) acid that showed antifungal inhibition while monoglycerides had no affect on the growth of *Candida albicans*.

However, using of fatty acids in food system have some disadvantages to illustrate for instance, acids longer than 10 carbons were difficult to solve in water solution (Baird-Parker, 1980; Woolford, 1975). Furthermore, they contribute to the sensory quality of fermented foods (Earnshaw, 1992a).

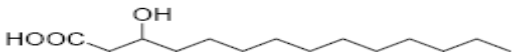


3-hydroxydecanoic acid



3-hydroxytetradecanoic acid

3-hydroxydodecanoic acid

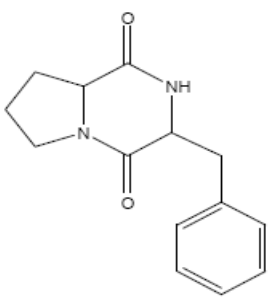


3-hydroxy-5-cis-dodecenoic acid.

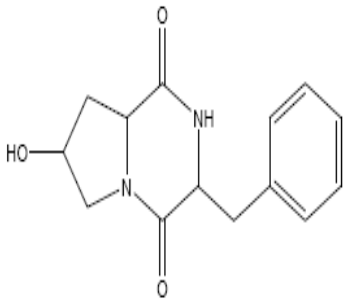
Figure 13. Structure of four fatty acids detected in the supernatant of *Lactobacillus plantrum* strain MiLAB 14 (Sjogren *et al.*, 2003)

3.4. Cyclic dipeptides

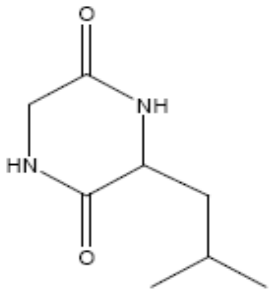
The production of the antifungal cyclic dipeptides cyclo (L-Phe–L-Pro) and cyclo (L-Phe–*trans*-4-OH-L-Pro) by lactic acid bacteria is firstly reported by Ström *et al*.,(2002). *L. plantarum* strain MiLAB393 was isolated from grass silage which produced broad spectrum antifungal compounds active against food- and feed-borne filamentous fungi and yeasts *such as Fusarium sporotrichioides*, *Aspergillus fumigatus* and *Kluyveromyces marxianus*. The antifungal activity of the isolated cyclic dipeptides is most probably a secondary effect; the primary reason for production might instead be related to quorum sensing or other unknown mechanisms. It was found that cyclo (glycyl-L-leucyl) is produced by *L. plantarum* VTT E-78076 as shown in Figure 14A. (Niku-Paavola *et al.*, 1999). In addition, Magnusson (2003) also reported that both of cyclo (Phe-Pro) and cyclo (Phe-OH-Pro) are produced by strains of *Pediococcus pentosaceus* and *L. coryniformis* (Figure14B and 14C). The cyclic dipeptides have antifungal activity at mg/ml concentrations, and hence they are much less effective than the hydroxylated fatty acids (Magnusson, 2003).



A



B



C

Cyclo (Phe-Pro) cyclo (Phe-OH-Pro) cyclo (Gly-Leu)

Figure 14. Antifungal cyclic dipeptides produced by lactic acid bacteria (Magnusson, 2003)

3.5. Proteinaceous compounds

Some LAB strains produce antimicrobial proteins, especially bacteriocins which are against closely related bacterial species. A great number of bacteriocins produced from many LAB strains were published; conversely, a limited research about antifungal proteinaceous compound was reported.

Okkers *et al*., (1999) characterized pentocin TV35b, a bacteriocin like peptide isolated from *L. pentosus* with a fungi static effect on *Candida albicans. L. pentosus* was isolated from vagina of a prenatal patient. Pentocin TV35b totally inhibited *Candida albicans* but could not inhibit some bacteria such as *Clostridium sporogenes, C. tyrobutyricum, L. curvatus, L. fermentum, L. sake, Listeria innocua*, *Propionibacterium* sp. as well. The molecular size of pentocin TV35b was 3.932 KDa as indicated by electrospray ionization mass spectroscopy and consists of 33 amino acids detected by a Pico-Taq method. It was inactivated when treated with papain and proteinase K, but remained inhibitory activity after heating at 100^{o}C for 30 min and incubation at pH 1-10 for 2 h at 25^{o}C.

Magnusson and Schnürer, (2001) reported that *L. coryniformis* subsp. *coryniformis* strain Si3 produced broad-spectrum proteinaceous antifungal compound. By the dual-culture agar plate assay, this strain shown inhibitory effect on several fungi such as *Aspergillus fumigatus*, *A. nidulans*, *Penicillium roqueforti*, *Mucor hiemalis*, *Talaromyces flavus*, *Fusarium poae*, *F. graminearum*, *F. culmorum* and *F. sporotrichoides*, while a weaker activity was observed against the yeasts *Debaryomyces hansenii*, *Kluyveromyces marxianus* and *S. cerevisiae*. Molecular size of this substance which is approximately 3 KDa was evaluated by gel filtration chromatography. It retained antifungal activity after autoclaving at 121^{o}C for 15 min and the maximum activity was observed at pH range 3.0-4.5, but the activity was lost by treating with proteinase K, trypsin and pepsin.

4. Bacteriocin

Bacteriocins are proteinaceous bacterial products which have bactericidal activity. They are produced by various lactic acid bacteria (LAB) (Choi *et al.*, 2000). Many bacteriocins produced by LAB inhibit not only species closely related to the producer strain (Tagg et al. 1976), but also the growth of food-borne pathogens such *as Listeria monocytogenes* and spoilage bacteria (Cintas *et al.*, 1998). Bacteriocin-producing strains can be used as starter cultures for fermented foods in order to improve safety and quality (Bromberg *et al.*, 2004a).

Bacteriocins are commonly classified into 3 groups that also include bacteriocins from other Gram-positive bacteria (Klaenhammer, 1993; Nes *et al.*, 1996). Examples of bacteriocins from these 3 classes are shown in Table 3.

Class I bacteriocins, Lantibiotics (from lanthionine-containing antibiotic) are small (<5 kDa) and heat-stable peptides containing the unusual amino acids lanthionine (Lan), α-methyllanthionine (MeLan), dehydroalanine, and dehydrobutyrine. Class I is further subdivided into type A and type B lantibiotics according to chemical structures and antimicrobial activities (Guder *et al.*, 2000; Moll *et al.*, 1999). Type A lantibiotics are elongated peptides with a net positive charge that exert their activity through the formation of pores in bacterial membranes. Type B lantibiotics are the smaller globular peptides which have a negative or no net charge; antimicrobial activity is related to the inhibition of specific enzymes (Chen and Hoover, 2003).

Class II contains small (<10 kDa), heat-stable, nonmodified peptides (Nes and Holo, 2000). The largest group of bacteriocins in this classification system. These peptides are divided into 3 subgroups. Class IIa includes pediocin-like peptides having an N terminal consensus sequence -Tyr-Gly-Asn-Gly-Val-Xaa-Cys. This subgroup has attracted lot of the attention due to its anti- Listeria activity (Ennahar *et al.*, 2000). Class IIb contains bacteriocins requiring 2 different peptides for activity, and class IIc contains the remaining peptides of the class, including sec-dependent secreted bacteriocins.

Class III contains large (>30 kDa) heat-labile proteins. This group is not well documented. Only few large bacteriocins produced by LAB are described at the

molecular level, such as helveticin J produced by *Lactobacillus helveticus* 481 (Joerger and Klaenhammer, 1986) and helveticin V-1829 produced by *Lactobacillus helveticus* V-1829 (Vaughan *et al.*, 1992).

There is a special class of bacteriocin (Class IV) which requires carbohydrate or lipid moieties for activity (Klaenhammer, 1993); however, bacteriocins in this class have not been characterized adequately at the biochemical level to the extent that the definition of this class requires additional descriptive information (Chen and Hoover, 2003).

Table 3. Bacteriocins produced by Gram-positive bacteria (Adopted from Chen and Hoover, 2003)

Bacteriocins	Producer
Class I-type A lantibiotics	
nisin	*Lactococcus lactis*
lactocin	*Lactobacillus sake*
epidermin	*Staphylococcus epidermidis*
gallidermin	*Staphylococcus gallinarum*
Class I-type B lantibiotics	
mersacidin	*Bacillus subtilis*
cinnamycin	*Streptomyces cinnamoneus*
duramycin	*S. cinnamoneus*
actagardin	*Actinoplanes* ssp.
Class IIa	
pediocin PA-1/AcH	*Pediococcus acidilactici*
sakacin A	*L. sake*
mesentericinY105	*Leuconostoc mesenteroides*
enterocin A	*Enterococcus faecium*
divercin V41	*Carnobacterium divergens*
lactococcin MMFII	*L. lactis*
Class IIb	
lactococcin G	*L. lactis*
plantaricin A	*Lactobacillus plantarum*
plantaricin JK	*L. plantarum*
plantaricin EF	*L. plantarum*
plantaricin JK	*L. plantarum*
Class IIc	
acidocin B	*Lactobacillus acidophilus*
divergicin A	*C. divergens*
enterocin B	*E. faecium*
enterocin B	*E. faecium*
Class III	
helveticin J	*Lactobacillus heleveticus*
helveticin V-1829	*L. helveticus*

5. *Gracilaria fisheri*

Gracilaria fisheri is red seaweed in division Rhodophyta and class Rhodophyceae. The common characteristics are erect thalli, reddish brown to maroon color when fresh, terete to slightly compressed measuring 1 mm or less in diameter, up to 16 cm tall constricted at bases of branches, tapering at ends, branching is alternate and crowd at or near the terminal, growing on sandy to rocky bottom and on dead corals (Lewmanomont, 1994).

It is commonly found in the seashore of the Indian Ocean and the gulf of Thailand. *Gracilaria fisheri* in Thailand has several names such as Sarhai Phomnang, Sai, Sarhai Kao Kwang and Sarhai Won. In Songkla province, south of Thailand, local people have consumed this seaweed for a long time. Moreover, it is an important raw material for agar production (Praiboon *et al.*, 2006; Werawanich *et al.*, 2000). Physical and chemical characterizations of agar polysaccharide extracted from this seaweed was revealed by Praiboon *et al.*,(2006). Fresh and dried form of *Gracilaria fisheri* is shown in Figure 15 and 16, respectively.

For the medicinal benefit of this seaweed, Amornlertpison *et al.*, (2007) and Amornlertpison *et al.*, (2007) found the antioxidant and hypotensive activities of *G. fisheri*. Moreover, Zandi *et al.*, (2007b) found the antiviral activity (herpes simplex virus type 2) of the seaweed in *Gracillaria* sp.

Kantachote *et al*., (2005) found that *G. fisheri* in a fermented beverage form exhibited antibacterial activity against pathogenic bacteria such as *S. aureus*, *V. parahaemolyticus*, *E. coli*, and *Salmonella* sp. In addition, Kongkiattikajorn and Pongdam, (2006) isolated and characterized a vanadium-haloperoxidase produced by *G. fisheri* collected on the Southern Thailand coast, at Koh-Yor (Yor island) in Songklha province. This compound is important for pharmaceutical and industrial applications (Littlechild, 1999).

Figure 15. Fresh form of *Gracilaria fisheri* from Koh-Yor, Songkhla, Thailand

Figure 16. Dried form of *Gracilaria fisheri* from Koh-Yor, Songkhla, Thailand.

CHAPTER 3

MATERIALS AND METHODS

I. Materials

1. Chemicals and Media

Chemicals	Company
Acetic acid	Nacalai tesque
Acetonitril	Nacalai tesque
Amylase	Fluka
Beef extract	Difco
Bismuth Sulfite agar	Difco
Baird-Parker medium	Difco
Catalase	Fluka
Cellobiose	Fluka
α-Chymotrypsin	Fluka
Cyclohexamide	Fluka
DL-phenyllactic acid (DL-PLA)	Sigma
D-phenyllactic acid (D-PLA)	Wako
de Man Rogasa Sharp medium (MRS)	Merck
Endo medium	Difco
Eosin Methylene Blue medium (EMB)	Difco
Ethyl acetate (EtOAc)	Nacalai tesque
Ethanol (EtOH)	Nacalai tesque
Glucose	Fluka
Galactose	Fluka
Hydrochloric acid	Nacalai tesque
Inulin	Fluka
L-phenyllactic acid (L-PLA)	TCI

Chemicals and Media (continued)

Chemicals	Company
Lactic acid	Nacalai tesque
Lactose	BD
Lipase	Fluka
Maltose	Fluka
Malt extract (ME)	Merck
Methanol (MeOH)	Nacalai tesque
Nutrient agar (NA)	Difco
N, O-bis (trimethylsilyl)-acetamide (BSA)	Nacalai tesque
Perfringens agar	Difco
Pepsin	Fluka
Plate count agar (PCA)	Difco
Potato dextrose agar (PDA)	Difco
Potassium metabisulfite (KMS)	Nacalai tesque
Pronase E	Fluka
Raffinose	Fluka
Sodium hydroxide (NaOH)	Nacalai tesque
Starch	Fluka
Sucrose	Fluka
Sulfuric acid (H_2SO_4)	Lab Scan
Trehalose	Fluka
Tris hydrochloride	Fluka
Tryptic soy agar (TSA)	Merck
Tryptic soy broth (TSB)	Merck
Trypsin	Fluka
Xylose	Fluka

2. Equipments

Equipments	Model/Company
Analytical balance 5 digits	AT201, Mettler
Analytical balance 5 digits	Libror L-200 SM, Shimadzu
API 50 CH kit	BioMérieux
Autoclave	HG50, Hirayama
Auto-pipettes	Gilson
Centrifuge	Kubota 6500, Kubota
Desiccator	Iwaki
Fermented bucket	Japin
Freeze dryer	N.Y.12484, STS system
Cellulose acetate filter (0.22 and 0.45µm)	Sartorius
Filter paper	Whatman No.1
Gas chromatography (GC)	HP5890 seriesII, HP
Haemacytometer	Boeco, Germany
HPLC	Agilent 1100 series, HP
Laminar flow	DF-11AK, Dalton
Light microscope	model G, Nikon
Microplate Reader	Power $wav_{x,}$ Biotek
Micro-titerplate 96 wells	Falcon
Mass Spectrometer (MS)	JMS automass20, JEOL
Water bath	Mag-Mixer4, Yamato
pH meter	IQ 125, IQ
Rotary evaporator	EYELA
Plate heater	Eupa
Vacuum pump	SVR 16F, Hitachi
Vortex	G-560E, Scientific industry

3. Microorganisms

Microorganism	Source
Aspergillus flavus TISTR 3041	Thailand Institute of Scientific and Technological Research
Aspergillus nidulans TISTR 3169	Thailand Institute of Scientific and Technological Research
Aspergillus niger PSSCMI 3001	Department of Microbiology, PSU
Bacillus cereus ATCC 11178	Department of Microbiology, PSU
Candida albicans PSSCMI 7010	Department of Microbiology, PSU
Cryptococcus neoformans PSSCMI 7011	Department of Microbiology, PSU
Endomycopsis sp. PSSCMI 7004	Department of Microbiology, PSU
Escherichia coli PSSCMI 0001	Department of Microbiology, PSU
Fusarium moniliforms PSSCMI 3011	Department of Microbiology, PSU
Hansenula sp. PSSCMI 7007	Department of Microbiology, PSU
Klebsiella pneumoniae PSSCMI 0031	Department of Microbiology, PSU
Listeria monocytogenes DMST 4553	Department of Medical Sciences Collection, Thailand
Pennicillium sp. PSUNMI	Department of Microbiology, PSU
Proteus vulgaris PSSCMI 0041	Department of Microbiology, PSU
Pseudomonas aeruginosa PSSCMI 0048	Department of Microbiology, PSU
Rhodotorula mucilaginosa PSSCMI 7006	Department of Microbiology, PSU
Salmonella thyphi PSSCMI 0034	Department of Microbiology, PSU
Schizosaccharomyces sp. PSSCMI 7008	Department of Microbiology, PSU
Shigella sonneii PSSCMI 0032	Department of Microbiology, PSU
Staphylococcus aureus PSSCMI 0004	Department of Microbiology, PSU
Vibrio parahaemolyticus PSSCMI 0064	Department of Microbiology, PSU

4. Fermented plant beverages

Commercial samples of FPBs from various sources of Thailand were kindly supported by Assistant professor Dr. Chaiyavat Chaiyasut. For laboratory FPBs, plant materials were purchased from local markets in Hat-Yai, Songkhla province. The plant materials were well mixed with cane sugar and potable water in the ratio of 1:3:10 (w/w/v).

5. Fermented food products

Fermented food products such as fermented fish, fermented pork, yoghurt, fermented vegetables were purchased from local markets in Hat-Yai, Songkhla province.

II. Methods

1. Isolation and identification of contaminated yeasts in FPBs

Twenty seven FPBs commercial products were collected from various sources in Thailand and thirteen FPBs products were also produced in Department of Microbiology, Faculty of Science, Prince of Sonkla University (PSU). Each sample of FPBs was fermented from different raw materials such as noni (*Morinda coreias* Ham), *Phyllanthus emblica* Linn (Malacca fruit), *Houttuynia cordata* Thunb (Heartleaf), *Kaempferia parviflora* Wall (Krachai-Dam), *Benincasa hispida* Thunb (Green pumpkin), *Salacca* sp. (Salacca), *Momordica charantia* Linn (Bitter cucumber), Zea mar (Babi corn), *Capsicum annuum* Linn (Chili spur pepper), *Carica papaya* Linn (Papaya), *Citrus aurantifolia* (Lime), *Glycine max* (L.) Merr (Soybean), *Cucarbita moschata* (Pumkin), *Citrullus lanatus* (Water melon), *Vigna radiate* (Mung bean), and *Bruguiera Parviflora* (Kidney bean). To isolate yeasts, 0.1 ml of each sample was spread on the Malt extract agar (MEA), pH 3.5 and then incubated at 30^{o}C for 48-72 h. Purity of each distinguished colony was checked by using a microscope and the pure culture was maintained on MEA slant at 4^{o}C. Identification

was conducted by the conventional methods as described in Standard Taxonomic Manuals as follows: biochemical test; sugar assimilation, sugar fermentation, and morphological observation on agar by a light microscope (Barnett *et al*., 2000) and investigated in genera and species levels by 26S rDNA sequencing. In brief, the DNA of yeasts were extracted and amplified by PCR (Polymerase Chain Reaction). The amplified products were further purified and directly sequenced in a DNA sequencer and compared to 26S rDNA gene data obtained from Genbank by using BLASTN program from National Center for Biotechnology Information (NCBI). Detail of DNA sequencing is described in Appendix B.

2. Isolation and selection of AFLAB from Thai fermented foods and FPBs

2.1. LAB isolation

LAB were isolated from 150 samples of Thai fermented food such as fermented fish, Nham, Somfuk, Esan sausage, pickles and Budo and FPBs. A 10% food sample in diluent (0.85% NaCl) was homogenized. One loopful of a diluted sample or undiluted of FPB was streaked on MRS plate (0.02% bromocresol purple) and incubated at 30^{o}C for 48 h. LAB colonies, which changed the medium color around colonies to yellow were isolated and purified by streaking on MRS plates. Purified culture was tested by a microscope after Gram staining and it was also checked for catalase negative. The purified cultures were kept at -20^{o}C in MRS broth containing 15% glycerine.

2.2. Primary screening (Combination of organic acids and H_2O_2 production)

All isolates of LAB were primary screened for their antifungal activity by using agar spot assay (Harris *et al.*, 1992) and no attempt was made to control the production of either H_2O_2 or organic acids, Overnight, 10 µl of LAB's cultures were spotted onto 2% glucose-MRS agar plates. The cultures were incubated under aerobic conditions at 30^{o}C for 24 h before being overlaid with 10 ml of MEA (0.75% agar) inoculated with 10^4 cells/ml of 48 h culture of the indicator strain (*Rhodotorula*

mucilaginosa DKA). After further incubation for 48 h, colonies were examined for clear zones of inhibition surrounding them.

2.3. Secondary screening (Limitation of both organic acids and H_2O_2 production)

The selected strains of LAB from the primary screening were further selected by dual culture assay (Strom *et al.*, 2005), streaking two 2-cm lines of each selected strain, 18 h on 0.2% glucose-MRS agar plates and allowing to grow in an anaerobic jar to achieve anaerobic conditions at 30^{o}C for 48 h. The plates were then overlaid with 10 ml of malt extract soft agar (MEA) (2% malt extract) containing 10^4 cells per ml of the target yeast selected as previously mentioned in 2.2 and incubation at 30^{o}C for 48 h. The radius of inhibition zone was measured. Yeast inhibition was graded by following scales: (-) no inhibition zone, (+) inhibition zone 1-3 mm; (++) inhibition zone >3-10 mm, and (+++) inhibition zone > 10mm. The inhibition test was done in duplicate and the group which showed strong inhibitory effect (+++) was selected for investigation of antifungal spectrum.

3. Antifungal spectrum

The antifungal spectrum of selected AFLAB strains was tested with several food spoilage molds and some of pathogenic molds and yeasts by agar spot method as previously described in the step 2.2. However, in this study, 0.2% glucose MRS medium was used instead of 2% glucose MRS medium. The fungal indicators were following provided; *Hansenula* sp. PSSCMI 7007*, Rhodotorula mucilaginosa* PSSCMI 7006*., Schizosaccharomyces* sp. PSSCMI 700*8, Saccharomyces cerevisiae* PSSCMI 7002 *., Candida albicans* PSSCMI 7010*, Pichia* sp*., Cryptococcus neoformans* PSSCMI 7011*, Candida tropicalis* DKE, *Issatchenkia occidentalis* DKB, *Pichia membranifaciens* DKC, *Pichia anolama* DKD, *Rhodotorula mucilaginosa* DKA, and *Saccharomyces cerevisiae* DKF.

4. Identification of lactic acid bacteria

The selected strains were identified by a commercial test kit, API 50 CH kit (BioMérieux). In brief, the selected strains were streaked on MRS plate and incubated at 30°C for 48 h. The colonies were inoculated in 3 ml of sterile distilled water and adjusted to $9x10^8$ CFU/ml by comparing the turbidity with Mcfarland No. 3 and further adjusted to 6.0 $x10^8$ CFU/ml in 10 ml of API solution by comparing the turbidity with Mcfarland No. 2. Aliquot of inoculated API solution (120 µl) were added in test media and incubated at 37°C for 24-48 h. Positive result were interpreted when the color media were changed to yellow. Identification was performed by using API web stand alone V.1.1.0.

To confirm identification results from the test kit, the selected strains were identified by 16S rDNA sequencing technique (see details in Appendix B), in short, the DNA of bacteria were extracted and amplified by PCR (Polymerase Chain Reaction). The amplified products were further purified and directly sequenced in a DNA sequencer and compared to 16S rDNA gene data obtained from Genbank by using BLASTIN 2.0.5 program from National Center for Biotechnology Information (NCBI).

5. Partial characterization of antimicrobial substances produced by a selected strain

5.1. Preparation of cell free concentrated supernatant

The selected strain was inoculated in MRS broth incubated at room temperature for 24 h, adjusted to $1.5x10^8$ cells/ml by 0.5 McFarland and 8 ml of inoculant was added in 400 ml MRS broth (2% inoculant) left at room temperature for 48 h. Cell-free supernatant was obtained by centrifugation at 12,000 rpm for 15 min and filtration by 0.45 µm cellulose acetate filter and then concentrated by freeze drying. 10-fold concentration of supernatant (10X FDS) was prepared by dissolving 7 g of freeze dried filtrate in 20 ml distilled water. The 10X FDS was adjusted pH to 6.5 by 2M NaOH and sterilized by filtration though 0.45 µm cellulose acetate filter. Whilst 10X MRS broth was also prepared and served as the negative control.

5.2. Antimicrobial spectrum

The 10X FDS of LAB was tested for its antimicrobial spectrum by agar well diffusion by modified the method of Choi *et al*., (2000) and Magnusson and Schnurer (2001). The indicators were *Staphylococcus aureus* PSSCMI 0004, *Bacillus cereus* ATCC 11778, *Escherichia coli* PSSCMI 0001*, Salmonella typhi* PSSCMI 0034, *Shigella sonneii* PSSCMI 0032, *Pseudomonas aeruginosa* PSSCMI 0048*, Vibrio parahaemolyticus* PSSCMI 64*, Rhodotorula mucilaginosa* DKA, *Issatchenkia ocidentalis* DKB, *Pichia membranifaciens* DKC*, Pichia anomala* DKD*, Candida tropicalis* DKE*, Saccharomyces cerevisiae DKF***,** *Candida albicans* PSSCMI 7010, *Candida neoformans* PSSCMI 7011, *Aspergillus flavus* TISTR 3041*, Aspergillus nidulans* TISTR 3169*, Aspergillus niger* PSSCMI 3001*, Pennicillium sp.* PSUNMI and *Fusarium moniliforms* PSSCMI 3011**.** Bacterial indicators were cultured in TSA (Tryptic soy agar) for 24 h at room temperature. The colonies on TSA were inoculated in TSB (Tryptic soy broth) for 4 h, adjusted to 0.5 Mcfarland and diluted to $1.5x10^8$ cells/ml before testing while fungal indicators were grown on malt extract agar (MEA) slants (Merck) at 25°C for 7 days and then stored at 5°C. Inocula containing spores or conidia were prepared by growing the molds on MEA slants for 7 to 10 days (or until sporulation) and then collecting spores or conidia after vigorously shaking the slants with sterile peptone water (0.2% [w/v]). Yeast cell inocula were prepared from washed cultures grown in malt extract broth (Merck) as still cultures at 30°C for 24 h. Mold (spores or conidia) and yeast concentrations were determined using haemocytometer, and adjusted to 10^5 cells or spores/ml of sterile peptone water (0.2%).

For the agar well diffusion assay, TSA/MEA agar plates containing 10^4 cells or spores of indicators per ml were prepared. Wells, with a diameter of 5 mm, were then punch in the agar using a sterile cork-borer. Then, 50 µl samples were added to the wells and incubated at 30°C for 48 h. In order to eliminate the action of organic acids produced by LAB on the test organisms, the pH of the concentrated supernatant was adjusted to 6.5 with 1M NaOH. 10X MRS medium without inoculation was used as a negative control set whereas 10X FDS without adjustment of pH was used as a positive control.

5.3. Effects of enzymes, pH and temperature on antibacterial activity

The 10X FDS were treated with the following enzymes at a final concentration of 0.1 mg/mL: lipase in 0.05 Tris hydrochloride (pH 8.0) with 0.01 M $CaCl_2$, α-chymotrypsin in 0.05 M tris hydrochloride (pH 8.0), trypsin in 0.05 M Tris Hydrochloride (pH 8.0), pepsin in 0.2 M citrate (pH 6.0), pronase E in 20 mM Tris-HCl, pH (7.8), proteinase K in 1 N NaOH (pH 6.5), catalase in 10 mM potassium phosphate (pH 7.0), amylase in 1 N NaOH (pH 6.5). All these solutions were filter-sterilized through 0.22 µm filters and then added to sterile cell-free supernatants (v/v, 1/1). Controls consisted of enzyme solutions without cell-free supernatant in their respective buffer. The samples and controls were incubated at 37°C for 1 h, except for samples containing trypsin, α-chymotrypsin and catalase which were incubated at 25°C (Bromberg *et al.*, 2004a; Magnusson and Schnurer, 2001; Savadogo *et al.*, 2004). Before evaluating the antifungal activity, the pH of the supernatants was readjusted initial pH to 6.5.

The pH effect was investigated with 10X FDS, in 10-ml aliquots, adjusted to pH values of 3, 4, 5, 6, 7, 8, 9 and 10 with 1 M HCl and 2 M NaOH before evaluating the antifungal activity. Concentrated 10X MRS broth was separately adjusted to the same pH values, served as controls.

Aliquots (10 ml) of 10X FDS, prepared as described above to obtain pH 6.5 were heated at 40, 60, 80, 100 for 30 min or 121°C for 15 min. The samples were allowed to cool and then tested for antifungal activity. The 10X MRS broth treated with different temperature served as controls. The remaining activity was determined by agar well diffusion assay as described above (5.2), whereas *S. aureus* PSSCMI 0004 and *E. coli* PSSCMI 0001 were used as indicator strains. The activity was expressed in arbitary units (AU/ml). One AU was defined as the reciprocal of the highest serial dilution showing a clear zone of growth inhibition of the indicator strain (Schillinger and Lucke, 1989).

$$\text{Activity (AU/ml)} = \frac{1000\ \mu l \times \text{dilution fold}}{\text{Volume of supernatant (50 } \mu l)}$$

5.4. Effect of storage conditions on antibacterial activity

The 10X FDS in a dried form as freeze dry supernatant (FDS) and in a form of FDS solution were kept at -20, 4 and 37°C for 30 days. The remaining antibacterial activity was determined at days 0, 1, 14, and 30 by agar well diffusion assay as described above (5.2). *S. aureus* PSSCMI 0004 and *E. coli* PSSCMI 0001 were used as indicator strains.

6. Identification of the antifungal substance produced by a selected strain

6.1. Preparation of 10X FDS

The selected AFLAB strain was inoculated in MRS broth incubated at room temperature under static condition as no shaking for 24 h, adjusted to $1.5x10^8$ cells/ml by 0.5 McFarland and 8 ml of inoculant was added in 400 ml MRS broth (2% inoculant) left at room temperature for 48 h. Cell-free supernatant was obtained by centrifugation at 12,000 rpm for 15 min and filtration by 0.45 µm cellulose acetate filter. The cell free supernatant was examined for antifungal activity by the microtitre plate well method. The 10X FDS was prepared as previously described in the step 5.1.

6. 2. Antifungal assay

Antifungal activity was measured by the microtitre plate well method (Magnusson *et al*., 2003). Briefly, 100 µl of ME broth (Malt extract) containing 10^4 fungal spores or yeast cells per ml were added to each well of a microtitre plate. 100 µl of test samples were transferred to the corresponding wells and incubated at 30^oC for 48 h while 10X MRS was served as negative control. The fungal growth was measured by a microplate reader at optical density 550 nm. The inhibition was calculated as follows.

$$\% \text{ Inhibition} = \frac{(100 - \text{test sample OD}) \times 100}{\text{Negative control OD}}$$

6. 3. Enzyme treatment of 10X cell free supernatant

The 10X FDS were treated with various enzymes as described in the step 5.3. Before evaluating the antifungal activity the pH of the supernatants was readjusted to initial pH value 3.6 because this pH was a final pH of cell free supernatant. The 10X MRS broth treated with various enzymes used served as negative controls whereas the positive control was a 10XFDS without addition of enzyme. The remaining antifungal activity was determined by microtiter plate well method as described above (6.2) and *R. mucilaginosa* DKA was used as the fungal indicator.

6. 4. High performance liquid chromatography (HPLC)

In order to investigate other antifungal active substance besides proteinacious compounds, Phenyllactic acid (PLA), one of effective marker for AFLAB and starter was reviewed (Chapter 2) and preliminary checked by HPLC. HPLC analysis of PLA was performed using a rapid method described by Armaforte *et al* (2006). The HPLC condition was slightly modified from the paper. In brief, the filtrated supernatant was fractionated by R-HPLC, equipped with a quanternary pump, diode array detector set at 210 nm, a Hypersil ODS column C18 RP (250 x 4.0 mm, HP, particle size 5 um) at room temperature, linear gradient from 25% acetronitrile in 75% water (pH 3) (at 0-3.0 min, flow rate 1ml/min) to 50% acetronitrile in 50% water (pH 3) (in 4 min-6min, flow rate 1 ml/min) and acetronitrile was adjusted to 100% (in 8 min-12 min, flow rate 1.3 ml/min). Chem Station for LC 3D. Rev.A.10.02 (1757) was used for data acquisition and processing on a personal computer. The chromatogram of filtrated supernatant was compared with DL-PLA standard (Sigma).

6.5. Preparation of the freeze-dried sample (FDS) of the cell-free supernatant

Cell free supernatant was prepared as described in the step 6.1 and was frozen by freeze dryer for 24 h to obtain 10X FDS.

6.6. Extraction of PLA

An aqueous solution of FDS (1g/ml in distilled water) was adjusted pH to 2 by 1 N HCl and then extracted three times with a half volumes of ethyl acetate. The organic extract was dried over Na_2SO_4 and concentrated in vacuum to obtain the ethyl acetate fraction (EA fraction).

6.7. Thin layer chromatography (TLC)

TLC analysis of the EA fraction was performed on a silica gel plate (Merck, Kieselgel, Germany; 60 F254, 0.25 mm). The extract solution was spotted on the TLC plate by a capillary tube and developed in ethyl acetate: acetic acid (9.5: 0.5, v/v). The spots were visualized by exposing the plates to UV radiation at 254 nm and spraying with anisaldehyde: sulfuric acid: ethanol (0.5:0.5:9, v/v/v) followed by heating for 1 min.

6.8. Gas chromatograph-Mass spectrometer (GC-MS)

Derivatization and GC-MS analysis were performed according to the method described by Chaves and Gianfagna (2006). Briefly, the dried EA fraction (corresponding to 16 mg of FDS) was treated with 50 µl of *N*, *O*-bis (trimethylsilyl) acetamide (BSA) at 70°C for 15 min and then injected in the GC without further purification. GC-MS analyses were performed using a Hewlet Packard (HP) gas chromatograph 5890 series, equipped with Agilent column DB-1 MS (30m_ 0.25 mm i.d., 0.25 µm film thickness) using helium as a carrier gas. Oven temperature was set at 70^{o}C, held for 2 min and increased to 240°C at 10^{o}C per min and held for 30 min. The MS source temperature was set at 230^{o}C. The compounds corresponding to the peaks in total ion chromatogram were tentatively identified by library search of the JEOL data system (Lucy version 5.0 software, JEOL). This preliminary identification was confirmed by co-injection of an authentic compound. Amount of PLA was quantified by using four difference concentrations of authentic DL-3-PLA (4 -16 µg) and *p*-hydroxybenzoic acid was served as the internal standard (Appendix C).

7. Characterization of antifungal activity of PLA

7.1. Determination of MIC_{90}

MIC_{90} were determined for concentrated supernatant, ethyl acetate extract (EA layer), water extract (water layer) and 3 forms of commercial phenyllactic acid (D-, L- and DL-PLA).

Concentrated supernatant (20 fold) were prepared by dissolving 7 g of freeze dried sample in 10 ml water which corresponded to 700 mg freeze dried sample/ml and then diluted to 525, 350, 175, 87.5, 35, and 17.5 mg/ml, respectively by distilled water and adjusted pH to 4 by 2 N NaOH before filtrated through 0.22 μm filter.

EA layer was prepared by dissolving 10 g of freeze dried sample in 125 ml distilled water and extraction by 60 ml EtOAc for three times (60x3). The EA layer (150 ml) was separated from a water layer by centrifugation at 6000 rpm for 10 min. For this step, 150 ml of a EA layer contained 10,000 mg of FDS (1000 mg/15 ml). In order to obtain the amount which corresponded to the concentrated FDS (700 mg/ml), 31.5 ml of the EA layer was evaporated and resuspended in 3 ml distilled water. The concentrated sample was diluted to 525, 350, 175, 87.5, 35 and 17.5 mg/ml, respectively by distilled water and adjusted pH to 4 by 2 N NaOH before filtrated through 0.22 μm filter.

After removing the EA layer, 120 ml of the water layer was obtained. In order to prepare concentration of water layer equal to EA layer, 25.2 ml of the water layer was evaporated and dissolved in 3 ml of distilled water. The concentrated sample was diluted to 525, 350, 175, 87.5, 35 and 17.5 mg/ml, respectively by distilled water and adjusted pH to 4 by 2 N NaOH before filtrated though 0.22 μm filter.

Each form of PLA stock solution was prepared by dissolving 100 mg PLA in 1 ml of 8% methanol and adjusted pH to 4 by 2 N NaOH. The stock solution was diluted to 20, 15, 10, 7.5, 5, 3.75, 2.5 and 1.25 mg/ml by 8% MeOH and adjusted pH to 4 by 2 N NaOH and then sterilized by filtration though 0.22 μm cellulose acetated filter. Cell growth in the 8% methanol-water solution and ME broth without PLA was used as a negative control. MIC_{90} determinations were performed in triplicates as

serial two fold dilutions using the microtiter plate well method described by Strom *et al*, (2002) and with *R. mucilaginosa* as a target organism. In the microtiter plate well method, a 100 µl sample and 100 µl of malt extract broth (2%) containing 10^4 cells/ml were added to each well. After 48 h incubation at 30°C, the inhibition was measured by optical density at 550 nm. The MIC_{90} was determined as the lowest concentration where 90% inhibition of cell growth compared to the negative controls was observed.

7.2. Influence of pH on PLA activity

PLA solution 5 mg/ml in 8% MeOH was adjusted to pH 2.6, 4.0, 4.5, 5.0, and 5.5 with 2 N NaOH. The influence of pH on PLA activity was assayed by using the microtiter plate well method. *R. mucilaginosa* DKA was used as a target organism. The pH adjusted of 8% MeOH solutions were used as control sets.

7.3. Influence of temperature on PLA activity

PLA solution 5 mg/ml (pH 4) in 8% MeOH was separately incubated at 40, 60, 80, 100 for 30 min and 121°C for 15 min before assay by using the microtiter plate well method, and *R. mucilaginosa* DKA was used as a target organism. The adjusted pH 4 of 8% MeOH solution was used as controls.

7.4. Antifungal spectrum

DL-PLA solution 5 mg/ml (pH 4) in 8% MeOH was tested its antifungal spectrum by using the microtiter plate well method. The following target fungal strains were used *Rhodotorula mucilaginosa* DKA, *Issatchenkia occidentalis* DKB, *Pichia membranifaciens* DKC, *Pichia analoma* DKD, *Candida tropicalis* DKE, *Saccharomyces cerevisiae* DKF, *Hanseniaspora clermontiae* PKG, *Pichia ferinosa* PKC, *Endomycopsis* sp.PSSCMI 7004, *Candida albicans* PSSCMI 7010, *Schizosaccharomyces* sp. PSSCMI 7008**,** *Aspergillus niger* PSSCMI 3001, *Fusarium moniliforms* PSSCMI 3011, *Aspergillus flavus* TISTR 3041, *Aspergillus nidulans*

TISTR 3169 and *Pennicillium* sp. PSUNMI. The adjusted pH 4 of 8% MeOH solution was used as a control set.

8. Effect of the selected AFLAB strain used as inoculants on the quality of fermented seaweed beverage (FSB)

8.1 Seaweed fermentation

Lactobacillus plantarum DW3 was used as a starter inoculum in this study. The bacterium was inoculated into 100 ml MRS broth and incubated at 30^{o}C until the culture reached the late log phase. The cells were harvested by centrifugation at 6000 rpm/min for 15 min. The cell pellet was washed twice with 0.85% NaCl and then resuspended approximately to 1.5 x 10^{8} CFU/ml based on the McFarland number 0.5 for use as the inoculum. Dried seaweed (*Gracilaria fisheri*) was purchased from a local market at Yor island, Songkhla province. The experimental design comprised four different treatments. Two sets of treatments used seaweed without pre-treatment by potassium metabisulfite (KMS). One set of these was inoculated with a 5% starter culture to obtain an initial cell density of approximately 7.5 x 10^{6} CFU/ml (N-S). The other set, with no added starter (N-N) served as a natural fermentation control. In another two sets, the seaweed was subjected to partial sterilization, by pre-treating with 0.5% KMS, overnight, followed by cleaning with tap water. One set was inoculated with 5% of the starter culture to provide 7.5 x 10^{6} CFU/ml (P-S) and the last set had no added starter (P-N) to act as a negative control. The seaweed was well mixed with cane sugar and potable water in the ratio of 3:1:10 (w/w/v). The mixture had a total volume of 12 L in a 15 L plastic bucket and the starter was added to achieve an initial cell concentration as mentioned above. The space above the fermentation liquid was filled with a water containing plastic bag and covered with the bucket's lid (Kantachote and Charernjiratrakul, 2008a). All fermentation buckets were incubated in a controlled room at 30 ± 2^{o}C by air conditioner for 60 days (Figure 17). All sets of fermented seaweed beverage (FSB) were monitored to evaluate their physicochemical properties, role of microorganisms, microbiological quality, antimicrobial activity and a sensory test.

8.2 Microbial populations

Total bacterial counts (TBC) and lactic acid bacteria (LAB) were enumerated on Plate Count Agar (PCA) and de Man Rogosa Sharpe (MRS agar), respectively by the pour plate method, whereas yeasts were counted on MEA by the spread plate technique (APHA, 1995) at days 0, 1, 2, 4, 7, 14, 21, 30, 45 and 60.

8.3. Microbial quality

Standard microbiological methods (APHA, 1995) were used to enumerate microbes at days 0, 14, 30, 45 and 60. Additionally, microbiological quality after 3 months storage was investigated. Mold and yeast numbers were counted on MEA by the spread-plate method. Total coliform bacteria and *E. coli* were counted on Endo medium and EMB (Eosin Methylene Blue medium) using a membrane filter technique, while *Salmonella* sp., *Stabphylococcus aureus* and *Clostridium perfringens* were counted on Bismuth Sulfite agar (BS), Baird-Parker medium and Perfringens agar, respectively.

8.4. Physicochemical quality

The following parameters were monitored in every FSB set at days 0, 1, 2, 3, 4, 5, 6, 7, 14, 21, 30, 45 and 60; pH and electrical conductivity (pH-EC meter), total sugar as glucose (Dubois *et al*., 1956), total acidity as lactic acid (AOAC, 1990). Amounts of ethanol, methanol, lactic acid and acetic acid were assayed at days 0, 14, 30, 45 and 60 by gas chromatography according to the method of Yang and Choong, (2001). While Cu, Zn, Fe, Na, K, As and Pb were also analyzed at these times by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) following the define methods provided by the company.

8.5. Antibacterial activity

Several foodborne pathogens were provided by Department of Microbiology, Faculty of Science, Prince of Songkla University as follows: *Bacillus*

cereus ATCC 11778, *Staphylococcus aureus* PSSCMI 0004, *Escherichia coli* PSSCMI 0001, *Salmonella typhi* PSSCMI 0034 and *Vibrio parahaemolyticus* PSSCMI 0064. Antibacterial activity of the FSB (125 µl filtrate using a 0.45 µm Millipore filter) was conducted by the agar well diffusion technique (Schillinger and Lucke, 1989) at days 0, 30, 60 and after storing for 3 months.

8.6. Sensory quality

All treatments of the FSB at day 60 and after storage for 3 months were organoleptically evaluated by 30 panels with Hedonic-5-scale on flavor, odor, color, clearness and overall acceptation (Chambers and Wolf, 1996). The scores 1, 2, 3, 4 and 5 define as dislike extremely, dislike, moderate acceptable (neither like nor dislike), like, like extremely respectively.

9. Experimental design and statistical analysis

In this study, all of experiments had been conducted in duplicate. One-way analysis of variance was used for most of experiments that needed statistics analyses, except for seaweed fermentation. After that the Duncan multiple comparison test was used to analyze significant differences between treatments. The seaweed fermentation in steps 8.3, 8.4, 8.5 and 8.6, were statistical analyzed by using the multiple general linear model in order to establish whether the monitored parameters were affected by the variable conditions used for the fermentation. The Duncan's method was also used with the monitored factors for investigating their significant differences. The means, standard deviations and least significant difference (LSD) values ≤ 0.05 were presented.

Figure 17. Photo illustrating the seaweed (*Gracilaria fisheri*) in the fermenting plastic bucket.

CHAPTER 4

RESULTS AND DISCUSSION

Part I. Isolation and Identification of FPBs contaminated yeasts

Fifty nine yeast strains were isolated from 40 samples of FPBs (Table 4). Based on biochemical characteristics such as carbon assimilation, sugar fermentation, and observation of spore shapes and pigment appearance, all strains were classified into 7 groups (Tables 5-6). Contaminated yeasts were named as follows: DKA, DKB, DKC, DKD, DKE, DKF (DK means Duangporn Kantachote). Figure 18 demonstrates cells of 6 main contaminated yeast strains. However, they were a few unclassified groups because they can not be placed in any group (Tables 5 and 6). Except unclassified group, the representatives of each group were further identified by 26S rDNA sequence (see details in Appendix B) and results are shown in Table 7. From total 59 isolates, the most frequent group (DKA), was *Rhodotorula mucilaginosa* (14 isolates, 23.7%), following by DKC as *Pichia membranifaciens* (11 isolates, 18.6%). The third group, DKD, was *Pichia anomala* (9 isolates, 15.3%) and the rest groups (DKB, DKE and DKF) were *Issatchenkia occidentalis*, *Candida tropicalis* and *Saccharomyces cerevisiae*, respectively (see more details in Table 7).

R. mucilaginosa occurs intensively on fresh fruits and vegetables. Leaves and plant stems are its major habitats, and as a film yeast species, it sometimes occurs in cereals, flours, dough, malting barley, olive, soaking soybean, citrus products and fruit juice concentrate (Harrison, 1997). Plant or fruit is a major component of FPB. Therefore, it was not surprised that *R. mucilaginosa* was the major contaminated yeast in FPBs. This species sometime survived heating at 62.5^{o}C for 10 min (Put *et al.*, 1976). The ability to grow rapidly at refrigeration temperature means that *R. mucilaginosa* is commonly associated with food products and may cause spoilage in dairy products (Harrison, 1997). Whilst *P. membranifaciens and P. anomala* are extensively found in olive brines (Duran *et al.*, 1994) and variety of acetic acid preserves including onion, pickles, beetroot and sauerkraut (Hansen, 1997a) including brine meat product, fermented sausage, spoilage fermented olive, beer and candy

(Adams and Moss, 2000). There are some reports related to human pathogenicity of *P. anomala* such as neonate infection (Wong *et al.*, 2000) and urinary tract infection (Qadri *et al.*, 2001).

Table 4. Fermented plant beverages (FPBs) collected from various sources in Thailand and our laboratory

FPBs	Common name	Fermentation time	Number of sample
1. *Morinda citrifolia* Linn.	Indian mulberry	> 6 months	3
2. *Phyllantanthus emblica* Linn.	Malacca fruit	> 6 months	6
3. *Houttuynia cordata* Thunb.	Heartleaf	> 6 months	6
4. *Kaempferia parviflora*	Krachai-Dam	> 6 months	8
5. *Syzygium cumin* (L.)	Jambolan	> 6 months	4
6. *Benincasa hispida* (Thunb.)	Green pumkin	1 month	1
7. *Salacca* sp.	Salacca	1 month	1
8. *Momordica Charantia* Linn.	Bitter cucumber	1 month	1
9. *Zea mar*	Baby corn	1 month	1
10. *Capsicum annuum* Linn.	Chili spur	1 month	1
11. *Carica papaya* Linn.	Papaya	1 month	1
12. *Citrus aurantifolia*	Lime	1 month	1
13. *Glycine max* (L.) Merr.	Soybean	1 month	1
14. *Cucarbita moschata*	Pumpkin	1 month	1
15. *Morinda citrifolia* Linn.	Indian mulberry	1 month	1
16. *Citrullus lanatus*	Water melon	1 month	1
17. *Vigna radiata*.	Mung bean	1 month	1
18. *Bruguiera Parviflora*	Kidney bean	1 month	1
Total			40

FPBs: numbers 1-5 were commercial products from various sources of Thailand while numbers 6-18 were produced in the laboratory of Microbiology Department, Faculty of Science, Prince of Songkla University (PSU)

Table 5. Characteristics of the FPBs contaminated yeasts based on ability to grow in

various carbon sources or tolerate some compounds

Group	No	Mal	Gal	Suc	Lac	Raf	1.5% Eth	1.5% Mth	10% NaCl	16 % NaCl	0.01% Cyc	0.1% Cyc	Cit	Ure
DKA	14	+	+	+	-	+	+	-	+	+	+	+	+	+
DKB	5	+	-	+	-	+	-	-	-	-	-	-	-	-
DKC	11	+	+	+	-	+	+	-	+	+	d/-	-	+	-
DKD	9	-	-	-	-	-	+	-	-	-	+	+	+	-
DKE	4	+/d	+/d	+	-	-	+	-	+	-	+	+	+	-
DKF	6	+	+	+	-	+	+	-	+	-	-	-	-	-

No = Number of isolate; Mal, Gal, Suc, Lac, Raf, Eth, Mth, Cyc, Cit, Ure mean maltose, galactose, sucrose, lactose, raffinose, ethanol, methanol, cyclohexamine, citrate and urease respectively. + = utilize or growth, - = non utilize or no growth, d = delay

Group	Xyl	Raf	Mal	Inu	Glu	Gal	Suc	Lac	Cel	Sta	Tre	Pigm	Spore
DKA	-	-	-	-	-	-	-	-	-	-	-	pink	Chla
DKB	-	-	-	-	+	-	-	-	+	-	-	-	Asco
DKC	-	+	+	-	+	+	+	-	+	d	d	-	Asco
DKD	-	-	-	-	+	-	-	-	-	-	-	-	Asco
DKE	-	-	+	-	+	+	+	-	-	d	-	-	Chla
DKF	-	+	+	d	+	+	+	-	-	+	d	-	Asco

Table 6. Fermentation, pigment and spore shape of the FPBs contaminated yeasts

Xyl, Raf, Mal, Inu, Glu, Suc, Lac, Cel, Sta, Tre and Pigm mean xylose, raffinose, maltose, inulin, glucose, galactose, sucrose, lactose, cellubiose, starch, trehalose and pigment, respectively; Chla = chlamydospore, Asco = ascospore; + = utilize or growth, - = non utilize or no growth, d = delay

Table 7. Identification of yeasts which contaminated in FPBs by 26S rDNA sequence

Group	Yeast species	Number of isolate from commercial product (>6 months)	Number of isolate from laboratory product (1 month)	Number of isolate (%)
DKA	*Rhodotorula mucilaginosa*	12	2	14 (23.7)
DKB	*Issatchenkia occidentalis*	-	5	5 (8.5)
DKC	*Pichia membranifaciens*	9	2	11 (18.6)
DKD	*Pichia anolama*	9	-	9 (15.3)
DKE	*Candida tropicalis*	3	1	4 (6.8)
DKF	*Saccharomyces cerevisiae*	3	3	6 (10.1)
Unidentified			10	10 (17)
Total		36	23	59

Both *S. cerevisiae* and *C. tropicalis* are always found in partially and fully processed fruits, vegetables and orange juice (Arias *et al.*, 2002; Torok and King, 1991). Naturally, *S. cerevisiae* is widespread in nectars and exudates, on leaves and fruits. It has been known that *S. cerevisiae* plays an important role in the manufactures of bread and alcoholic beverage. Although it is infrequently food spoilage strain, *S. cerevisiae* sometime deteriorates fruit juice and dairy products (Hansen, 1997b). Whilst *I. occidentalis* has been known as olive spoilage yeast which is resistant to lactic acid and citric acid (López *et al.*, 2006; López *et al.*, 2007) and it normally is found in mangaba frozen pulp (Trindade *et al.*, 2002). Four of six main groups, *R. mucilaginosa*, *P. membranifaciens*, *S. cerevisiae* and *P. anomala*, are able to produce pectinase (Panon *et al.*, 1995). Therefore, if these strains contaminated in FPBs which have high pectin contained in plant materials, high level of methanol might be occurred because methanol is produced from pectin degradation by pectinase (Whittaker, 1990). Besides, ethanol produced by contaminated yeast is also a critical problem for FPBs production.

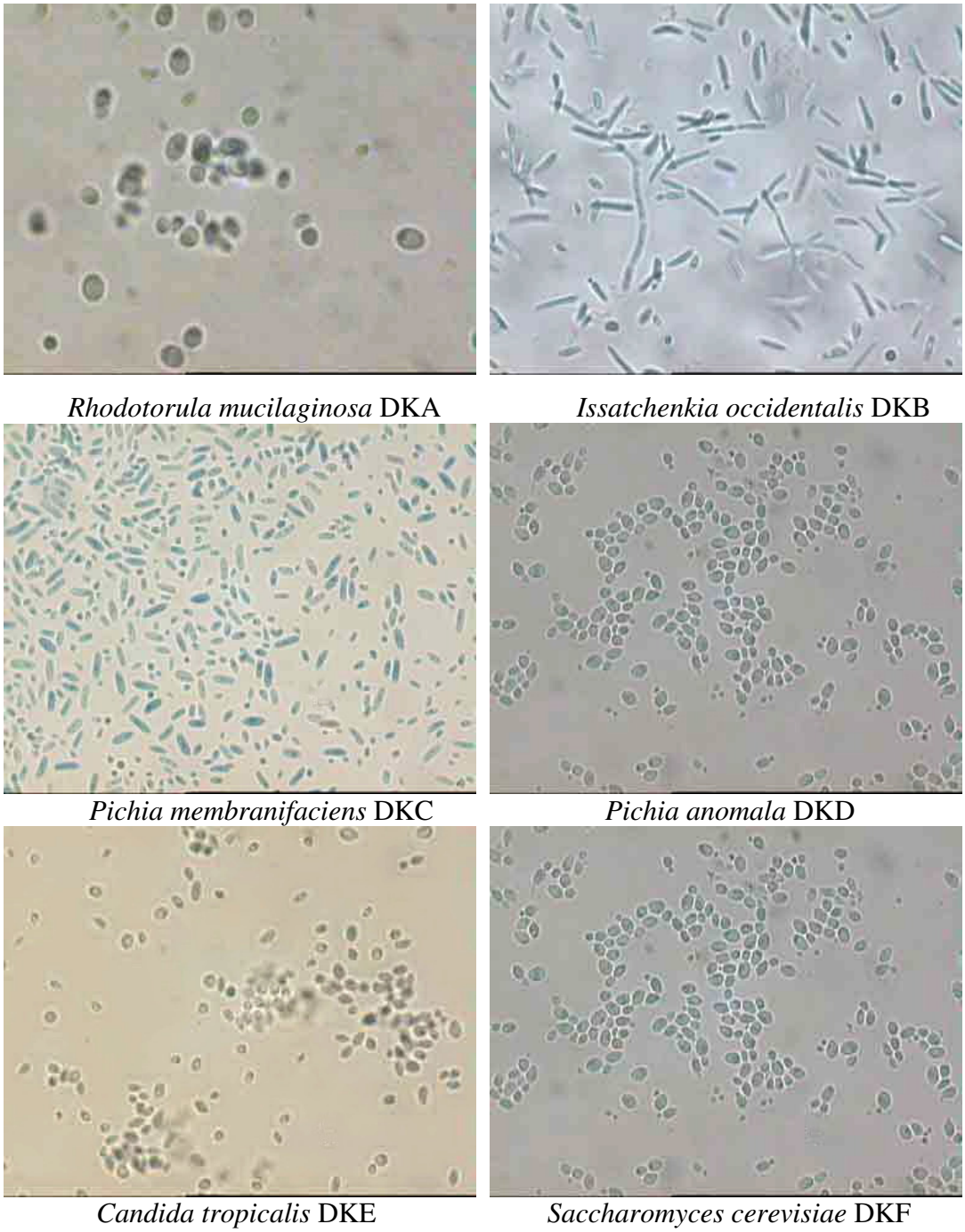


Figure 18. The major contaminated yeast strains isolated from fermented plant beverages (FPBs) (400X magnification)

The last group, was unidentified yeast strains (10 isolates: 17%), was not attractive for further studied due to differences in biochemical results among groups. It was noticeable that this group and *I. occidentalis* (DKB) were only found in the

sample prepared in our laboratory (data not shown) while they were not found in samples obtained from the commercial products. It might be because samples prepared in the laboratory were fermented only 1 month; thereby, varieties of contaminated yeasts still survived in these samples while the samples obtained from the commercial products were fermented more than 3 months and ready to be drunk, thus there were a few yeast strains that survived in the finished products. In addition, the unidentified group might be inhibited by the other groups in FPBs such as *P. membranifaciens* and *P. anomala* as they are killer yeast (Santos and Marquina, 2004; Sawant and Ahearn, 1990; Wang et al., 2007).

In this study, the major group of contaminated yeasts which is widespread in food product and FPBs was *R. mucilaginosa*. Thereby, it was used as the target yeast for screening antifungal lactic acid bacteria (AFLAB).

Part II. Isolation and selection of AFLAB from Thai fermented foods and FPBs

Five hundred LAB strains isolated from Thai fermented foods including FPBs were screened for their antiyeast activity by agar spot technique on MRS medium under aerobic conditions and *R. mucilaginosa* DKA was used as the indicator. Total 369 isolates (73.8%) inhibited the yeast indicator but only 261 isolates (52.2%) gave strongest inhibitory activity (score = 3) as shown in Table 8. Figure 19 shows inhibitory levels of LAB against the yeast indicator by the agar spot technique. Antiyeast activity of LAB in the primary step might be combination of their antimicrobial metabolites which the major metabolite is organic acids and some H_2O_2. LAB isolates growing aerobically can produce both organic acids and H_2O_2 as metabolites (Adams and Moss, 2000). It has long been recognized that LAB produces a variety of organic acids or pH-reducing fermentation products such as lactic and acetic acids, hydrogen peroxide, formic acid, propionic acid, diacetyl and so on (Lindgren and Dobrogosz, 1990).

To investigate whether LAB produce other antiyeast substances besides organic acids, only 261 strong inhibitory activity strains were secondary investigated their antiyeast activity by dual culture assay on 0.2% glucose MRS medium (To limit acid production) under anaerobic conditions in order to limit the amount of organic

acids and H_2O_2 productions. It was found that only 6 isolates showed inhibitory activity (Figure 20). This result indicates that a limited organic acids production, there were a few LAB isolates which produced other antiyeast substances. Recently, other antifungal substances produced by LAB such as cyclic dipeptides, phenyllactic acid, reuterin and proteinaceous compounds have been reported (Lavermicocca *et al.*, 2000; Lindgren and Dobrogosz, 1990; Magnusson and Schnurer, 2001; Sjogren *et al.*, 2003; Strom *et al.*, 2002). It means that 6 isolates might have other antiyeast compounds comparing to organic acids.

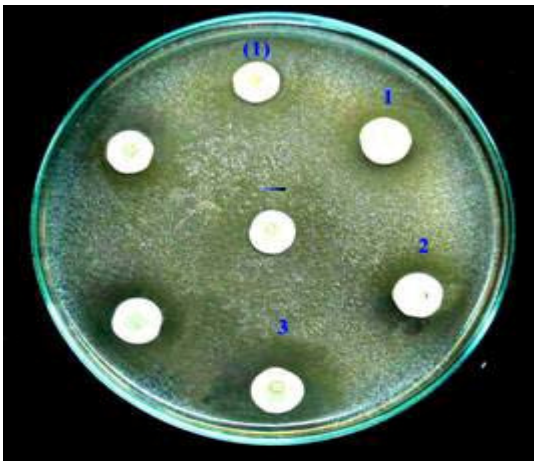


Figure 19. Degree of inhibition of *Rhodotorula mucilaginosa* DKA by LAB isolates. The degree of inhibitions were expressed as (1), 1, 2 and 3 which were inhibition radius less than 1, 2, 3 and more than 3 mm, respectively.

Table 8. Inhibitory effect of LAB isolates against *R. mucilaginosa* DKA by agar spot technique on 2% glucose MRS medium

Degree of inhibition (inhibitory radius)	Number of isolates (%)
- = no inhibition	131 (26.2)
(1) = less than 1 mm.	7 (1.4)
1 = 1-2 mm.	20 (4)
2 = 2-3 mm.	81 (16.2)
3 = more than 3 mm.	261 (52.2)

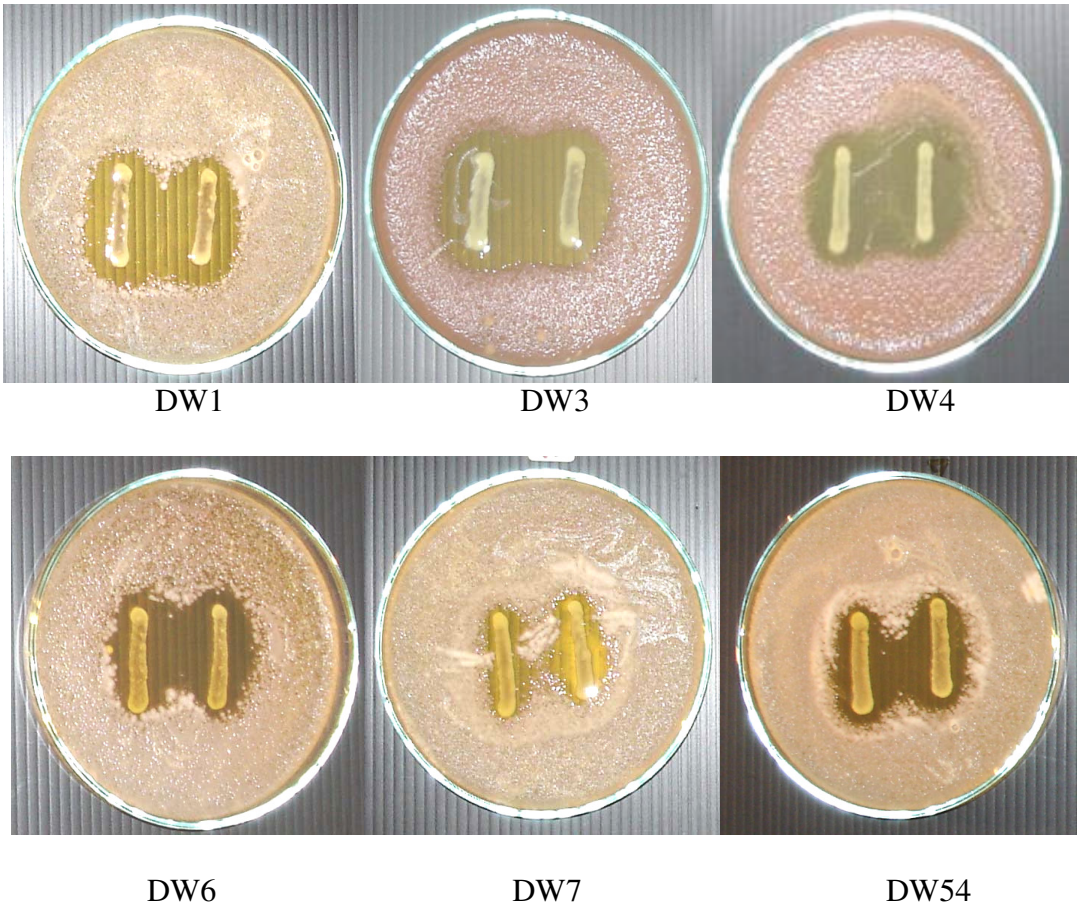


Figure 20. Inhibitory activity of selected LAB isolates against *R. mucilaginosa* DKA by dual culture assay on 0.2% glucose MRS medium
*DW = Duangporn and Wilawan

All 6 LAB isolates were further selected for their antiyeast spectra using the agar spot technique and yeast indicators were 6 dominant contaminated yeasts including the strains that obtained from Department of Microbiology, PSU. The antiyeast spectra of selected LAB were showed in Table 9. All selected LAB isolates showed broad inhibitory spectra against yeast indicators and the DW3 was the most effective strain. The 6 dominant contaminated yeasts isolated from FPBs were almost inhibited by DW3, DW4 and DW6 strains, except *S. cereviaiae* DKF which was only inhibited by DW3 strain. *Rhodotorula mucilaginosa* DKA and *R. mucilaginosa* PSSCMI 7006 were sensitive strains while *Cryptococcus neoformans* PSSCMI 7011, *Pichia* sp. PSSCMI 7003, *Saccharomyces cerevisiae* PSSCMI 7002, and

Schizosaccharomyces sp. PSSCMI 7008 were not inhibited by any selected LAB strains.

The following strains; DW1, DW3, and DW4 were identified as *Lactobacillus plantarum* by API 50 CH test kit due to their strong inhibitory activity against yeasts (Figure 20 and Table 9) and only DW3 was confirmed by 16S rDNA technique (see the result in Appendix B). The results from The DNA sequencing technique indicate that strain DW3 was *Lactobacillus plantarum* as well.

Several researchers have reported that *L. plantarum* has an ability to control fungi(Magnusson, 2003; Magnusson *et al.*, 2003; Sjogren *et al.*, 2003; Strom *et al.*, 2002). *L. plantarum* strain MiLAB 393 from the grass silage could produce compounds with a broad spectrum of antifungal activity against the food- and feed-borne filamentous fungi and also yeasts; *P. anomala, Kluyveromyces marxianus, R. mucilaginosa, Debaromyces hansenii, Candida albicans* and *S. cerevisiae* (Strom *et al.*, 2002). Ström *et al.,* (2005) reported that *Aspergillus nidulans* was inhibited by *L. plantarum* MiLAB393 after co-cultivation because of decreasing of biomass, and secretion of protein. Laitila *et al*., (2002) found great anti-fusarium activity of *L. plantarum* VTTE-78076 both in vitro and in malting barley. Lavermicocca *et al* (2000) demonstrated that *L. plantarum* 20B successfully controlled *Aspergillus niger* FTDC3227 in bread. Sjögren *et al*., (2003) discovered four types fatty acids which produced from *L. plantarum* MiLAB14. These fatty acids showed broad inhibition against fungi, however, yeasts seem to be more sensitive to fatty acids than molds.

Table 9. Antiyeast spectra of selected LAB isolates using agar spot technique on 0.2% glucose MRS medium

Yeast indicator	Selected LAB isolates					
Yeasts isolated from FPBs	DW1	DW3	DW4	DW6	DW7	DW54
Candida tropicalis DKE	1	1	2	1	2	-
Issatchenkia occidentalis DKB	2	2	1	2	2	-
Pichia membranifaciens DKC	2	3	2	-	-	2
Pichia anolama DKD	2	2	2	2	2	1
Rhodotorula mucilaginosa DKA	2	3	3	2	1	2
Saccharomyces cerevisiae DKF	-	1	-	-	-	1
Yeasts from culture collection						
Candida albicans PSSCMI 7010	-	1	1	-	1	-
Cryptococcus neoformans PSSCMI 7011	-	-	-	-	-	-
Endomycopsis sp. PSSCMI 7004	2	2	-	2	2	3
Hansenula sp. PSSCMI 7007	1	1	1	1	1	1
Pichia sp. PSSCMI 7003	-	-	-	-	-	-
Rhodotorula mucilaginosa PSSCMI 7006	3	3	2	3	2	2
Saccharomyces cerevisiae PSSCMI 7002	-	-	-	-	-	-
Schizosaccharomyces sp. PSSCMI 7008	-	-	-	-	-	-

- = no inhibition, 1= inhibition zone 1-2 mm, 2 inhibition zone 2-3 mm, 3 inhibition zone more than 3 mm

According to the previous studies, *L. plantarum* DW3 was considered to use as inoculants for producing FPBs. A promising strain *L. plantarum* DW3 was isolated from a fermented plant beverage (Phomnang seaweed) (Kantachote and Charernjiratrakul, 2004), and it was proved as a probiotic strain (Duangjitcharoen ,2006). Moreover, the isolate DW3 showed inhibitory effect against various of foodborn bacteria such as *S. aureus, E. coli*, *S. typhymurium* and *V. parahaemolyticus* although its organic acids and H_2O_2 productions were limited (Kantachote and Charernjiratrakul, 2008c). In this study, we demonstrated that *L. plantarum* DW3 inhibited many yeast strains (10 of 14 yeast indicators, table 9) therefore, we decided

to use *L. plantarum* DW3 as inoculants for improving the quality of a fermented seaweed beverage (FSB).

Table 10. Identification results of *Lactobacillus plantarum* DW3 by API 50 CH kit

Test	mg/cup	Test sample
Control	-	-
Glyceral	1.64	-
Erythritol	1.44	-
D-arabinose	1.4	-
L-arabinose	1.4	+
D-ribose	1.4	+
D-xylose	1.4	-
L-xylose	1.4	-
D-adonitol	1.36	-
Methyl-BD-xylopyranoside	1.28	-
D-galactose	1.4	+
D-glucose	1.56	+
D-fructose	1.4	+
D-mannose	1.4	+
L-sorbose	1.4	-
L-rhamnose	1.36	-
Dulcitol	1.36	-
Inositol	1.4	-
D-mannitol	1.36	+
D-sorbitol	1.36	+
Methyl-αD-mannopyranoside	1.28	+
Methyl-αD-glucopyranoside	1.28	-
N-acetylglucosamine	1.28	+
Amygdalin	1.08	+
Esculin	1.16	+
Salicin	1.04	-
D-cellobiose	1.32	+
D-maltose	1.4	+
D-lactose	1.4	+
L-lactose	1.4	+
D-melibiose	1.32	-
D-saccharose	1.32	+
D-treharose	1.32	+
D-melezitose	1.32	-
D-raffinose	1.56	-
Amidon(starch)	1.28	-

Table 10. continue

Test	mg/cup	Test sample
Xylitol	1.4	-
Gentiobiose	0.5	+
D-lyxose	1.4	-
D-tagatose	1.4	-
D-Fucose	1.28	-
L-fucose	1.28	-
D-arabitol	1.4	+
L-arabitol	1.4	-
Potassium Gluconate	1.84	-
Potassium-2-keto Gluconate	2.12	-
Potassium-5-keto Gluconate	1.8	-

Figure 21. Identification results of *Lactobacillus plantarum* DW3 by API 50 CH kit.Yellow and purple media indicated positive and negative results, respectively.

III. Partial characterization of antimicrobial substances produced by strain DW3

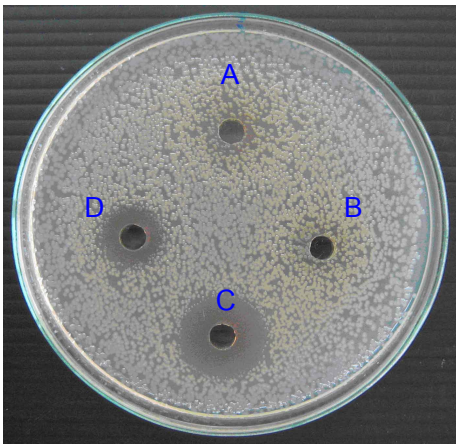
1. Antimicrobial spectra

Antimicrobial spectra of 10X FDS were demonstrated in Table 11 and also Figures 22 and 23. The results indicate that acidic condition of FDS (pH 3.6) showed broad inhibition, especially with bacterial indicators. After neutralization to prevent the inhibitory effect of organic acids, FDS (pH 6.5) still inhibited bacterial indicators, except *B. cereus* ATCC 11778 and *S. typhi* PSSCMI 0034. In neutral pH, *V. paraheamolyticus* PSSCMI 0064 was the most sensitive strain (16.4 mm), while all of fungal indicators were not inhibited by the neutralized FDS. This clearly demonstrated that under conditions which eliminate the possible effect of organic acids, *L. plantarum* DW 3 produced some antimicrobial compounds since FDS showed inhibitory activity against almost of pathogenic foodborne bacteria tested although the pH was adjusted to 6.5.

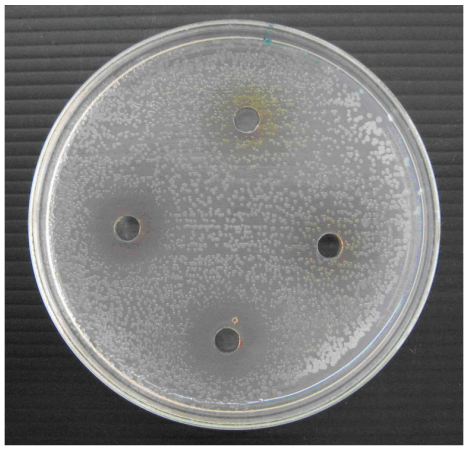
Table 11. Antimicrobial spectra of 10X FDS produced by *L. plantarum* DW3

	Inhibition zone (mm)			
Indicator	Non adjusted pH 3.6		Adjusted pH 6.5	
	Control	FDS	Control	FDS
Staphylococcus aureus PSSCMI 0004	0	20.3	0	14
Bacillus cereus ATCC 11778	0	19.8	0	0
Escherichia coli PSSCMI 0001	0	16.5	0	8.9
Salmonella typhi PSSCMI 0034	0	20.0	0	0
Shigella sonneii PSSCMI 0032	0	21.6	0	14.2
Pseudomonas aeruginosa PSSCMI 0048	0	19.6	0	8.5
Vibrio parahaemolyticus PSSCMI 64	0	18.3	0	16.4
Rhodotorula mucilaginosa DKA	0	11	0	0
Issatchenkia ocidentalis DKB	0	6	0	0
Pichia membranifaciens DKC	0	9	0	0
Pichia anomala DKD	0	8.3	0	0
Candida tropicalis DKE	0	0	0	0
Saccharomyces cerevisiae DKF	0	0	0	0
Candida albicans PSSCMI 7010	0	0	0	0
Candida neoformans PSSCMI 7011	0	0	0	0
Aspergillus flavus TISTR 3041	0	0	0	0
Aspergillus nidulans TISTR 3169	0	7.2	0	0
Aspergillus niger PSSCMI 3001	0	0	0	0
Pennicillium sp. PSUNMI	0	14.0	0	0
Fusarium moniliforms PSSCMI 3011	0	0	0	0

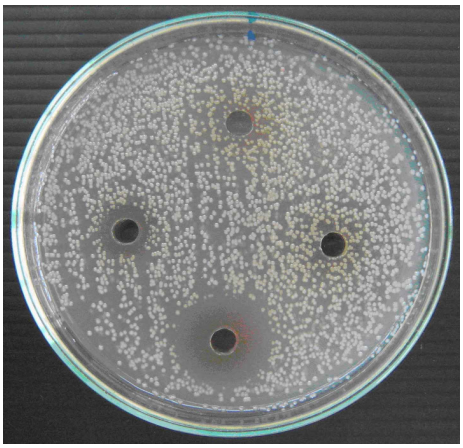
Control = 10X freeze dried MRS medium



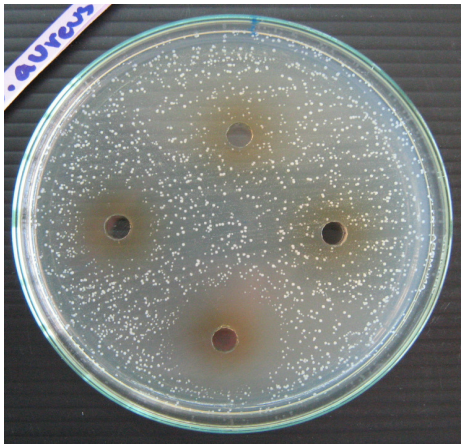
Escherichia coli PSSCMI 0001



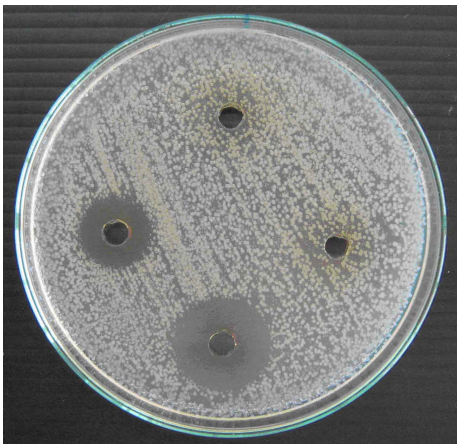
Vibrio parahaemolyticus PSSCMI 0064



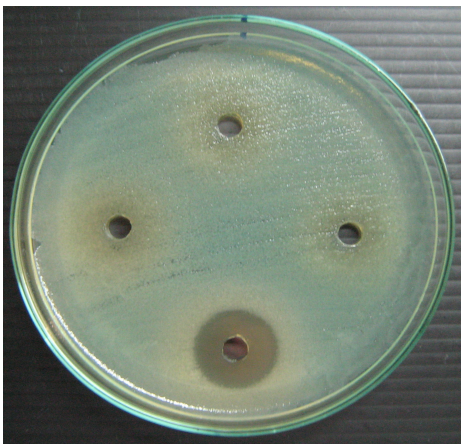
Pseudomonas aeruginosa PSSCMI 0048



Staphylococcus aureus PSSCMI 0004

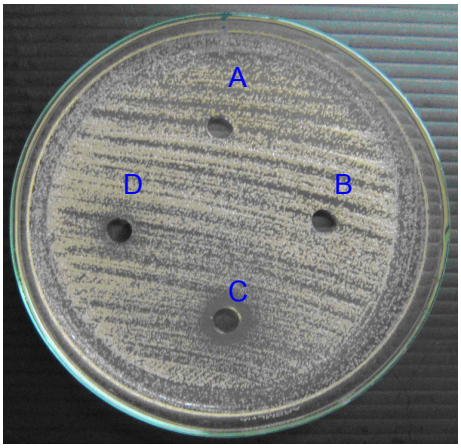


Shigella sonneii PSSCMI 0032

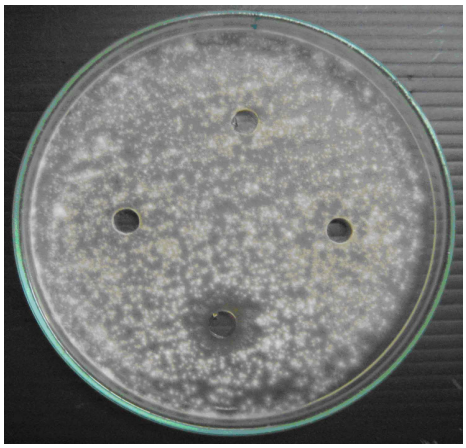


Salmonella typhi PSSCMI 0034

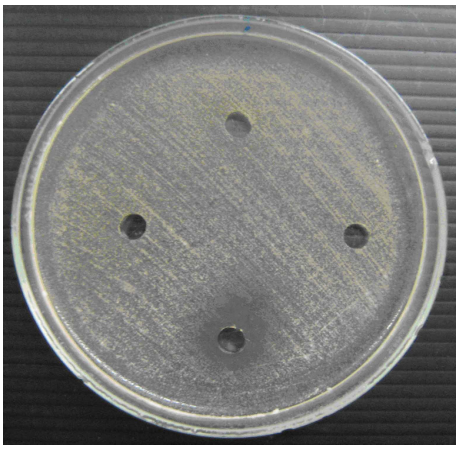
Figure 22. Antibacterial spectra of 10X FDS tested by agar well diffusion in TSA incubated at room temperature for 24 h; A, 10X Freeze dried MRS (pH 3.6); B, 10X Freeze dried MRS (pH 6.5); C, 10X FDS (pH 3.6) and D, 10X FDS (pH 6.5)



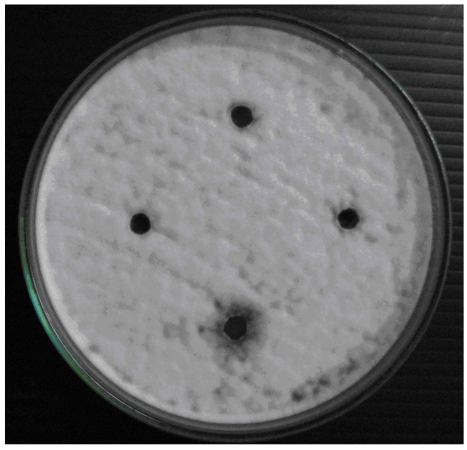
Rhodotorula mucilaginosa DKA



Aspergillus nidulans TISTR 3169



Pennicillium sp. PSUNML



Aspergillus flavus TISTR 3041

Figure 23. Antifungal spectrum of 10X FDS tested by agar well diffusion in MEA incubated at 30°C for 48 h; A, 10X Freeze dried MRS (pH 3.6); B, 10X Freeze dried MRS (pH 6.5); C, 10X FDS (pH 3.6) and D, 10X FDS (pH 6.5)

2. Effect of enzymes on the antibacterial activity of 10xFDS

Figure 24 shows that amylase, lipase and catalase did not affect the activity of antimicrobial substances produced by *L. plantarum* DW3. This finding suggests that the substances were not lipid and carbohydrate moieties or hydrogen peroxide. On the contrary, the substances were inactive after treatment with proteases namely pepsin, trypsin, chymotrypsin, pronase E and protenase K. The sensitivity of the found substances to all proteases is a proof of their proteinaceous nature, which allows us to consider their bacteriocins. Furthermore, it was noticeable that Gram positive bacteria like *S. aureus* was more sensitive to the bacteriocins than to Gram negative bacteria

like *E. coli*. It is confirmed that the substances were one of bacteriocins because bacteriocins are active against a narrow spectrum of closely related bacteria (Vuyst and Vandamme, 1994). The active substances were named bacteriocin DW 3 and the activity was expressed in arbitory units (AU/ml). The activity of bacteriocin DW 3 was 80 AU/ml and 60 AU/ml by testing with *S. aureus* PSSCMI 0004 and *E. coli* PSSCMI 0001, respectively.

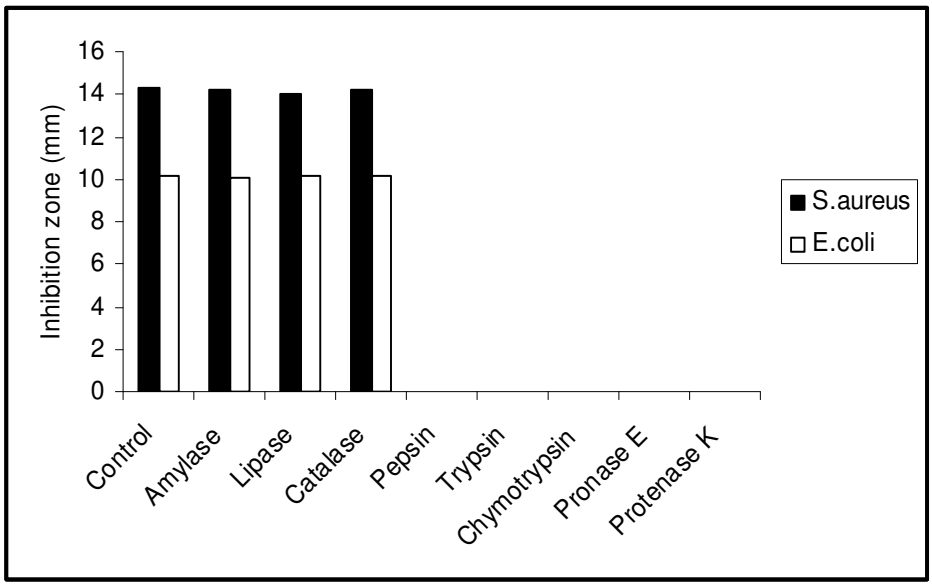


Figure 24. Effect of various enzymes on antibacterial activity of substances produced by *L. plantarum* DW3

3. Effects of pH, temperature and storage condition on the activity of 10X FDS

The pH stability of the 10X FDS was examined in a range of 3-10. It was found that the inhibitory effect of 10X FDS sharply decreased as pH increased (Figure 25). The results reveal that the 10X FDS was more active in an acidic condition than a basidic condition. Regarding food industry, application of 10X FDS, or the use of producing strain DW 3 as a starter culture in food or beverage is advantage in term of preserved food from foodborne pathogen in fermented or acidic food products.

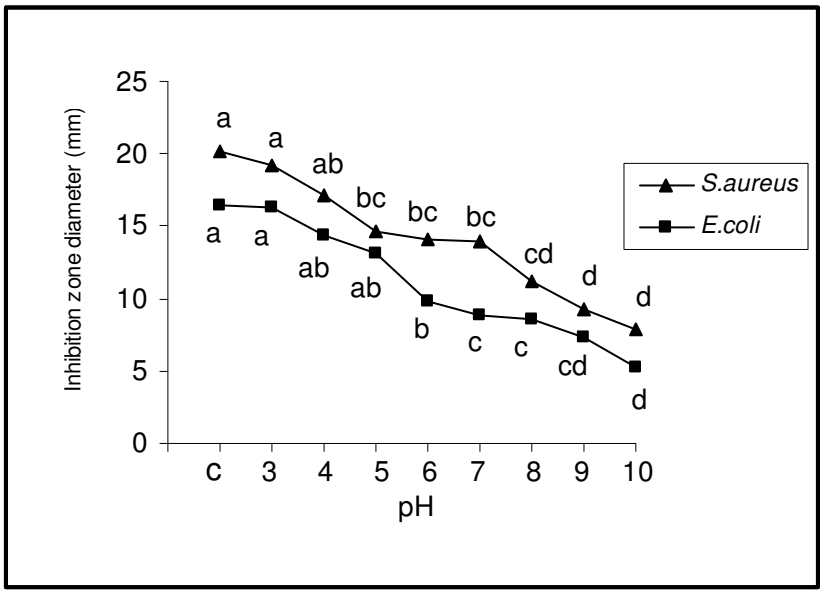


Figure 25. Effect of pH on antibacterial activity of 10X FDS produced by *L. plantarum* DW3 against *S. aureus* PSSCMI 0004 and *E. coli* PSSCMI 0001, the same letter on each point indicates no significant difference (P > 0.05). C = control (unadjusted pH 10X FDS, pH 3.6)

The 10X FDS produced by *L. plantarum* DW3 resisted on pasteurization (60^{o}C, 30 min). In respect to temperature sensitivity, the activity of the 10X FDS against *S. aureus* PSSCMI 0004 significantly dropped when the temperature reached 80^{o}C and 100^{o}C for 30 min as well as 121^{o}C for 15 min. Whilst temperature up to 80^{o}C for 30 min did not make significant change on the inhibitory effect against *E. coli* PSSCMI 0001; however, a little decrease of the activity was observed when temperature was up to 100 and 120^{o}C. Nevertheless, the results indicate that *S. aureus* PSSCMI 0004 was more sensitive than *E. coli* PSSCMI 0001 (Figure 26). If bacteriocin DW3 was the main antibacterial substance produced by *L. plantarum* DW3, It might be class III bacteriocin (large-heat-labile protein). This bacteriocin Generally, bacterial inhibitory activity of class III bacteriocin is reduced when the temperature increases to 60-100^{o}C for 10-15 min (Klaenhammer, 1993). Many bacteriocins in this class i.e. Helvitacin J, acidophilucin A, lactacin A&B have been studied (Klaenhammer *et al*., 1993; Vaughen *et al*., 2004).

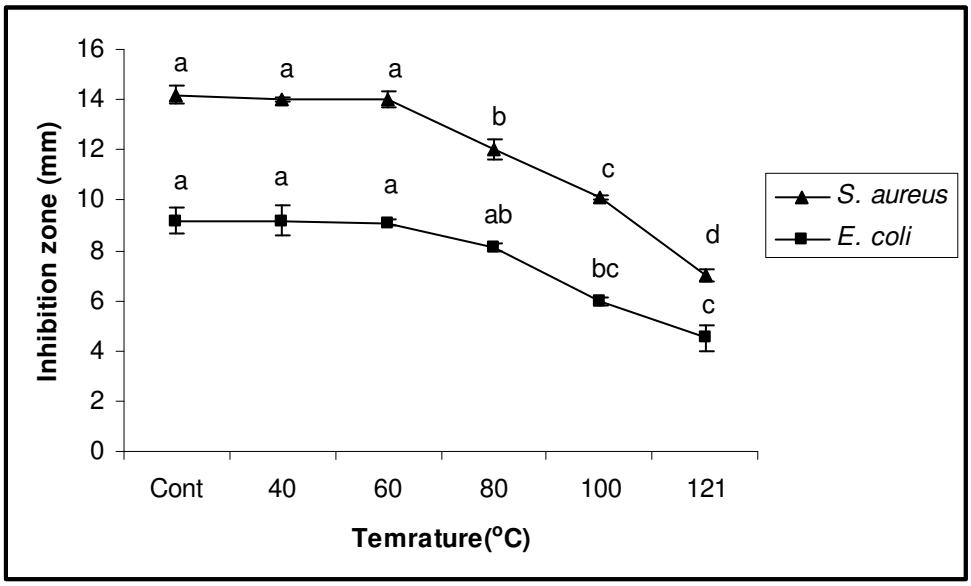


Figure 26. Effect of temperature on the activity of 10X FDS (pH 6.5) against *S. aureus* PSSCMI 0004 and *E. coli* PSSCMI 0001. The same letter on each point indicates no significant difference (P > 0.05); Cont = control (10XFDS in room temperature, pH 3.6)

Storage of concentrated supernatant of DW 3 in 10X FDS solution at -20°C for 30 days did not affect its activity against *S. aureus* PSSCMI 0004 and *E. coli* PSSCMI 0001. In contrast, storage at 4°C and 37°C, the inhibitory activity of bacteriocin DW 3 significantly decreased after 2 weeks (Figure 27: A and B).

Unlike a concentrated solution, the activity of freeze dried sample did not change when keeping at -20°C and 4°C for 30 days. However, at 37°C, the remarkable decrease of its activity was observed over 2 weeks storage (Figure 28: A and B). As aspect, low temperature storage is suggested for stability of the activity of bacteriocins, especially class III, heat sensitive bacteriocin.

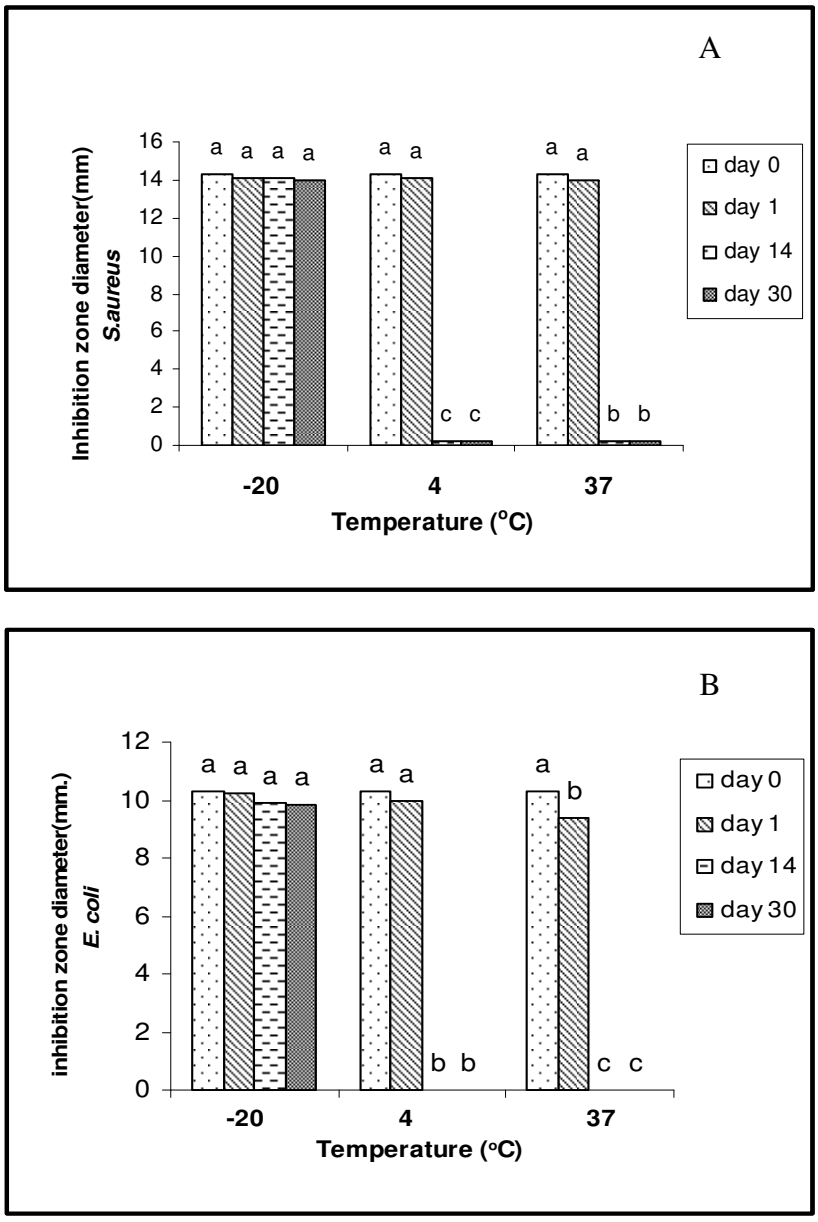


Figure 27. Effect of storage conditions on activity of 10X FDS solution produced by *L. plantarum* DW 3 against *S. aureus,* [A] and *E. coli*, [B]. The different letters on the bars indicate significant differences in each checking time ($P < 0.05$).

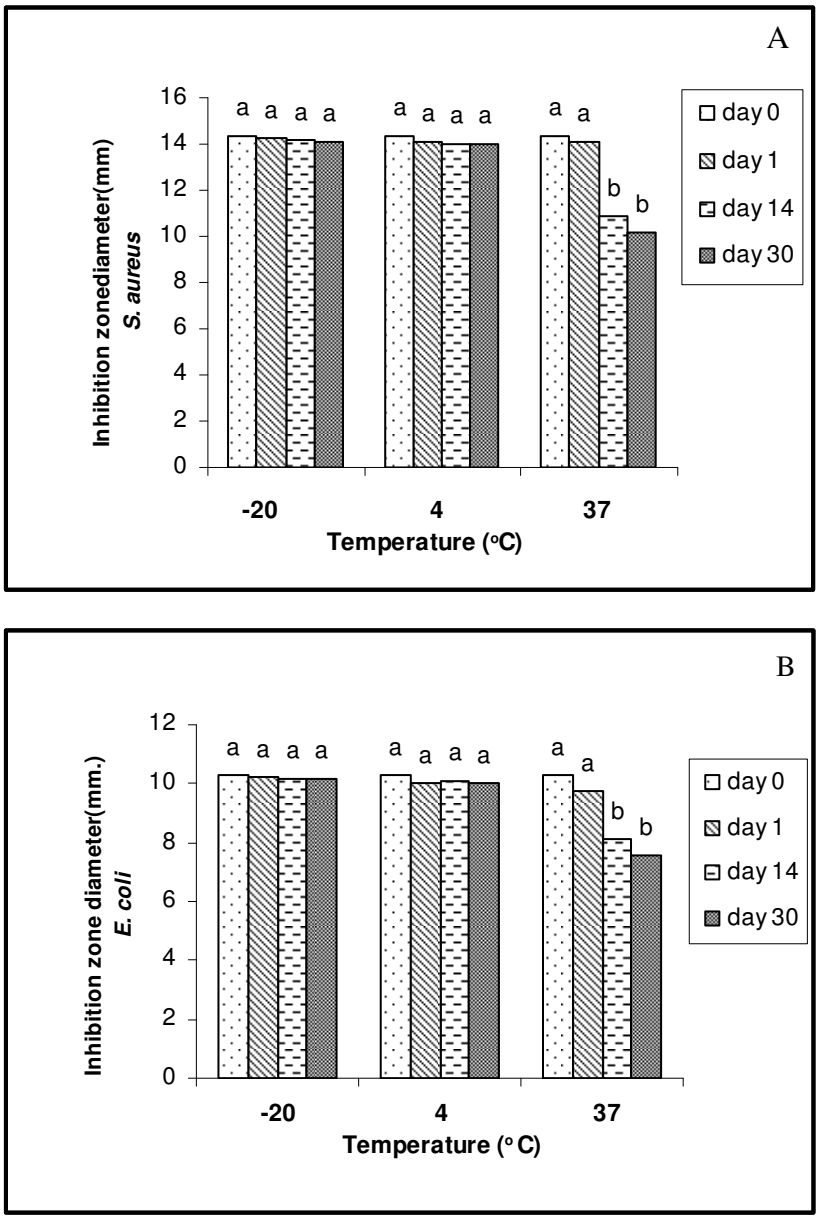


Figure 28. Effect of storage conditions on activity of 10X FDS produced by *L. plantarum* DW 3 (freeze dried form) against *S. aureus* PSSCMI 0004, [A] and *E. coli* PSSCMI 0001, [B]. The different letters on the bars indicate significant differences in each checking time ($P < 0.05$).

The bacteriocin DW3 were not further studied due to the fact that the main aim of this study was to explore antifungal substances produced by *L. plantarum* DW 3 for controlling contaminated yeasts in FPBs. Hence, antifungal substance whether produced by *L. plantarum* DW 3 was further investigated.

Part IV. Identification of the antifungal substance

Effect of enzyme treatment on antifungal substances

The antifungal activity of the cell free supernatant produced by *L. plantarum* DW3 in a form 10X FDS was determined by microtiter plate well method and *R. muciaginosa* was used as a target organism. The 10X FDS showed inhibition of 55.3%, *R. mucilaginosa* DKA, in the positive control set without treatment by enzymes (Figure 29). After treatment with various proteases, the full antifungal activity still remained. Similarly, treatment with amylase, lipase and catalase did not affect the antifungal activity of the supernatant produced by *L. plantarum* DW3.

The results indicate that the antifungal components of the supernatant were not proteins and were not related to carbohydrate or lipid moiety. In addition, catalase had no effect on the antifungal activity; therefore, hydrogen peroxide (H_2O_2) was not one of active compounds as well. Most LAB possess flavoprotein oxidases (and NADH peroxidases), which enables them to produce hydrogen peroxide in the presence of oxygen. However, this study, *L. plantarum* DW3 was grown under the static condition with no oxygen; therefore, no hydrogen peroxide was produced. To prove that *L. plantarum* DW3 may produce other antifungal compounds unlike organic acids such as lactic acid and acetic acid, HPLC was used to investigate antifungal compounds produced by this organism.

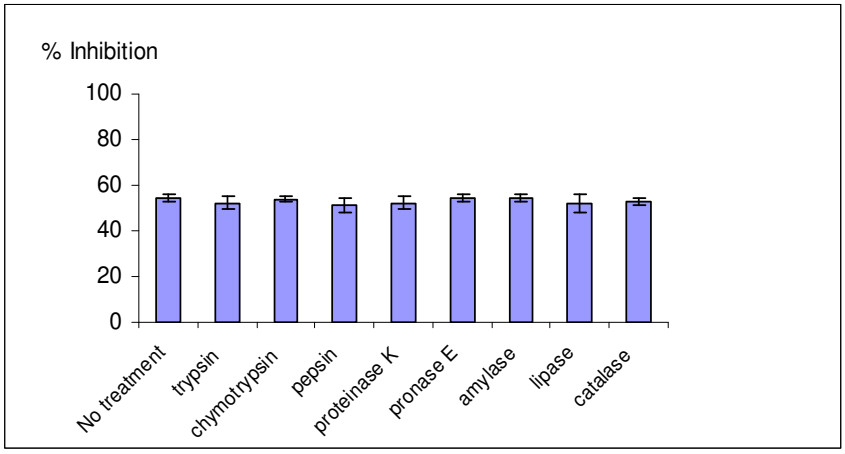


Figure 29. Effect of enzymes on antifungal activity of 10X FDS, pH 3.6 produced by *L. plantarum* DW3 against *R. mucilaginosa* DKA, Results are expressed as inhibition percent from the negative control. Error bars indicate SD (n = 3).

PLA identification

In this study, PLA (phenyllactic acid) in the cell free supernatant of *L. plantarum* DW3 was preliminary detected by HPLC as the peak A in Figure 30 shows the same retention time (4.714 min) with the authentic PLA. To confirm the presence of PLA in the supernatant, TLC and GC-MS analyses were performed. In TLC analysis, EA fraction showed one spot with the same R_f value (0.55) as that of the authentic PLA under 254 nm UV light. This spot and the authentic PLA spot gave similar blue color by spraying with 5% methanolic *p*-anisoldehyde-sulfuric acid reagent followed by heating (Figure 31).

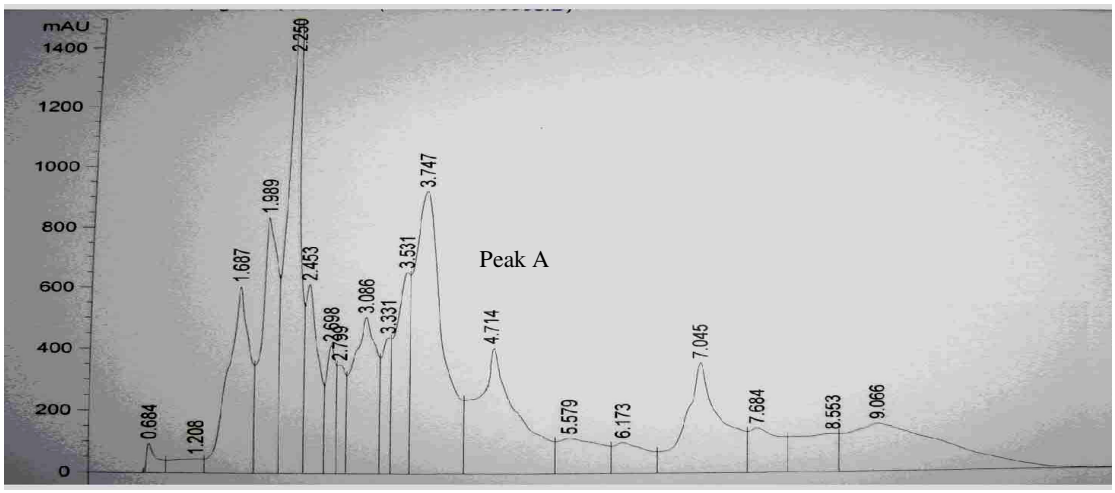


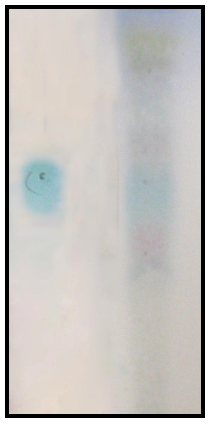
Figure 30. HPLC profile of the cell free supernatant of *L. plantarum* DW3
Column: Hypersil ODS column C18 RP (250 x 4.0 mm, HP, particle size 5 um)
Elution: linear gradient from 25% acetronitrile in 75% water (at 0-3.0 min, flow rate 1ml/min) to 50% acetronitrile in 50% water (in 4 min-6min, flow rate 1ml/min) and acetronitrile was adjusted to 100% (in 8 min-12 min, flow rate 1.ml/min).
Detection: diode array detector set at 210 nm

The EA fraction was analyzed by GC-MS after TMS-derivatization with BSA (N, O-bis (trimethylsilyl)-acetamide). The compound corresponding to the peak with retention time of 13.4 min was identified as PLA by comparing its electron ionization

(EI)-MS spectrum with that recorded in the mass spectrum library. GC-MS analysis of the authentic PLA showed the peak with the same retention time and EI-MS spectrum as the peak (t_R 13.7 min) observed in GC-MS analysis of the EA fraction. The total ion chromatograms in GC-MS analysis of EA fraction and authentic PLA were demonstrated in Figure 32. The EI-MS spectra of the peak with retention time of 13.4 min in GC-MS analysis of EA fraction and the authentic PLA were demonstrated in Figure 33. The identification was also confirmed by co-chromatography with the authentic PLA (Figure 34).

As shown in Figure 34A, one peak area of the compound (t_R = 13.4 min) in EA fraction was significantly increased when co-injected with the authentic PLA (Figure 34 B). Besides, PLA peak, we found the suspected peak (t_R = 15.1 min) in both of EA fraction and authentic PLA, therefore we suspected that this compound was not naturally contained in FDS. Co-chromatography analysis obviously indicates that the suspected peak in both EA fraction and authentic PLA was the same compound because the significant increasing of peak area was observed after co-injection. Hence, this suspected compound might be occurred in the derivatization step.

PLA content was determined by GC-MS analysis using *p*-hydroxybenzoic acid as an internal standard (IS). The PLA content was found to be 0.85 µg/mg FDS, namely 31 mg/L of supernatant. The calculation and the standard curve were demonstrated in Appendix C



PLA EA fraction

Figure 31. Thin layer chromatograph of the authentic PLA and EA fraction of *L. plantarum* DW3

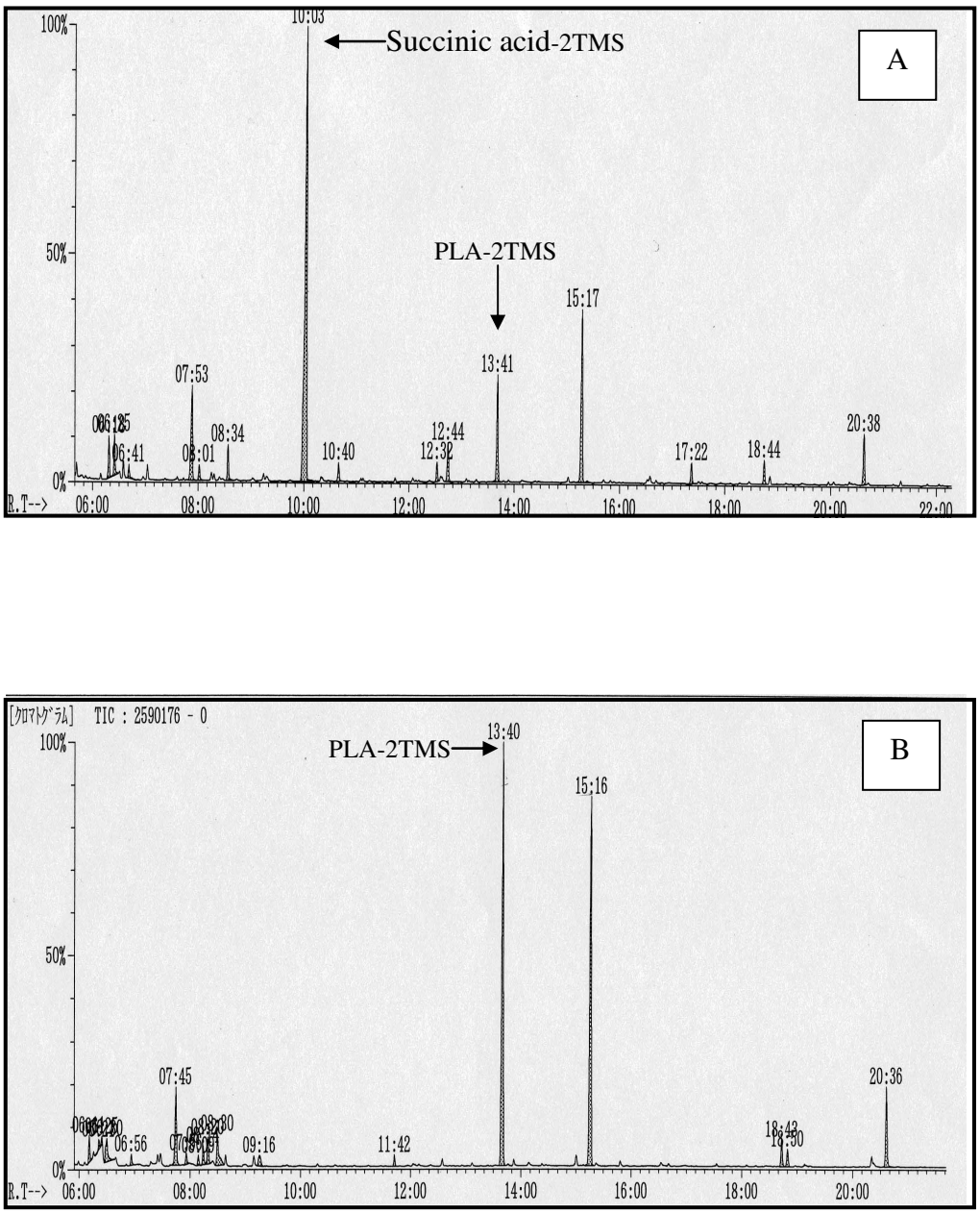


Figure 32. Total ion chromatograms of (A), EA fraction of *L. plantarum* DW3 and (B), authentic PLA

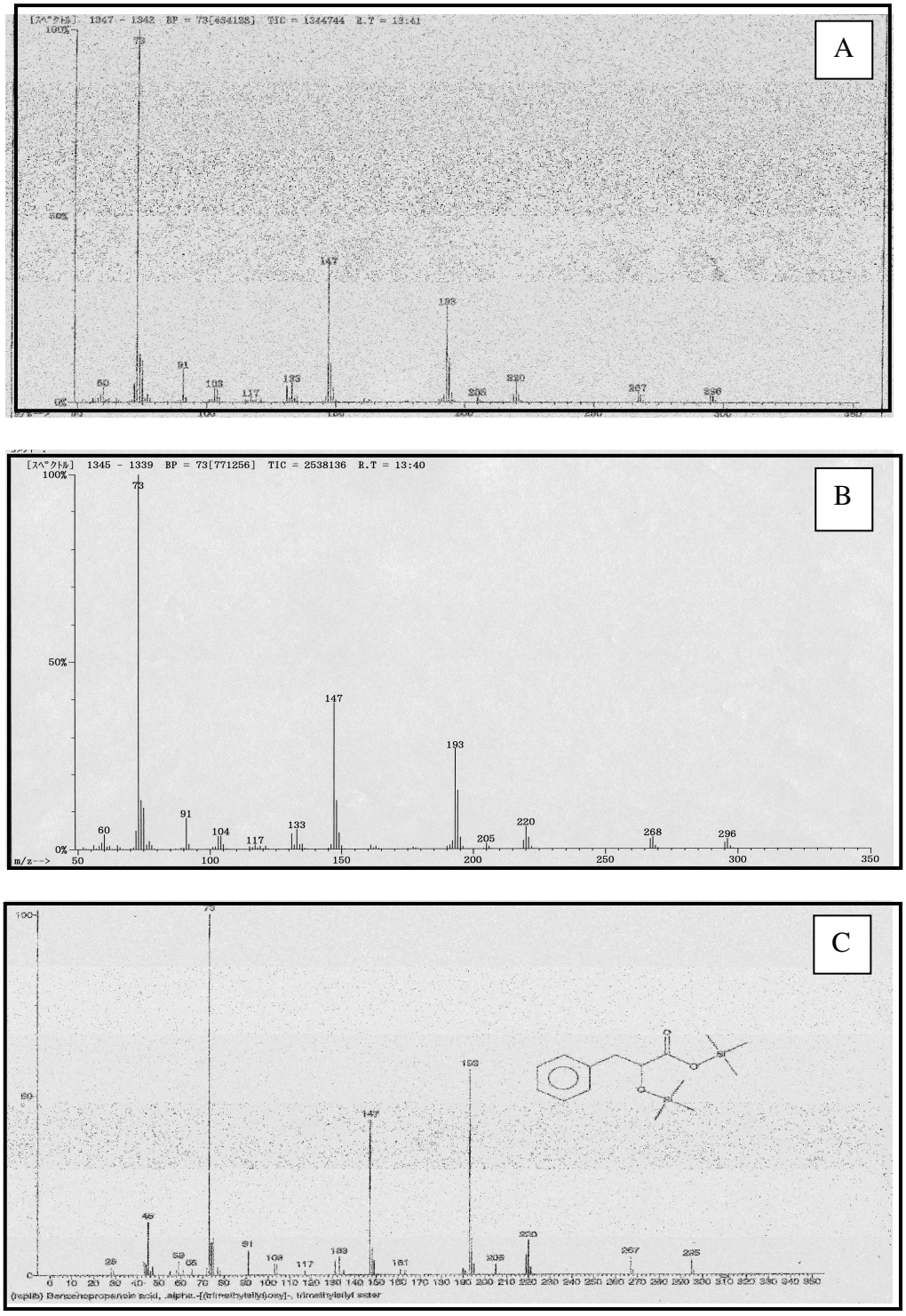


Figure 33. The EI-MS spectra of the peak with retention time of 13.4 min in GC-MS analysis of EA fraction of *L. plantarum* DW3, A; authentic PLA, B; and the mass spectrum from the library, C

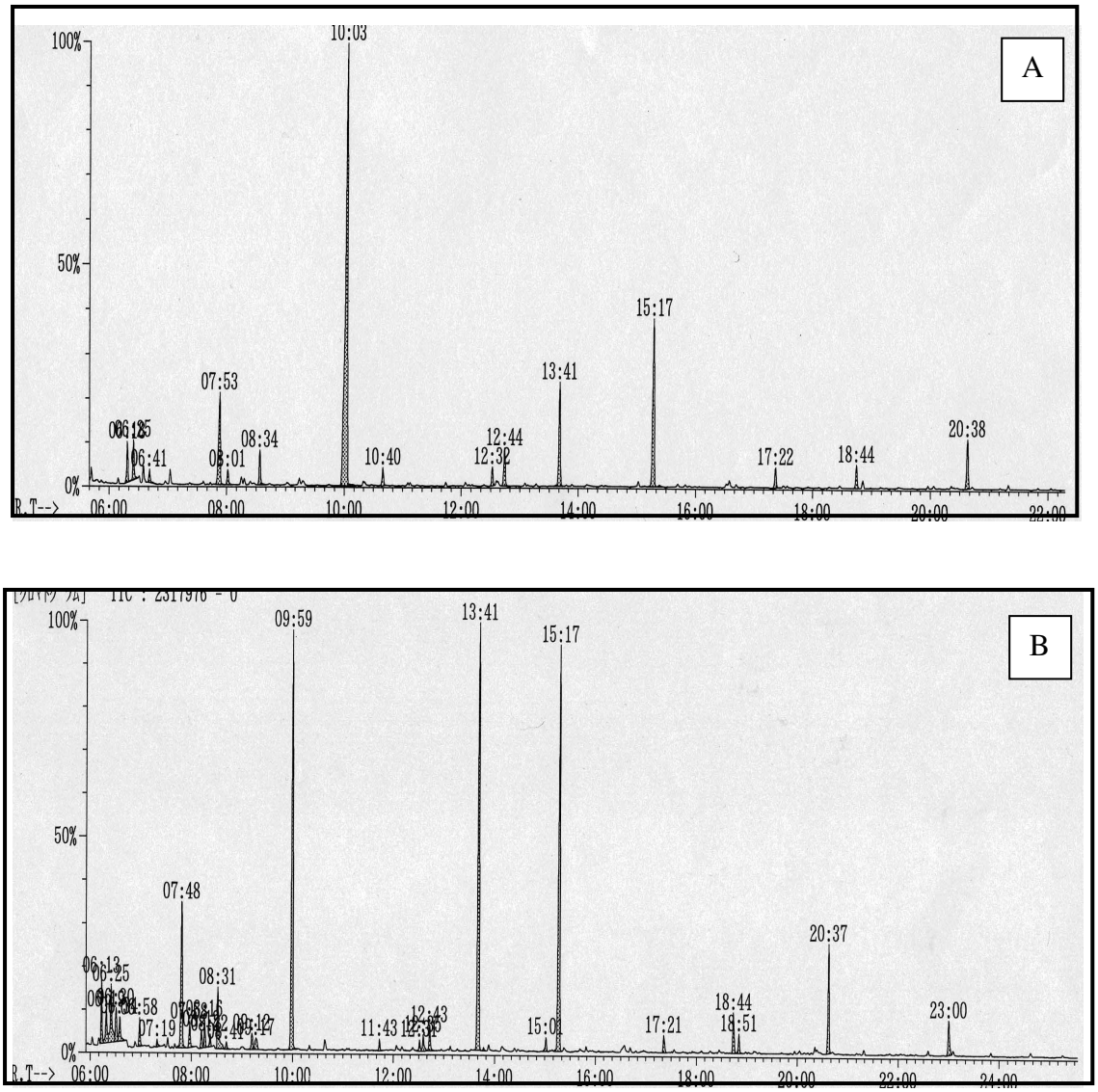


Figure 34. Total chromatogram of EA fraction of *L. plantarum* DW3, A; Total chromatogram of the mixture of EA fraction and authentic PLA, B

Characterization of the antifungal activity of Phenyllactic acid (PLA)

The MID_{90} of FDS (freeze dried supernatant) was 8.8 mg while MID_{90} of EA (ethyl acetate) fraction and Water fraction had the same amount and was equivalent to 18 mg of FDS. The antifungal activity of FDS, EA fraction and Water fraction is shown in Table 12. This reveals that both fractions contained antifungal active compounds of the culture supernatant of *L. plantarum* DW3. It has been known that

LAB can produce variety of antifungal substances such as organic acids, fatty acids, cyclic dipeptides, phenyllactic acid, reuterin and proteinaceous compounds (Lavermicocca *et al.*, 2000; Magnusson, 2003; Magnusson and Schnurer, 2001; Okkers *et al.*, 1999; Sjogren *et al.*, 2003; Strom *et al.*, 2002; Talarico and Dobrogosz, 1989).

Compared to the finding of Li *et al.*, (2007), 60 of 112 strain LAB produced 16-61 mg/L PLA, our LAB strain, *L. plantarum* DW3 is a moderate producer of PLA (31 mg/L, previously mentioned). There have been reported that PLA was produced by *L. plantarum* 21B (Lavermicocca *et al.*, 2000), *L. plantarum* MiLAB 393 (Strom *et al.*, 2002), *L. corynifomis* Si3, *Pediococcus pentosaceus* and *L. sake* (Magnusson *et al.*, 2003), *L. plantarum* TMW1 468 and *L. sanfranciscensis* DSM 20451T (Valerio *et al.*, 2004), and *Lactobacillus* sp. SK 007 (Li *et al.*, 2007). Moreover, PLA was also produced by yeast, *Geotrichum candidum* (Dieuleveux *et al.*, 1998).

Table 12. Antifungal activity of *Lactobacillus plantarum* DW3 in forms of FDS, EA fraction and Water fraction against *Rhodotorula mucilaginosa* DKA

Concentration (mg/ml)	% Inhibition		
	FDS	EA fraction	Water fraction
350	100	100	100
265	100	100	100
175	100	100	100
90	100	**95.5**	**97.5**
44	**92.1**	73.9	69.3
17.5	28.0	20.1	18.2
9.0	19.1	12.5	10.2

Doses of EA and water fractions are expressed in equivalent mg of FDS

D-, L- and DL-PLA showed inhibitory activity against growth of *R. mucilaginosa* DKA as MID_{90} and MIC_{90} values of 1 mg and 5 mg/ml, respectively (Table 13). No significant difference in inhibitory activity was observed among three forms of PLA (P = 0.05).

Table 13. Inhibitory activity of commercial PLA in various forms (D-, L-, and DL-) against *Rhodotorula mucilaginosa* DKA

Dose (mg)	Final concentration (mg/ml)	% Inhibition		
		D-PLA	L-PLA	DL-PLA
2	10	100	100	100
1.5	7.5	100	100	100
1	5	95.7	95.7	98
0.75	3.75	76.1	71.7	71.7
0.5	2.5	58.7	43.5	56.5
0.33	1.65	37.0	37.0	43.5
0.25	1.25	28.2	32.6	34.8
0.13	0.65	28.3	23.9	26.1

As shows in Table 12, MIC_{90} of FDS was 44 mg/ml, whilst the concentration of PLA containing in the FDS was only 0.039 mg/ml. Compared to the MIC_{90} of authentic PLA (5 mg/ml) (Table 13), it can be concluded that PLA was not the main antifungal active compound in the FDS of *L. plantarum* DW 3.

Effect of pH and heat treatment on antifungal activity of DL-PLA activity

Inhibition assay of DL-PLA (5 mg/ml) against *R. mucilaginosa* DKA in pH conditions from 2.6 to 5.5 showed that the greatest antifungal effect was obtained at unadjusted pH (2.1), while the dramatic decrease of activity was observed at pH 5.5 (36.9%$\pm$2.4) (Figure 35). The inhibitory effect of DL-PLA against *R. mucilaginosa* DKA showed no significant difference (97.2-98.5%) in a range of pH 3.5-4.5.

For heat treatment, we found that high temperature had no effect antifungal activity of DL-PLA at 5 mg/ml since the growth of indicator had not detected by OD 550 although PLA was treated with 121^{o}C for 30 min (Figure 36). From this study, PLA was effective in acidic system (pH 2-5) which covering pH range of fermented foods. This agrees with the report of Lavermicocca *et al.,* (2003) that the greatest antifungal effect was obtained in unbuffered medium (pH 2.6), while a significant

decrease in PLA activity occurred at pH 5. Similar to the others, weak acid used as a preservative compound such as propionic acid, benzoic acid, sorbic acid , lactic acid, citric acid and acetic acid, etc. The pH is the most important factor for the activity of PLA , therefore its mode of action is related to the lipophilic properties which enable the undissociated form to cross microbial membranes (Gould, 1991; Lavermicocca *et al.*, 2003).

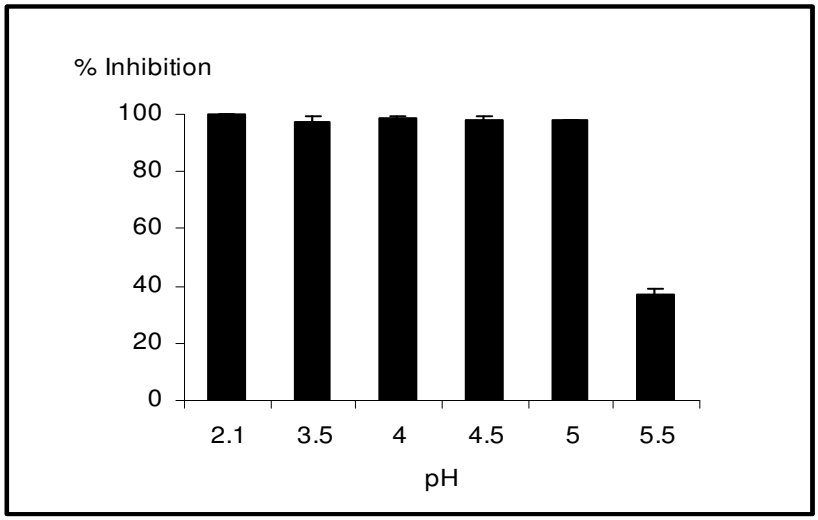


Figure 35. Effect of pH on antifungal activity of DL-PLA (5 mg/ml) against *Rhodotorula mucilaginosa* DKA

Different from yeast, the effect of PLA on bacterial cells has been studied. In comparison to anti-listeria activity between lactic acid and PLA, it was found that inhibitory properties of lactic acid are due to its acid nature, not to the molecule itself. Lactic acid (120mM) at pH 5.6 had no action against *L. monocytogenese* in the agar well test, while the same concentration and pH, distinguish activity of PLA were observed (Dieuleveux *et al.*, 1998). However, the exact mode of action of PLA needs to be investigated. In this study, we demonstrated that the full activity still remained after treatment at 121^{o}C for 30 min. Temperature stability is important if PLA is to be used as a food preservative because many procedures of food preparation involve a heating step.

Because fungi are responsible for contamination of a variety of food products such as dairy and meat products, fruit, vegetables, and fermented foods especially

FPBs which was focused on this work. Using PLA-producing LAB strain might be overcome the contamination of undesirable fungi, particularly yeasts in FPB product.

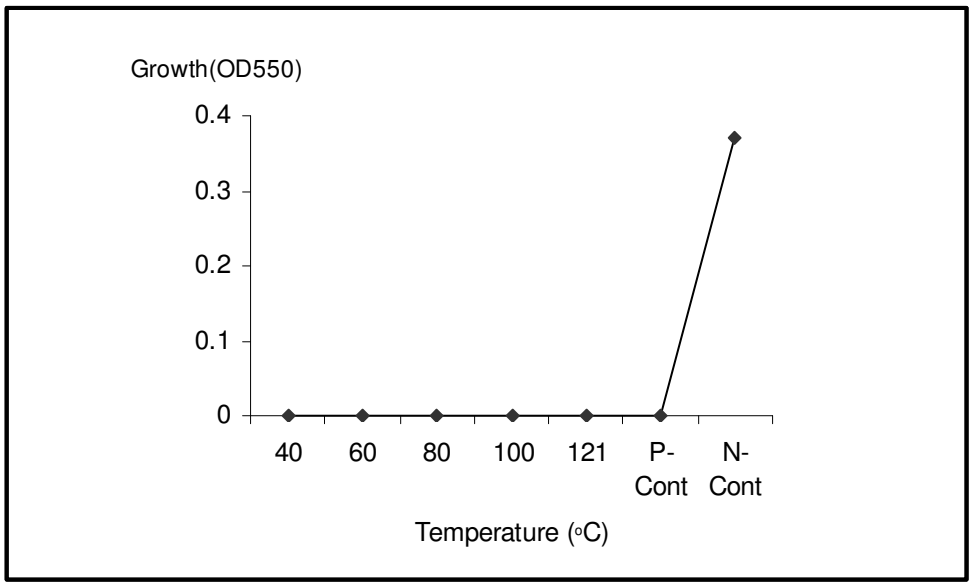


Figure 36. Effect of temperature on antifungal activity of DL-PLA (5 mg/ml) against *Rhodotorula mucilaginosa* DKA; P-Cont: positive control and N-Cont: negative control

Antifungal spectrum of DL-PLA

DL-PLA (5 mg/ml) showed strong inhibitory activity (more than 90% inhibition) against all fungal strains tested, except *Pichia ferinosa* PKC and *Aspergillus niger* PSSCMI 3001, which were inhibited 81% and 26%, respectively (Table 14). Although it was not the main active antifungal compound, broad antifungal spectrum of PLA is attractive to study. Many yeast indicator strains were isolated from fermented plant beverages and most of them were inhibited by DL-PLA. As a consequence, application of PLA or the use of PLA producing strain in fermented plant beverage is considerable. In addition, in this study, PLA showed inhibitory activity against the growth of mold indicator strains including toxic producing strains like *Aspergillus flavus* TISTR 3041. Lavermicocca *et al.,* (2003) found that 23 fungal strains isolated from bakery products were strongly inhibited (90% growth inhibition) by PLA at the concentration of 7.5 mg/ml. Strom *et al.*, (2002) reported that *L. plantarum* MiLAB393 produced PLA which is active against

food-and feed borne filamentous fungi and yeast. At 7.5 mg/ml of PLA showed total inhibition against the growth of *Aspergillus fumigatus* and *Penicillium roqueforti*.

Apart from fungi, pathogenic bacteria such as *L. monocytogenese, Staphylococcus aureus, Enterococcuss faecalis*, *Providencia stuartii* and *Klebsiella oxytoca* were also inhibited by PLA (Dieuleveux *et al.*, 1998).

Table 14. Antifungal spectra of DL-PLA (5 mg/ml) tested by the microtiter plate well Method

Target fungi	% Inhibition	SD (n=3)
Rhodotorula mucilaginosa DKA	97.5	0.25
Issatchenkia occidentalis DKB	95.3	0.10
Pichia membranifaciens DKC	98.3	0.20
Pichia ferinosa PKC	81.6	4.07
Pichia analoma DKD	95.9	0.56
Candida tropicalis DKE	96.5	2.05
Saccharomyces cerevisiae DKF	96.9	1.24
Hanseniaspora clermontiae PKG	97.5	0.14
Endomycopsis sp. PSSCMI 7004	98.5	0.04
Candida albicans PSSCMI 7010	96.6	0.04
Aspergillus niger PSSCMI 3001	26.0	8.30
Fusarium moniliforms PSSCMI 3011	94.3	1.13
Aspergillus flavus TISTR 3041	97.4	0.76
Aspergillus nidulans TISTR 3169	91.2	0.76
Pennicillium sp. PSUNMI	95.2	0.86

Part V. Effect of *L. plantarum* DW3 used as inoculants on the quality of fermented seaweed beverage (FSB)

Microbial populations

Significant differences in the numbers of total bacteria, LAB and yeasts were found in the four procedures (Figure 37A–C) and (Table 15). At the start of the fermentation sets, with no pre-sterilization and no starter inoculums of LAB (N-N), the TBC was approximately 3.6 log CFU/ml, and the LAB approximately 1.5 log CFU/ml, while after treatment with 0.5%KMS overnight the TBC was reduced to about 1.5 log CFU/ml and the LAB were not detectable (P-N). After addition of 7.5 x 10^6 CFU/ml of LAB starter, TBC increased by only about 4 log CFU/ml for both untreated (N-S) and partially sterilized seaweed (P-S) so only 4 log CFU/ml of LAB survived when plated on PCA medium (Figure 37A). In contrast, after addition of the starter, the LAB count measured on MRS agar was initially about 7 log CFU/ml on P-S and about 8 log CFU/ ml on N-S (Figure 37B). This shows that MRS agar is far more suitable for counting LAB than PCA agar.

In the early phase of the fermentation (days: 0-7), TBC, LAB and yeast levels in most treatments increased in a similar pattern with the highest number found at day 7, except that the N-N set peaked for TBC and LAB at day 4. The results did imply that LAB was initially the predominant bacteria in all the fermentations. After day 7 all counts decreased steadily to reach a steady low state from day 30 onwards. At day 60 the TBC counts were mostly about 4 log CFU/ml, except the N-S was at about 5 log CFU/ml while the LAB counts of P-S and N-S were 4 and 4.73 log CFU/ ml, respectively whereas the LAB, N-N and P-N count was only 2.4 and 2.6 log CFU/ml, respectively the higher values presumably being due to the addition of KMS and continuing presence of the starter *L. plantarum* DW3.

In the absence of added LAB without sterilization (N-N), yeast numbers increased rapidly from 3.5 log CFU/ ml to about 6 log CFU/ml at day 7. Although treatment with KMS (P-N) reduced the yeast numbers to 1.2 log CFU/ml they rapidly

increased to 4.68 log CFU/ml at day 7 in the absence of added LAB (Figure 37C). Treatment with KMS without inoculation had reduced the yeast numbers by 2.3 log CFU/ml. In the presence of added LAB, in the non-sterilized preparation (N-S), over the first 4 days there was a decrease in yeast numbers from 3 log CFU/ml to 2.5 log CFU/ml followed by an increase to day 7 to 3.5 log CFU/ml whereas in the sterilized preparation (P-S) the numbers increased over the first 7 days from 1.5 log CFU/ml to 2.5 log CFU/ml.

The presence of the added LAB was obviously inhibiting the growth of the yeast. After day 60, yeast numbers reduced at a uniform rate to 1.2 log CFU/ml in N-S and P-N and to 2.63 log CFU/ml in N-N but after sterilization and in the presence of LAB (P-S) the rate of loss was faster and yeast were not detected at all after day 45. The presence of the added LAB was obviously inhibiting the growth and survival of the yeast and at the end of the fermentation no yeast were detected. From this study, it appears that the most effective way to control yeast contamination is to use a suitable starter culture and to partially sterilize the raw material with 0.5% KMS (P-S).

Microbial quality

Based on Thai Microbiological Quality Guidelines, Section of Food Analysis, Division of Medical Science, Ministry of Public Health, yeast counts must not be more than 100 CFU/ml and therefore in the situations here only the natural fermentation set N-N did not pass the guideline. It also states that inoculating with an anti-yeast Lactobacillus or partial sterilization is required for achieving the goal to control yeast contamination. Treatment with KMS certainly had a major effect on the normal LAB population. At the beginning of the fermentation (day 0), no LAB were detected (P-N). After treatment with 0.5% KMS and numbers did not show an increase until day 4 then reached a peak at only 3.6 log CFU/ml on day 7. In contrast, the N-N natural LAB grew quickly from day zero and the count increased from 1.34 log CFU/ml to 6.48 log CFU/ml on day 4. The behavior of the starter LAB cultures added to the N- and P- cultures was very similar from day zero except that the total LAB was always less in the P-S preparation (Figure 37B).

Previously we have shown that *L. plantarum* DW3 was a probiotic strain (Duangchitchareon, 2006), the increased numbers of LAB, as a result of the inoculation, may make this supplemented fermented plant beverage a more attractive functional food as a source of nutrients, bioactive compounds, essential elements and probiotics. The microbiological quality of all FSB treatments was also investigated. Bacterial indicators such as *E. coli* and coliforms including food borne pathogenic bacteria such as *S. aureus*, *Salmonella* sp. *and C. perfringens* were not detected throughout the fermentation process. Moreover, the finished products of the FSB after storing for 3 months passed the standard microbiological quality tests. Results indicate that lactic fermentation preserves raw materials and provides a safe fermented beverage for drinking.

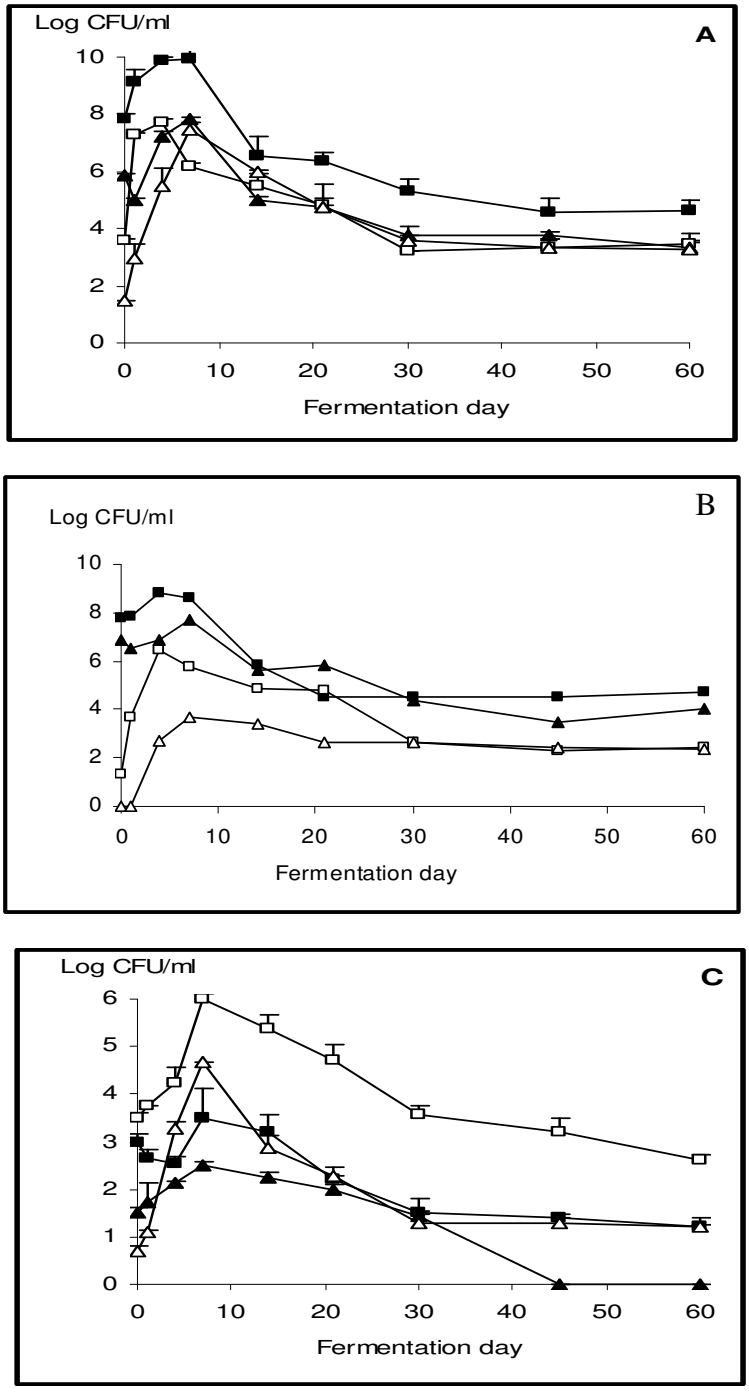


Figure 37. Microbial growth in seaweed beverage fermentation procedures. (A) total bacterial count (TBC), (B) lactic acid bacteria (LAB) and (C) yeast; ■, N-S; □, N-N; ▲, P-S and △, P-N ; Bars indicate range of duplicated result.

Table 15. Statistical analysis of parameters detected in the FSB treatments

Parameter	Seaweed			
Treatment	N-S	N-N	P-S	P-N
Total bacterial count: TBC				
0 day	a1	b1	a1	c1
30 days	a2	b1	b2	b2
45 days	a2	b1	b2	b2
60 days	a2	b1	b2	b2
150 days	nd3	nd2	nd3	nd3
Lactic acid bacteria: LAB				
0 day	a1	b1	a1	c1
30 days	a2	a2	b2	b2
45 days	a2	b2	c2	c2
60 days	a2	b2	c2	c2
150 days	nd3	nd3	nd3	nd3
Yeast				
0 day	a1	a1	b1	b1
30 days	a2	b1	a1	a1
45 days	a2	b1,2	a2	c1
60 days	a2	b2	c2	a1
150 days	nd3	nd3	nd2	nd1
Total sugar				
0 day	a1	a1	a1	a1
30 days	a2	a2	a2	a2
45 days	a2	a2	a2	a2
60 days	a2	a2	a2	a2
150 days	na	na	na	na
Total acidity				
0 day	a1	a1	a1	a1
30 days	a2	b2	a2	c2
45 days	a3	a3	a3	b3
60 days	a4	a3	a4	b4
150 days	na	na	na	na
pH				
0 day	a1	a1	a1	a1
30 days	a2	a2	a2	a2
45 days	a2	a2	a2	a2
60 days	a2	a2	a2	a2
150 days	na	na	na	na
Acetic acid				
0 day	a1	a1	a1	a1
30 days	a2	a2	a2	a2
45 days	na	na	na	na
60 days	b3	a3	c3	c3
150 days	na	na	na	na
Lactic acid				
0 day	a1	a1	a1	a1

Table 15 continued

Parameter	Seaweed			
Treatment	N-S	N-N	P-S	P-N
30 days	a1	a1	a1	a1
45 days	na	na	na	na
60 days	a2	d2	b2	c2
150 days	na	na	na	na
Ethanol				
0 day	a1	a1	a1	a1
30 days	a1	a1	a1	a1
45 days	na	na	na	na
60 days	b2	a2	c2	b2
150 days	na	na	na	na
Methanol				
0 day	nd	nd	nd	nd
30 days	nd	nd	nd	nd
45 days	nd	nd	nd	nd
60 days	nd	nd	nd	nd
150 days	nd	nd	nd	nd
Electrical conductivity: EC				
0 day	a1	a1	a1	a1
30 days	a2	a2	a2	a2
45 days	a3	a3	a3	a3
60 days	a4	a4	a4	a4
150 days	na	na	na	na
Cu (Cupper)				
0 day	a1	a1	a1	a1
30 days	a1	a1	a1	a1
45 days	a2	a2	a2	a2
60 days	a2	a2	a2	a2
150 days	na	na	na	na
Fe (Iron)				
0 day	a1	a1	a1	a1
30 days	a2	a2	a2	a2
45 days	a2	a2	a2	a2
60 days	a2	a2	a2	a2
150 days	na	na	na	na
K (Potassium)				
0 day	a1	a1	a1	a1
30 days	a2	a2	a2	a2
45 days	a2	a2	a2	a2
60 days	a2	a2	a2	a2
150 days	na	na	na	na
Na (Sodium)				
0 day	a1	a1	a1	a1
30 days	a1	a1	a1	a1
45 days	a1	a1	a1	a1

Table 15 continued

Parameter	Seaweed			
Treatment	N-S	N-N	P-S	P-N
60 days	a1	a1	a2	a2
150 days	na	na	na	na
Zn (Zinc)				
0 day	a1	a1	a1	a1
30 days	a2	a2	a2	a2
45 days	a2	a2	a2	a2
60 days	a2	a2	a2	a2
150 days	na	na	na	na
Inhibition				
***Bacillus cereus* ATCC 11778**				
0 day	a1	a1	a1	a1
30 days	a2	a2	a2	a2
45 days	na	na	na	na
60 days	a3	a3	a3	a3
150 days	a2	a2	a2	b4
***S. aureus* PSSCMI 0004**				
0 day	a1	a1	a1	a1
30 days	a2	b1	a2	b1
45 days	na	na	na	na
60 days	a3	b2	a3	b2
150 days	a2	a2	a2	a2
***Escherichia coli* PSSCMI 0001**				
0 day	a1	a1	a1	a1
30 days	a1	a1	a1	a1
45 days	na	na	na	na
60 days	a2	b2	a2	b2
150 days	a3	b2	a3	b2
***Salmonella* sp. PSSCMI 0034**				
0 day	a1	a1	a1	a1
30 days	a1	a1	a1	a1
45 days	na	na	na	na
60 days	a2	b2	a2	b2
150 days	a3	a3	a3	b3
***V. parahaemolyticus* PSSCMI 0064**				
0 day	a1	a1	a1	a1
30 days	a2	b1	a2	b1
45 days	na	na	na	na
60 days	a3	b2	a3	b2
150 days	a4	a3	a2	a3

Different letters in row indicate significant differences (P=0.05)
Different numbers in column indicate significant differences (P=0.05)
na = not analyzed; nd = not detected
N-S = No partial sterilization-starter, N-N = No partial sterilization-no starter
P-N = Partial sterilization-no starter, P-S = Partial sterilization-starter

Physicochemical properties

Changes in sugar concentrations, measured as glucose, were similar in all treatments. During the first 2 days sugar concentrations decreased at a rapid rate from 11.25– 11.76% to about 9%, then the rate decreased until day 21 to a level of about 8%, accelerated again to a level of about 5% at day 45 and then changed very little until day 60 at 4.21–4.72% (Figure 38A). As the dominant organisms in the seaweed fermentation were LAB, the end products of the sugar loss were acids and ethanol. Over the first 7 days acid production in all cases was low and almost identical from 0 to 0.10% but then there were significant changes (Figure 38B). The fermentations inoculated with LAB, N-S and P-S produced identical rates of acid production from 0.10 at day 7 to 0.95 at day 60. The highest rate of acid production was by the preparation that had shown the highest rate of LAB production (N-N), (Figure 37B), but this rate decreased as the LAB count fell (Figure 37B) to give a final acidity of 0.80%. On the other hand, the lowest rate of acid production and final acidity (0.63%) was found in (P-N). At the end of the fermentation (day 60), the pH value of all treatments was in a range of 2.9–3.1 (Figure 38C). This profile is general in lactic fermentations, and similar to the findings of Ruiz-Barba *et al* (1994) that used *L. plantarum* LPCO10, a bacteriocin producer, as a starter culture in a Spanish-style green olive fermentation.

Soluble elements (Cu, Fe, Zn, K and Na) increased following fermentation time, and they had no significantly different among treatments, except for K and Na (Table 15 and Figure 39). The elements such as Cu, Fe and Zn in the final product were less than the limitation required by the USFDA for drinking water (FDA, 2003) while toxic elements such as As (arsenic) and Pb (lead) were not found as their detectable limit was 10 µg/L (Table 15). It is well recognized that Cu, Zn and Fe have their own bio-functions i.e. Cu and Zn are cofactors of superoxide dismutases, that protects oxygen-using cells against toxic reactive oxygen species produced as by products of respiration, oxidative metabolites and radiation (Woodruff *et al.*, 2004). Iron deficiency is one major cause of anemia and a world-wide nutritional problem (WHO, 2002).

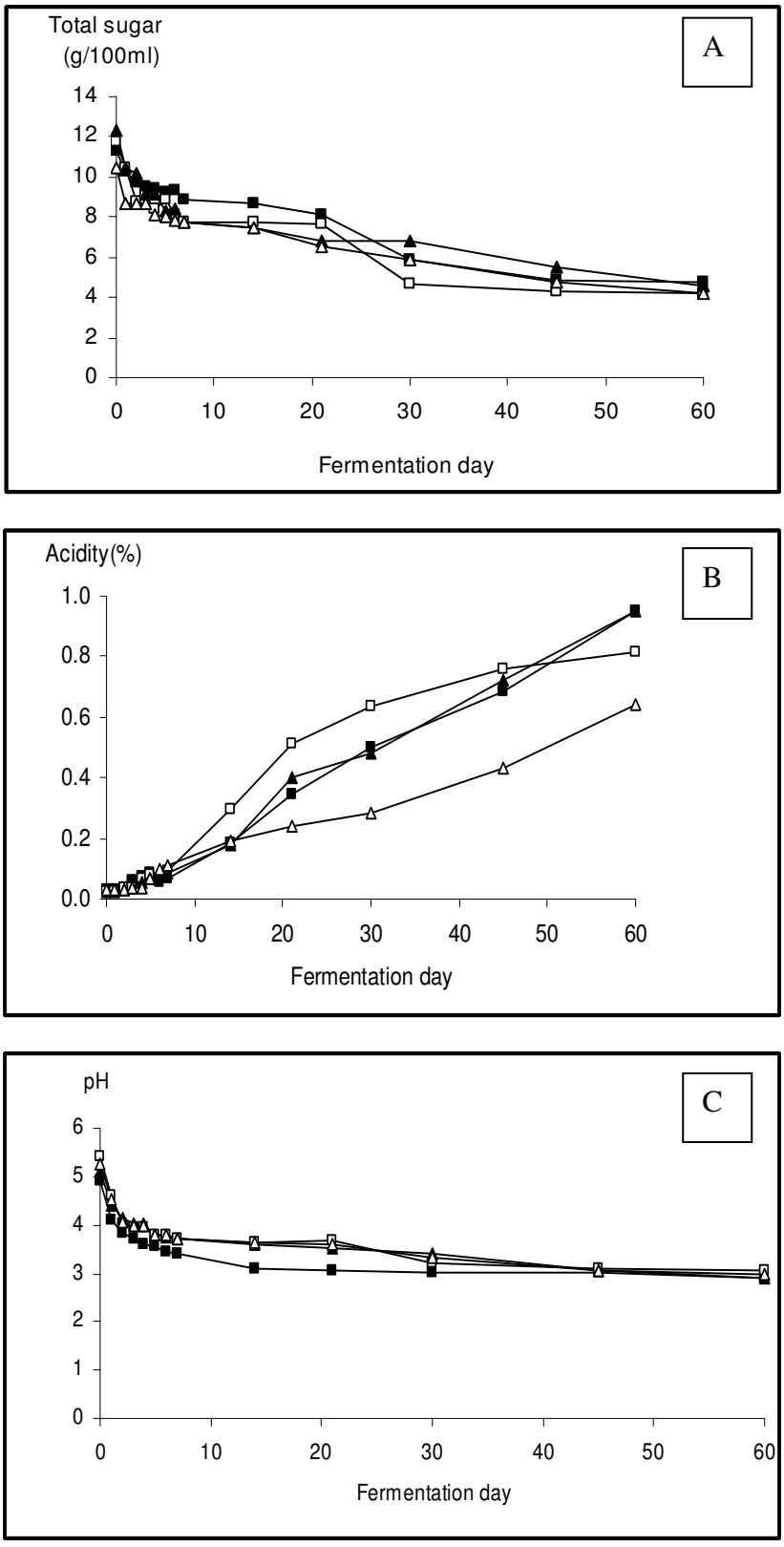


Figure 38. Changes of (A) total sugar, (B) acidity and (C) pH in seaweed beverage fermentations. ■, N-S; □, N-N; ▲, P-S and △, P-N

In this study, we have found that the seaweed fermentation provides more soluble forms of elements, particularly Fe, which was not found at zero time but increased up to 0.16–0.18 mg/L in the drinkable end product. There was a positive relationship between total acidity and soluble elements (Figure 38A and Figure 39) and therefore the organic acids produced by LAB improve the availability of elements. This has been supported by Bergqvist *et al.*, (2005) who reported that lactic acid fermentation strongly increases Fe solubility in carrot juice with the increase related to the levels of organic acids.

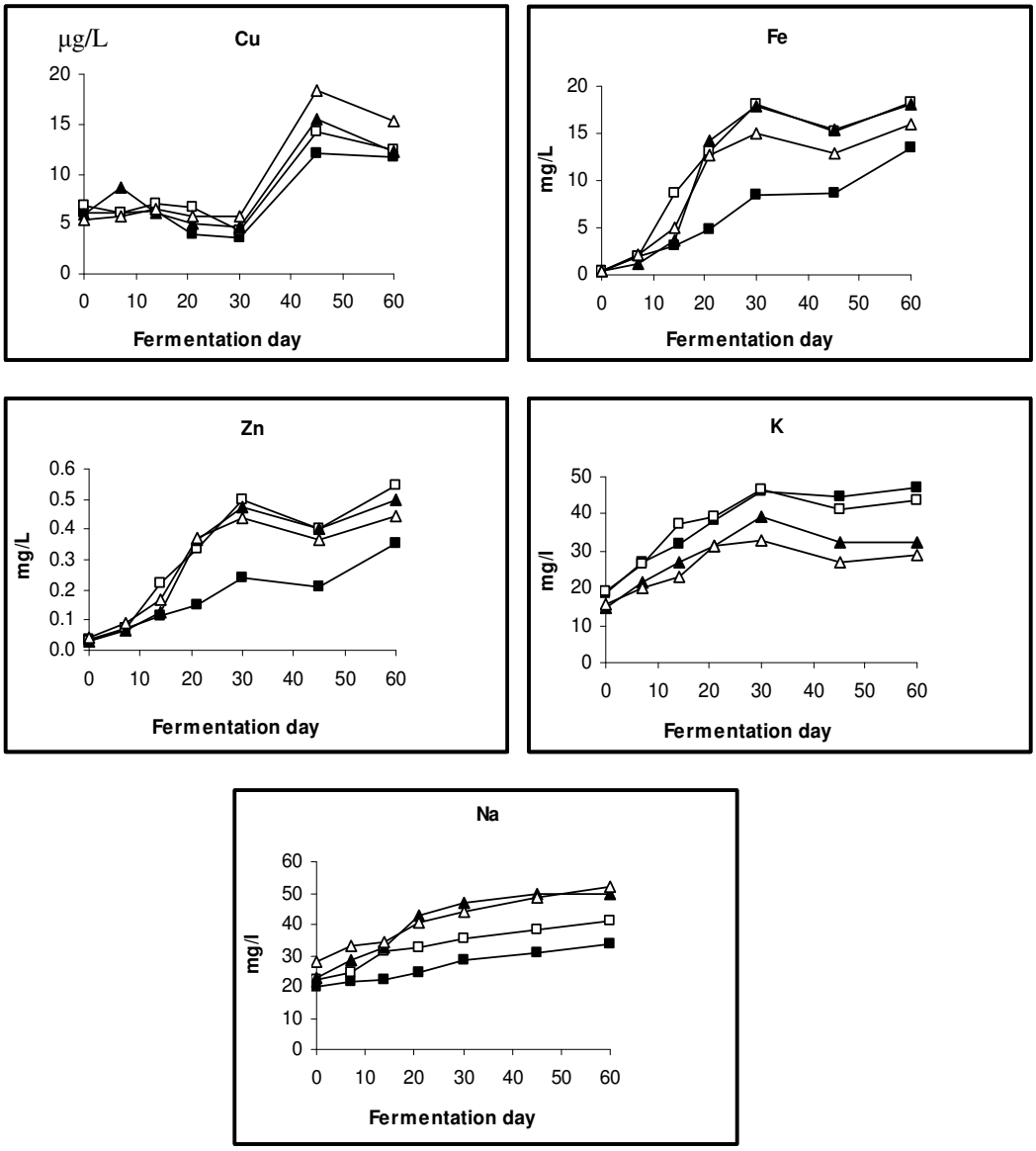


Figure 39. Changes of elements in seaweed beverage fermentations

■, N-S; □, N-N; ▲, P-S and △, P-N

Electrical conductivity (EC: A measure of the total concentration of dissolved salts in water or solution) of all treatments increased following fermentation day and there were no significant difference among treatments (Table 15 and Figure 40). At the beginning, EC values were approximately 100 µS/cm (0.10 mS/cm) and at day 60 increased to about 1,000 µS/cm (1.0 mS/cm). The increasing of EC values agreed with the result of acidity and soluble elements (Figure 38B, Figures 39 and 40). The result of EC values indicate that soluble substances such as elements from plant were extracted from the seaweed used by fermentation process. However, USFDA recommends that the EC in drinking water must be lower 900 µS/cm. Normally FPBs are used as a functional food therefore the FPBs are diluted before drinking by consumers. In addition, some products of FPBs are diluted before packing into bottles and they are ready to drink.

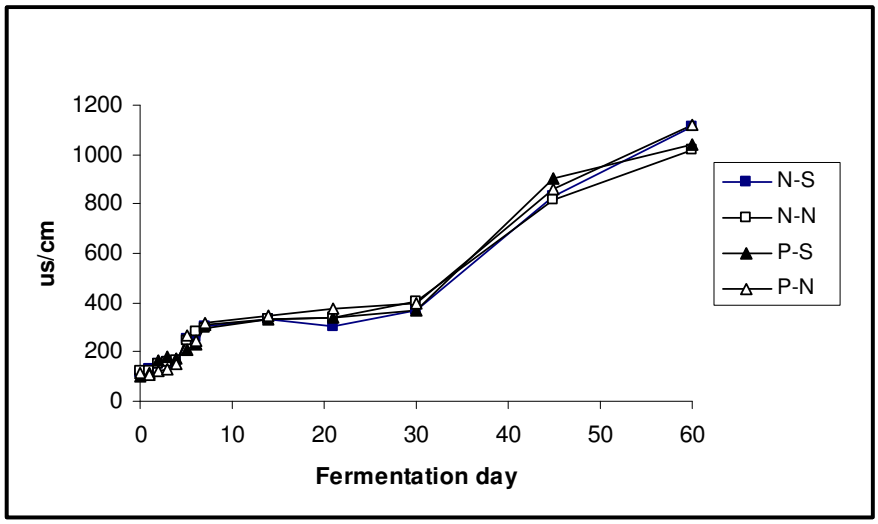


Figure 40. Changes of electrical conductivity in seaweed beverage fermentations
■, N-S; □, N-N; ▲, P-S and △, P-N

Methanol was less than the detectable level (0.003 g/L) in all treatments, while ethanol was present in a range of 0.78–1.84 g/L (Table 16). In general, yeasts such as *S. cerevisiae* produce ethanol from sucrose under oxygen limitation. As our finding, *S. cerevisiae* is a common contaminant of FPBs. It was not surprising therefore to find

that the amount of ethanol detected was determined by the yeast count. The N-N set (natural fermentation) had the highest amount of yeasts (Figure 37C) and the highest amount of ethanol, while the P-S set had the lowest yeast numbers and the lowest ethanol content. This confirms that the ethanol in the FSB is produced by contaminating yeast.

Lactic acid is the dominant acid produced by LAB so it was not surprising that the amount of lactic acid in the final beverage (Table 16) in general reflected the numbers of LAB present during the fermentation (Figure 37B). Those inoculated with LAB (N-S and P-S) had higher levels (7.48 and 6.22 g/L) than those (N-N and P-N) with natural LAB i.e. 3.72 and 5.86 g/L, respectively. The only slight anomaly was the lowest level in the N-N sample but this also had the highest acetic acid level, so the rapid increase in the LAB count in this preparation was probably due to a different population. The acetic acid in the final beverage was probably mainly produced by bacteria that originated from raw materials, especially seaweed. Some of these could have been heterofermentative LAB. These were mostly removed by the treatment with KMS. However, in some situations it is known that *L. plantarum* DW3 can be a facultative heterofermenter producing acetic acid.

Table 16. Comparison of the concentrations of some important elements present in the final fermentation products (day 60) with the limit guidelines of the Thai FDA for drinking water

Element level (mg/L)	N-S	N-N	P-S	P-N	Thai FDA
As[a]	nd	nd	nd	nd	0.05
Pb[a]	nd	nd	nd	nd	0.05
Cu	0.01±0.0	0.01±0.0	0.01±0.0	0.02±0.0	1.00
Fe	0.18± 0.1	0.18± 0.0	0.18± 0.0	0.16± 0.0	0.50
Zn	0.36± 0.1	0.54± 0.2	0.50± 0.0	0.45± 0.1	5.00
Na[b]	40.00±0.2	40.94±0.0	49.75±0.1	51.96±0.1	1-3 g/d[c]
K[b]	47.06±0.0	43.80± 0.2	42.00± 0.0	40± 0.1	2-4 g/d[c]

Values are given as mean ± standard deviation from duplicate determinations
[a] Detectable limit was 10 µg/L for Pb and As, nd = not detected; [b] Compared with USFDA, [c] Adult requires (g/day)

Table 17. Concentrations of alcohols and organic acids in the final fermented seaweed beverages (day 60) produced under different conditions

Organic compound	Treatment			
(g/L)	N-S	N-N	P-S	P-N
Methanol	nd	nd	nd	nd
Ethanol	1.57 ± 0.03^{b}	1.84 ± 0.05^{a}	0.78 ± 0.03^{c}	1.55 ± 0.07^{b}
Lactic acid	7.48 ± 0.79^{a}	3.72 ± 0.55^{d}	6.22 ± 0.55^{b}	5.86 ± 0.76^{c}
Acetic acid	2.24 ± 0.04^{b}	2.41 ± 0.12^{a}	1.81 ± 0.03^{c}	1.85 ± 0.07^{c}

Values are given as mean ± standard deviation from duplicate determinations.
a, b, c, d different superscripts in the same row denote a significant difference ($P<0.05$) nd = not detected (detectable limit = 0.003 g/L)

Antibacterial activity

The inhibition of the FSB against food borne pathogenic bacteria (*B. cereus* ATCC 11778, *S. aureus* PSSCMI 0004, *E. coli* PSSCMI 0001, *S. typhi* PPSCMI 0034 and *V. parahaemolyticus* PSSCMI 0064) was evaluated at days 0, 30 and 60. As no antibacterial activity was found at day 0, the antagonistic activity in the final FSB was a result of the lactic fermentation (Figure 41). The inhibitory activity of the final FSB (60 days) from the inoculated preparations was much higher than the others (Figure 41). The order of inhibitory activities was N-S>P-S>P-N>N-N (Table 17) and this correlated with the amounts of lactic acid in the FSB (Table 16). In terms of overall sensitivities, *B. cereus* ATCC 11778 and *V. parahaemolyticus* PSSCMI 0064 were equally sensitive, followed by *S. typhi* PPSCMI 0034, *S. aureus* PSSCMI 0004 and *E. coli* PSSCMI 0001. These antibacterial activities are mainly associated with the level of total acidity mainly lactic acid (Figure 38B and Table 17).

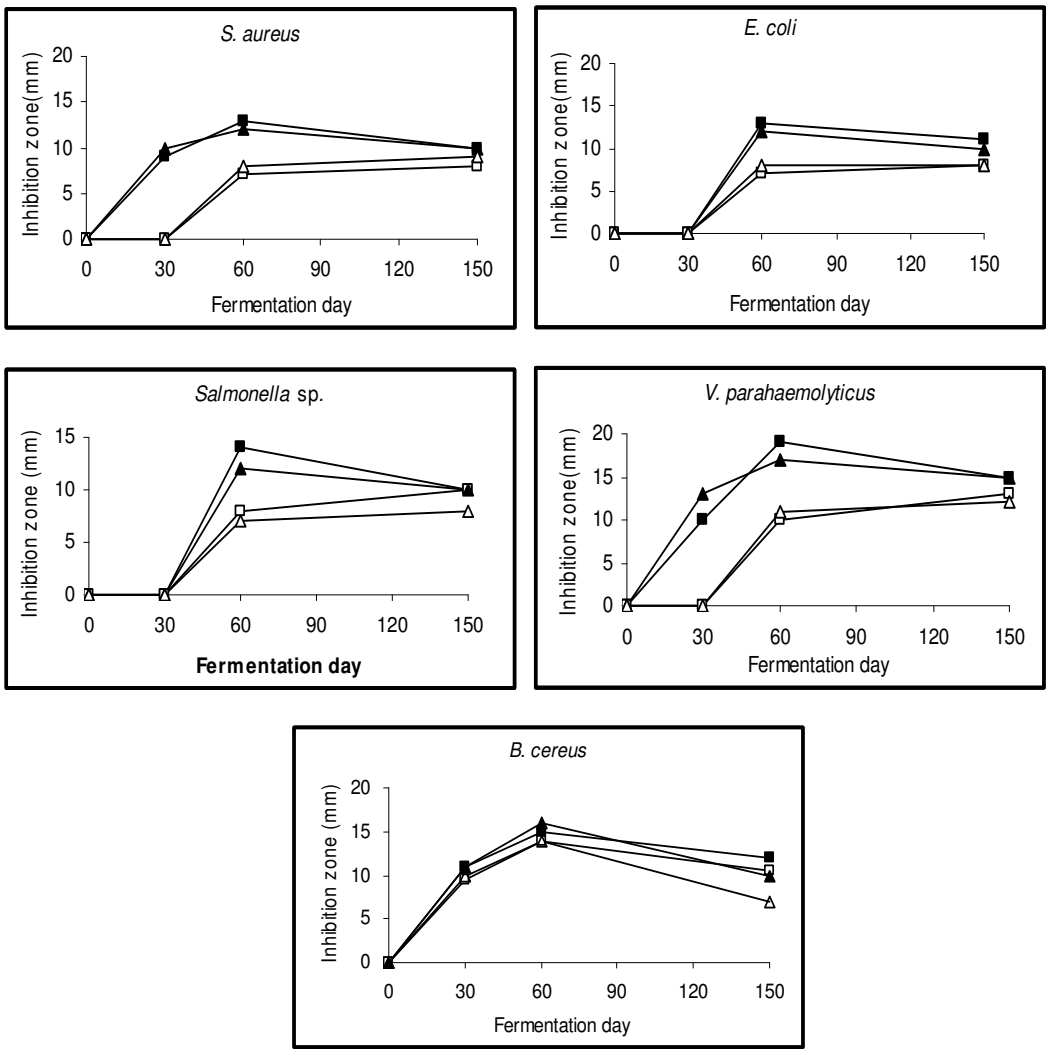


Figure 41. Antibacterial activity of fermented seaweed beverages produced under different conditions; ■, N-S; □, N-N; ▲, P-S and △, P-N

* day 60 shows inhibition of finished products

* day 150 shows the effect of storage time (90 days) on the inhibitory activity of treatments

Table 18. Inhibitory effect of the final fermented seaweed beverage (day 60) from various treatments against foodborne pathogens by the agar well diffusion

Foodborne pathogenic bacteria	Inhibition zone (mm)			
	N-S	N-N	P-S	P-N
B. cereus ATCC 11778	15.0±2^{b}	14.0±2.0^{c}	16.0±1.3^{a}	4.0±1.0^{c}
S. aureus PSSCMI 0004	13.0±0.8^{a}	7.2±0.8^{b}	12.0±1.7^{a}	8.1±0.4^{b}
E. coli PSSCMI 0001	13.2±0.3^{a}	7.1±0.4^{d}	11.8±0.8^{b}	8.1±0.3^{c}
S. typhi PSSCMI 0034	14.1±0.2^{a}	7.9±0.4^{c}	7.0±0.6^{c}	11.9±1.0^{b}
V. parahaemolyticus PSSCMI 0064	17.2±0.3^{b}	10.3±0.6^{c}	19.2±0.8^{a}	11.2±1.3^{c}

Values are given as mean ± standard deviation from duplicate determinations.
a, b, c, d; the different superscripts in the same row denote a significant difference ($P<0.05$)

The antibacterial activity of lactic acid and acetic acid has been documented (Doores, 1993). Ethanol can also inhibit bacteria, particularly *S. typhi* PSSCMI 0034 but even the largest amount detected (N-N set, 1.84 g/L) was probably below the concentration required for inhibition and this set had the lowest inhibitory activity but also the lowest lactic acid level. The inhibitory effects of the FSB against food borne pathogenic bacteria were retained throughout 3 months of storage with only minor reductions (Figure 41). A similar conclusion was reached from the finding that the inoculated treatment sets had a higher inhibiting activity than that in the uninoculated sets. The results demonstrate that LAB starter inoculants provided the best benefit to increase antibacterial activity of the fermented beverage and also partial sterilization supported a suitable condition for proliferation of LAB in seaweed fermentation (Figure 37B).

Sensory evaluation

In the sensory evaluation at day 60, there were no significant differences in the overall acceptance and flavor (Table 18). Assuming that a high value reflects a better acceptance then the most acceptable overall (adding together the values in each row in

Table 18) was the sterilized seaweed preparation inoculated with *L. plantarum* DW3, but the differences were only small (P> 0.05). However, the overall acceptance indicates that all beverages were only moderately acceptable (3.00–3.43). Compare to day 60, overall acceptable of FSB did not significantly change when they were kept at room temperature for 150 days. Moreover, all treatments did not significantly different in overall acceptable (Table 19). The characteristics of the FSB were; the solution was a clear yellow color, tasted a little sweet with sour flavor and a small amount of seaweed odor (Figure 42).

Based on personal communication after the sensory test, the main points were flavor and odor although evaluation of color produced the lowest score in almost all treatments, except a P-S set (Table 19). In addition, total acidity in the beverages (0.63–0.95%) (Figure 38B) was the main cause of the sour flavor while some seaweed odor was still retained. Generally, odor, color and clarity mainly depend on the types of plant used and sugar added (Kantachote *et al.*, 2005). Indeed, the beverages are not ready to drink unless they are diluted when they achieve higher scores. Further study will be required to prove the results of this sensory test of the FSB.

Table 19. Sensory analysis of seaweed plant beverage treatments at day 60

Treatment	flavor	odor	color	clearness	overall
N-S	3.47^{a}	3.67^{ab}	2.93^{a}	3.43^{a}	3.00^{a}
N-N	3.50^{a}	4.00^{a}	2.93^{a}	3.33^{ab}	3.03^{a}
P-S	3.57^{a}	3.43^{b}	3.70^{b}	3.30^{ab}	3.43^{a}
P-N	3.23^{a}	3.23^{b}	2.97^{b}	2.87^{b}	3.17^{a}

The different superscripts in the same column denote a significant difference (P<0.05)(n=30)

Table 20. Overall acceptable on seaweed plant beverage treatments at days 60 and150

day	N-S	N-N	P-S	P-N
60	3.13^{a}	3.13^{a}	3.07^{a}	3.40^{a}
150	3.50^{a}	3.40^{a}	3.40^{a}	3.57^{a}

The different superscripts in the same column and same row denote a significant difference (P<0.05) (n=30)

Based on the results of this study, we suggest that the inoculation set without pre-sterilization (N-S set) was the best strategy to produce fermented seaweed beverage since the quality of the finished product was improved as follows:

- Physico-chemical quality such as pH, ethanol, methanol and elements of the finished product met the standard of the community product for FPBs as shown in Table 21.
- The finished product passed microbiological quality based on the standard of the community product for FPBs (Table 21). It means that the use of the DW3 strain as inoculants was able to control yeast in FSB. Although partial sterilization by 0.5% KMS without inoculation (P-N set) also met the standard; however, using chemical in fermentation process is not accepted for functional food like FPBs.
- The finished product gave the best inhibitory effect against foodborne pathogenic bacteria after three months storage (Figure 41).
- The highest amount of LAB was found in the finished product (4.7 log CFU/ml) (Figure37B). The remaining of LAB in the finished product took an advantage in term of probiotic supplement.
- Inoculation had no adverse effect on sensory test

Figure 42. The finished product of fermented seaweed beverag

Table 21. Characteristics of the FSB at day 60 compared to the standard of the community product for FPBs and the standard of Thai FDA for drinking water

	Standard for FPBs community product (มผช 481/2547)	N-S set
Physico-chemical quality		
pH	≤ 4.3	2.9
Ethanol	≤ 3%	0.16%
Methanol	≤ 240 mg/L	nd
Microbial quality		
Lactic acid bacteria	No guildline	nd
Yeast and mold	100 CFU/ml	16 CFU/ml
Salmonella sp.	must not be detected in 50 ml	nd
S. aureus	must not be detected in 1ml	nd
C. perfringens	must not be detected in 0.1 ml	nd
E. coli	≤ 2.2 per 100 ml (by MPN method)	nd
	Standard for drinking water (Thai FDA) (mg/L)	
Elements		
Cu	1.0	0.01
Zn	5.0	0.36
Fe	0.5	0.18
Pb	0.05	nd
As	0.05	nd

nd = not detected, detectable limit of Methanol = 0.003 g/L, Pb and As = 0.001 g/L

CHAPTER 5

CONCLUSIONS

This final chapter of the thesis concludes the findings of the research study. It attempts to integrate the conclusions drawn from each experiment and identify future research needs. Finally, some general conclusion is drawn from the work.

1. Yeast contamination in finished products of Thai FPBs and fermenting plants beverages were isolated and identified as follows: *Rhodotorula mucilaginosa*, *Pichia membranifaciens*, *Pichia anomala*, *Issatchenkia occidentalis*, *Candida tropicalis*, *Saccharomyces cerevisiae* and unidentified yeasts. *R. mucilaginosa* was the most prevalent yeast species while all unidentified yeast species were isolated from fermenting plants. This result indicates that more diversity of yeast species was found during fermentation; however, they were completely inhibited as no detection is found in the finished products.

2. All of LAB produced organic acids, but a few isolates such as isolate DW3 also produced other antimicrobial compounds. A promising strain *L. plantarum* DW3 isolated from a fermented seaweed beverage gave the best inhibitory effects against foodborne pathogenic bacteria, pathogenic fungi and spoilage yeasts, including contaminated yeasts isolated from the FPBs. Hence, the isolate DW3 had a potential to be used as inoculants and its antimicrobial compounds were further investigated.

3. *L. plantarum* DW3 could produce bacteriocin with ability to inhibit pathogenic bacteria such as *S. aureus* PSSCMI 0004, *E. coli* PSSCMI 0001, *S. sonneii* PSSCMI 0032, *P. aeruginosa* PSSCMI 0048 and *V. parahaemolyticus* PSSCMI 0064. According to sensitivity of bacteriocin DW3 to proteases and high temperature (> 80°C), the bacteriocin DW3 was classified in class III: large heat-labile protein. This bacteriocin was strongly acted in an acidic condition (pH 2-6); however, its inhibitory activity is limited to only pathogenic bacteria. Therefore, the bacteriocin DW3 was not further studied while antifungal compounds were further determined.

4. *L. plantarum* DW3 also produced phenyllactic acid (PLA), broad spectrum antimicrobial compound. The quantification result from the GC-MS indicates that *L. plantarum* DW3 produced PLA approximately 31 mg/L in MRS broth. PLA showed inhibitory activity against growth of *R. mucilaginosa* DKA with MIC_{90} value of 5 mg/ml. Besides, at this concentration, PLA could inhibit more than 90% of several pathogenic fungi and spoilage yeasts as follows: *Issatchenkia occidentalis* DKB, *Pichia membranifaciens* DKC, *Pichia analoma* DKD, *Candida tropicalis* DKE, *Saccharomyces cerevisiae* DKF, *Hanseniaspora clermontiae* PKG, *Endomycopsis* sp. PSSCMI 7004, *Candida albicans* PSSCMI 7010, *Fusarium moniliforms* PSSCMI 3011, *Aspergillus flavus* TISTR 3041, *Aspergillus nidulans* TISTR 3169, *Pennicillium* sp. PSUNML. Moreover, the inhibitory activity of PLA was remained although it was heated at 121°C for 30 min. PLA was the most effective one in an acidic system (pH 2-5) which covering pH range of fermented foods.

5. Application of *L. plantarum* DW3, probiotic strain, as a starter culture was achievable to improve the quality of a fermented seaweed beverage in term of microbial quality (controlling contaminated yeast, pathogenic bacteria and spoilage organisms) as well as chemical quality i.e. level of elements (Cu, Fe, Na, K and Zn) and alcohols which met the standard guideline for the FPBs. In addition, the FSB was also a good source of available Fe. The results from the sensory test indicate that the FSB was only moderately acceptable by panelists. In addition, the FSB inoculated with the isolate DW3 had higher inhibitory effect against foodborn pathogenic bacteria such as *V. parahaemolyticus* PSSCMI0064, *B. cereus* ATCC 11178, *S. typhi* PSSCMI 0034, *S. aureus* PSSCMI 0004, and *E. coli* PSSCMI 0001 than that in both sets of uninoculated FSB.

The inoculation set without pretreatment by KMS As previously discussed in part V, we suggest that (N-S set) was the treatment that produced the best FSB based on its antibacterial activity, reduction of contaminated yeasts, remaining probiotic LAB and organoleptic properties.

Future research needs

It is clear from the above conclusions that many questions remain unanswered. Some specific suggestions for further research needs arising out of the current studies are listed as follows.

As variety of spoilage yeasts were detected in the finished FPB products. Good Manufacturing Practice (GMP) and Hazard Analysis Critical Control Point (HACCP) should be studied and then transferred the knowledge to producers.

Bacteriocin DW3 should be studied continuously due to the fact that it may be useful to use as a biopreservative compound in foods and beverages.

FSB provided valuable nutrients that derived from seaweed used and LAB during fermentation, therefore some useful compounds from seaweed i.e. antioxidant compounds and oligosaccharides as prebiotic should be investigated.

Due to the fact that *L. plantarum* DW3 was a probiotic LAB and seaweed may have prebiotic like oligosaccharide, it is interesting to consider the producing of FSB as a functional food drink and/or a source of probiotics.

Based on personal communication after the sensory test, the main points were flavor and odor whereas evaluation of color produced the lowest score in almost all treatments. Indeed, the beverages are not ready to be drunk unless they are diluted and achieve higher scores. Further study will be required to prove the results of this sensory test of the FSB.

General conclusion

In the light of the results presented in this study the questions posed in the introduction part can be answered.

Because contamination by yeasts always occurs, we used a starter culture that had inhibitory properties against yeast and a partial sterilization to halt or reduce this contamination in fermented plant beverages. Moreover, the finished products of the FSB after storing for 3 months passed the standard microbiological quality tests based on the standard of community product for FPBs (section microbiological quality). The inhibitory effects of the FSB against food borne pathogenic bacteria were retained throughout 3 months of storage with only minor reductions. A similar conclusion was reached from the finding that the inoculated treatment sets had a higher inhibiting

activity than that in the uninoculated sets because *L. plantarum* DW3 produced some substances other than organic acids. Base on this study, bacteriocin DW3 and phenyllactic acid were produced. Therefore, using a probiotic strain LAB as an inoculum is required to improve quality and increase nutrients of FPBs. Moreover, The FSB improved the availability of elements (Cu, Zn and Fe) and they were all in safety levels for drinking. According to above information, FPBs are a novel functional food product and this study provided information of how to improve the quality of FPB products and how to achieve the standard guideline for FPB products.

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APPENDIX A

Culture Media

1. Glucose fermentation medium for yeast

Peptone	5 g
Yeast extract	3 g
Glucose	20 g
Distilled water	1,000 ml

All ingredients were dissolved in distilled water and 4 ml of 0.2% bromothymol blue was added into the medium. 5 ml aliquot of the mixture was added in each test tube, which contained a Durham tube. They were sterilized using an autoclaved at 110°C (10 psi) for 10 min.

2. Fermentation media for yeast

- Prepare 6% (w/v) of individual sugar solution, except raffinose12% (w/v)
- Sterile the solution by filtration though a 0.45 μm filter

Basal medium

Peptone	4.5 g
Yeast extract	7.5 g
Distilled water	1000 ml

- The mixture was dissolved by adding 1000 ml distilled water and then 4 ml of 0.2% bromothymol blue into100 ml basal medium
- Pipette 4 ml of the mixture in a test tube containing a Durham tube, sterile by using autoclave at 121°C (15 psi) for 15 min
- Add 2 ml of each sugar solution in the basal medium (the final concentration of sugar is 2%)

3. Carbon assimilation media

- Dissolve 6.7 g of yeast nitrogen base and 5g of carbon source by CO_2 free distilled water 100 ml
- Boil the mixture until it was dissolved well and leave until the temperature decrease to 50°C

- Sterile the solution by filtration though a 0.45 μm filter
- Add 0.5 ml of prepared solution in 4.5 ml distilled water and mix well before test

4. MRS (de Man Rogosa and Sharpe)

Bromocresol purple	0.2 g	Peptone	10.0 g
Sodium azide	0.0014 g	Beef extract	10.0 g
Yeast extract	5.0 g	Glucose	20.0 g
K_2HPO_4	2.0 g	Tween 80	1.0 g
Sodium acetate.$3H_2O$	0.2 g	Diammonium citrate	2.0 g
$MgSO_4.7H_2O$	0.2 g	$MnSO_4.4H_2O$	0.005 g
Agar	15 g	Distilled water	1,000.0 ml

Dissolve the mixture by distilled water and boil until it was dissolved well and then sterilized by using an autoclave at 121°C (15 psi) for 15 min.

5. MRS broth

Peptone	10.0 g	Beef extract	10.0 g
Yeast extracts	5.0 g	Glucose	20.0 g
K_2HPO_4	2.0 g	Tween 80	1.0 g
Sodium acetate.$3H_2O$	5.0 g	Diammonium citrate	2.0 g
$MgSO_4.7H_2O$	0.2 g	$MnSO_4.4H_2O$	0.05 g
Distilled water	1,000.0 ml		

Dissolve the mixture by distilled water and boil until it was dissolved well and then sterilized by using an autoclave at 121°C (15 psi) for 15 min.

6. Malt Extract Agar

Malt extract	30 g
Agar	15 g
Distilled water	1,000.0 ml

Dissolve the mixture by distilled water and boil until it was dissolved well adjusted pH to 4 and then sterilized by using an autoclave at 121°C (15 psi) for 15 min.

7. Malt extracts broth

Malt extracts 20 g

Distilled water 1,000.0 ml

Dissolve the mixture by distilled water and boil until it was dissolved well and then sterilized by using an autoclave at 121°C (15 psi) for 15 min.

8. TSA (Trypticase (Tryptic) Soy Agar)

Trypticase peptone 17 g

Phytone peptone 3 g

NaCl 5 g

Agar 15 g

Distilled water 1,000.0 ml

Dissolve the mixture by distilled water and boil until it was dissolved well and then sterilized by using an autoclave at 121°C (15 psi) for 15 min.

9. TSB (Trypticase (Tryptic) Soy Broth)

Trypticase peptone 17 g

Phytone peptone 3 g

NaCl 5 g

K_2HPO_4 2.5 g

Dextrose 2.5 g

Distilled water 1,000.0 ml

Dissolve the mixture by distilled water and boil until it was dissolved well and then sterilized by using an autoclave at 121°C (15 psi) for 15 min.

10. McFarland standards

Formulas

Tube on	0.5	1	2	3	4	5	6	7	8	9	10
Barium Chloride (ml) 1.175 (ml)	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Sulfuric acid 1.0% (ml)	9.95	9.9	9.8	9.7	9.6	9.5	9.4	9.3	9.2	9.1	9.0
Approximate cell density ($x10^8$ cell/ml)	1.5	3	6	9	12	15	18	21	24	27	30

APPENDIX B

DNA sequencing

Bacterial or Yeast DNA Extraction

- Single colony from agar medium is cultured in broth for 6-12 h.
  (temperature condition is depended on microorganisms)
- Centrifuge broth culture at 10000 rpm for 2-5 min.
- Cell pellets are resuspended in 100 ml TE Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA)
- Vortex suspension and boil for 10-15 min.
- Centrifuge the suspension at 10,000-12,000 rpm for 10 min.
- Collect only DNA extract solution (DNA template)

Amplification DNA product

PCR Reaction for rDNA Gene

- 1x PCR Buffer pH 8.8 (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_2$ 0.1% Triton X-100)
- 2.0 mM $MgCl_2$
- DNA Template (2-5 µl)
- 0.4 µM dNTP
- 0.4 µM Primer
- 1 U Taq DNA Polymerase (Biolab)
- Adjust volume to 20 µl by deionized water

PCR Cycle

95°C	5	min	
95°C	1	min	25-30 cycles
60°C	1	min	
72°C	1	min	

72°C 3 min

DNA Sequencing PCR Reaction

Chemical reagent	Volume
BigDye Terminator v3.1 Cycle Sequencing Kit	8.0 µl
DNA Template	see DNA extraction
Primer	3.2 pmol
Deionized water	20 µl
Total volume	20 µl

DNA Sequencing PCR Cycle

95°C 5 min
95°C 30 min
50°C 10 min } 30 cycles
60°C 4 min
60°C 4 min

Purify Sequencing PCR product and compared the DNA sequence with data base

Chemical reagent	1X
H_2O (3A)	14.5 µl
Absolute Ethanol	62.5 µl
3M sodium acetate pH 4.6	3.0 µl

- Add 80 ml of prepared solution and leave at room temperature for 15 min
- Centrifuge at 12,000 rpm, room temperature for 15 min
- Wash the pellet with 70% EtOH by the same condition
- Dry pellets at 95°C for 2-3 min
- Dissolve DNA pellets by 10-15 µl Hi-Di Formamide 10-15 µl.
- Boil sample at 95°C for 2 min and cool down by suddenly deep in an ice chamber for 5 min
- Directly sequenced in a DNA sequencer and compared to 16S rDNA gene data obtained from Genbank by using BLASTIN 2.0.5 program from

National Center for Biotechnology Information (NCBI)

Rhodotorula mucilaginosa 26S ribosomal RNA gene, partial sequence

strain 25

Length=609

Score = 996 bits (539), Expect = 0.0

Identities = 539/539 (100%), Gaps = 0/539 (0%)

Strand=Plus/Plus

```
Query  1    TAGTAGCGGCGAGCGAAGCGGGAAGAGCTCAAATTTATAATCTGGCACCTTCGGTGTCCG 60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  44   TAGTAGCGGCGAGCGAAGCGGGAAGAGCTCAAATTTATAATCTGGCACCTTCGGTGTCCG 103

Query  61   AGTTGTAATCTCTAGAAATGTTTTCCGCGTTGGACCGCACACAAGTCTGTTGGAATACAG 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  104  AGTTGTAATCTCTAGAAATGTTTTCCGCGTTGGACCGCACACAAGTCTGTTGGAATACAG 163

Query  121  CGGCATAGTGGTGAGACCCCCGTATATGGTGCGGACGCCCAGCGCTTTGTGATACATTTT 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  164  CGGCATAGTGGTGAGACCCCCGTATATGGTGCGGACGCCCAGCGCTTTGTGATACATTTT 223

Query  181  CGAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAATTGGGTGGTAAATTCCATCTAAAGCT 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224  CGAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAATTGGGTGGTAAATTCCATCTAAAGCT 283

Query  241  AAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAGCACTTT 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  284  AAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAGCACTTT 343

Query  301  GGAAAGAGAGTTAACAGTACGTGAAATTGTTGGAAGGGAAACGCTTGAAGTCAGACTTGC 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  344  GGAAAGAGAGTTAACAGTACGTGAAATTGTTGGAAGGGAAACGCTTGAAGTCAGACTTGC 403

Query  361  TTGCCGAGCAATCGGTTTGCAGGCCAGCATCAGTTTTCCGGGATGGATAATGGTAGAGAG 420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  404  TTGCCGAGCAATCGGTTTGCAGGCCAGCATCAGTTTTCCGGGATGGATAATGGTAGAGAG 463

Query  421  AAGGTAGCAGTTTCGGCTGTGTTATAGCTCTCTGCTGGATACATCTTGGGGGACTGAGGA 480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  464  AAGGTAGCAGTTTCGGCTGTGTTATAGCTCTCTGCTGGATACATCTTGGGGGACTGAGGA 523

Query  481  ACGCAGTGTGCCTTTGGCGGGGGTTTCGACCTCTTCACACTTAGGATGCTGGTGGAATG  539
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  524  ACGCAGTGTGCCTTTGGCGGGGGTTTCGACCTCTTCACACTTAGGATGCTGGTGGAATG  582
```

Figure 43. The similarity of DNA sequence between the strain DKA and *R. mucilaginosa* strain 25

Issatchenkia occidentalis gene for 26S rRNA, partial sequence

strain: EB7

Length=559

Score = 981 bits (531), Expect = 0.0

Identities = 536/538 (99%), Gaps = 1/538 (0%)

Strand=Plus/Minus

```
Query  1    CGTTGTGCCAGCATCCGAGACAGATGTCGCAGTCCTCGGTCCCCGCACGCAGCATCTCGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547  CGTTGTGCCAGCATCCGAGACAGATGTCGCAGTCCTCGGTCCCCGCACGCAGCATCTCGC 488

Query  61   ACTGGCTATAACACTCCGAAGAGCCACATTCCAGCACGCCTTCTCCTGCGGCAAAAACCG 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487  ACTGGCTATAACACTCCGAAGAGCCACATTCCAGCACGCCTTCTCCTGCGGCAAAAACCG 428

Query  121  ATGCTGGCCCAGGAAAAGCACAGAGCGCCGCCCACAAGGAGCAGCGGTGCGCAAATCCCA 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427  ATGCTGGCCCAGGAAAAGCACAGAGCGCCGCCCACAAGGAGCAGCGGTGCGCAAATCCCA 368

Query  181  TGTCGAGCCCAATACCCTTCCCTTTCAACAATTTCACGTGCTGTTTCACTCTCTTTTCAA 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  367  TGTCGAGCCCAATACCCTTCCCTTTCAACAATTTCACGTGCTGTTTCACTCTCTTTTCAA 308

Query  241  AGTGCTTTTCATCTTTCCTTCACAGTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAG 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  307  AGTGCTTTTCATCTTTCCTTCACAGTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAG 248

Query  301  CCTTAGATGGAATTTACCACCCACTTAGAGCTGCATTCCCAAACAACTCGACTCGTCAGA 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  247  CCTTAGATGGAATTTACCACCCACTTAGAGCTGCATTCCCAAACAACTCGACTCGTCAGA 188

Query  361  AGGGCCTTACAGCTTCCGCACGCGTCTCACGGGGCTCTCACCCTCAATGGCACCCTGTTC 420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187  AGGGCCTTACAGCTTCCGCACGCGTCTCACGGGGCTCTCACCCTCAATGGCACCCTGTTC 128

Query  421  CAAGGGACTTAGACACACGCTTCCACAAAGACTCCAACCTGCAATCTACAACTCGTGCCG 480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  127  CAAGGGACTTAGACACACGCTTCCACAAAGACTCCAACCTGCAATCTACAACTCGTGCCG  68

Query  481  AAACACGATTTCAAATCTGAGCTTTGGCCGCTTCACTCGCCGCTACTGAGGCA-TCCC  537
            |||||||||||||||||||||||| |||||||||||||||||||||||||||| ||||
Sbjct  67   AAACACGATTTCAAATCTGAGCTTTTGCCGCTTCACTCGCCGCTACTGAGGCAATCCC  10
```

Figure 44. The similarity of DNA sequence between the strain DKB and *I. occidentalis* EB7

Pichia membranifaciens 26S ribosomal RNA gene, partial sequence

strain G46

Length=571

Score = 961 bits (520), Expect = 0.0

Identities = 529/533 (99%), Gaps = 2/533 (0%)

Strand=Plus/Minus

```
Query  1    CCAGCATTCGAGACAGAAGCCGCAGTCCTCGGTCCCCACACGCGGCATCTGGCCCCGGCT  60
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  532  CCAGCATCCGAGACAGAAGCCGCAGTCCTCGGTCCCCACACGCGGCATCTGGCCCCGGCT 473

Query  61   ATAACACTCCGAAGAGCCACTTTCCGGAGCCCCTTCTCCCGCAGCAGGAACCGATGCTGG 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  472  ATAACACTCCGAAGAGCCACTTTCCGGAGCCCCTTCTCCCGCAGCAGGAACCGATGCTGG 413

Query  121  CCCAGAGGGCGCCCAGAGCGCCGCCTACAAGAGACAGCGGTGCGCACCCCCCATGTCGAG 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  412  CCCAGAGGGCGCCCAGAGCGCCGCCTACAAGAGACAGCGGTGCGCACCCCCCATGTCGAG 353

Query  181  CCCAATACCCTTCCCTTTCAACAATTTCACGTGCTGTTTCACTCTCTTTTCAAAGTGCTT 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  352  CCCAATACCCTTCCCTTTCAACAATTTCACGTGCTGTTTCACTCTCTTTTCAAAGTGCTT 293

Query  241  TTCATCTTTCCTTCACAGTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAGCCTTAGA 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  292  TTCATCTTTCCTTCACAGTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAGCCTTAGA 233

Query  301  TGGAATTTACCACCCGCTTGGAGCTGCATTCCCAAACAACTCGACTCGTCAGCAGGGCCT 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  232  TGGAATTTACCACCCGCTTGGAGCTGCATTCCCAAACAACTCGACTCGTCAGCAGGGCCT 173

Query  361  CAAAAGCTTCGCGCAGCACCCTACGGGGCTCTCACCCTCTCAGGCACCCTGTTCCAAGGG 420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  172  CAAAAGCTTCGCGCAGCACCCTACGGGGCTCTCACCCTCTCAGGCACCCTGTTCCAAGGG 113

Query  421  ACTTGGACACCGCGCTCCACAGAGACTCCCGCCTACACTCTACAACTCGTGCCGAAACAC 480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  112  ACTTGGACACCGCGCTCCACAGAGACTCCCGCCTACACTCTACAACTCGTGCCGAAACAC  53

Query  481  GGATTTCAAATCTGAGCTCTTGGCGCTTCACTCGCCGCTACTGGGGCA-TCCC  532
            | |||||||||||||||||| ||||||||||||||||||||||||| |||| |
Sbjct  52   G-ATTTCAAATCTGAGCTCTTGCCGCTTCACTCGCCGCTACTGGGGCAATCCC  1
```

Figure 45. The similarity of DNA sequence between the strain DKC and *P. membranifaciens* isolate G46

Pichia anomala 26S ribosomal RNA gene, partial sequence

strain 8

Length=604

Score = 1037 bits (561), Expect = 0.0

Identities = 561/561 (100%), Gaps = 0/561 (0%)

Strand=Plus/Minus

```
Query  1    AGACGGGCGGCATTAGATCATTACGCCAGCATCCTAGTCAAAAGACGCAGCCCTCGATCC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  589  AGACGGGCGGCATTAGATCATTACGCCAGCATCCTAGTCAAAAGACGCAGCCCTCGATCC 530

Query  61   AGACAGGCAATATCAGCAGAAGCTATAACACTCCACCGAAGTGAAGCCACATTCAACTGC 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  529  AGACAGGCAATATCAGCAGAAGCTATAACACTCCACCGAAGTGAAGCCACATTCAACTGC 470

Query  121  CATTATCTTGCCATCCGAATCGATGCTGGCCCAGTGAAATACGAGTGCACAACTCAAGAA 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  469  CATTATCTTGCCATCCGAATCGATGCTGGCCCAGTGAAATACGAGTGCACAACTCAAGAA 410

Query  181  GAGAAGATAATCGTAAAACACCAAGTCTGATCTAATGCCCTTCCCTTTCAACAATTTCAC 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  409  GAGAAGATAATCGTAAAACACCAAGTCTGATCTAATGCCCTTCCCTTTCAACAATTTCAC 350

Query  241  GTACTTTTTCACTCTCTTTTCAAAGTTCTTTTCATCTTTCCATCACTGTACTTGTTCGCT 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  349  GTACTTTTTCACTCTCTTTTCAAAGTTCTTTTCATCTTTCCATCACTGTACTTGTTCGCT 290

Query  301  ATCGGTCTCTCGCCAATATTTAGCTTTAGATGGAATTTACCACCCACTTAGAGCTGCATT 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  289  ATCGGTCTCTCGCCAATATTTAGCTTTAGATGGAATTTACCACCCACTTAGAGCTGCATT 230

Query  361  CCCAAACAACTCGACTCTTCGATAGCACCTTACATAGGAATGGGCATCTCATCAGACGGG 420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  229  CCCAAACAACTCGACTCTTCGATAGCACCTTACATAGGAATGGGCATCTCATCAGACGGG 170

Query  421  ATTCTCACCCTCTATGACGTCCTGTTCCAAGGAACATAGACAAGAGCCAAACCCAAGGTT 480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  169  ATTCTCACCCTCTATGACGTCCTGTTCCAAGGAACATAGACAAGAGCCAAACCCAAGGTT 110

Query  481  ACCATCTTCAAATTACAACTCGAACACCGAAGGTGCTAGATTTCAAATTTGAGCTTTTGC 540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  109  ACCATCTTCAAATTACAACTCGAACACCGAAGGTGCTAGATTTCAAATTTGAGCTTTTGC  50

Query  541  CGCTTCACTCGCCGTTACTGA  561
            |||||||||||||||||||||
Sbjct  49   CGCTTCACTCGCCGTTACTGA  29
```

Figure 46. The similarity of DNA sequence between the strain DKD and *P. anomala* strain 8

Candida tropicalis 26S ribosomal RNA gene, partial sequence

strain XM07A

Length=607

Score = 1003 bits (543), Expect = 0.0

Identities = 555/560 (99%), Gaps = 4/560 (0%)

Strand=Plus/Minus

```
Query  1    CTAGGTATAAACCGCAGTCCTCAGTCTAGGCTGGCAGTATCGACGAAGGCTATAACACAC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  564  CTAGGTATAAACCGCAGTCCTCAGTCTAGGCTGGCAGTATCGACGAAGGCTATAACACAC 505

Query  61   AACCGAAGCCGTGCCACATTCCAACGCAATTCTCCTACCGCCCAAACTGATGCTGGCCCG 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  504  AACCGAAGCCGTGCCACATTCCAACGCAATTCTCCTACCGCCCAAACTGATGCTGGCCCG 445

Query  121  ATAAACTGTAGAGGCCACCCCCGAAGAAGTAACATACAAAATACCAAGTCTGATCTCAAG 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  444  ATAAACTGTAGAGGCCACCCCCGAAGAAGTAACATACAAAATACCAAGTCTGATCTCAAG 385

Query  181  CCCTTCCCTTTCAACAATTTCACGTACTTTTTCACTCTCTTTTCAAAGTTCTTTTCATCT 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  384  CCCTTCCCTTTCAACAATTTCACGTACTTTTTCACTCTCTTTTCAAAGTTCTTTTCATCT 325

Query  241  TTCCATCACTGTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAGCTTTAGATGGAATT 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  324  TTCCATCACTGTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAGCTTTAGATGGAATT 265

Query  301  TACCACCCACTTAGAGCTGCATTCCCAAACAACTCGACTCTTCGAAGGAACTTTACATAG 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264  TACCACCCACTTAGAGCTGCATTCCCAAACAACTCGACTCTTCGAAGGAACTTTACATAG 205

Query  361  GCCTGGATCATCTCATCGCACGGGATTCTCACCCTCTGTGACGTTCTGTTCCAAGAAACA 420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  204  GCCTGGATCATCTCATCGCACGGGATTCTCACCCTCTGTGACGTTCTGTTCCAAGAAACA 145

Query  421  TAGACAAGAGCCAGACCCAAAGATACCTTCTTCAAATTACAACTCGGACTCTGAAAGAGC 480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  144  TAGACAAGAGCCAGACCCAAAGATACCTTCTTCAAATTACAACTCGGACTCTGAAAGAGC  85

Query  481  CAGATTTCAAAATTTGAGCTTT-GCCGCTTCACTCGCCGCTACTAAGGCA-TCCCTGGTGG 538
            |||||||||||||||||||| |||||||||||||||||||||||||| |||||| |||
Sbjct  84   CAGATTTCAAAATTTGAGCTTTTGCCGCTTCACTCGCCGCTACTAAGGCAGTCCCTGTTGG  25

Query  539  GTTTCTTT-CCTCCGCTTAA  557
             ||||||| ||||||||||| 
Sbjct  24   -TTTCTTTTCCTCCGCTTAA  6
```

Figure 47. The similarity of DNA sequence between the strain DKE and *C. tropicalis* strain XM07A

Saccharomyces cerevisiae 26S ribosomal RNA gene, partial sequence

strain clone N224

Length=582

Score = 931 bits (504), Expect = 0.0

Identities = 513/517 (99%), Gaps = 2/517 (0%)

Strand=Plus/Plus

```
Query  1    ACGGCGAGTGAAGCGCCAAAGCTCAAA-TTGAAATCTGGTACCTTCGGTGCCCGAGTTGT  59
            ||||||||||||||| |||||||||| |||||||||||||||||||||||||||||||||
Sbjct  24   ACGGCGAGTGAAGCGGCAAAGCTCAAATTTGAAATCTGGTACCTTCGGTGCCCGAGTTGT  83

Query  60   AATTTGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTCA 119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  84   AATTTGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTATGTTCCTTGGAACAGGACGTCA 143

Query  120  TAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGAAGAG 179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  144  TAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTAAAGTGCCTTCGAAGAG 203

Query  180  TCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATT 239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  204  TCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATATT 263

Query  240  GGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGA 299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264  GGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGA 323

Query  300  GAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTTTT 359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  324  GAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGACATGGTGTTTT 383

Query  360  GTGCCCTCTGCTCCTTGTGGGTAGGGGAATCTCGCATTTCACTGGGCCAGCATCAGTTTT 419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  384  GTGCCCTCTGCTCCTTGTGGGTAGGGGAATCTCGCATTTCACTGGGCCAGCATCAGTTTT 443

Query  420  GGTGGCAGGATAAGTCCATAGGAATGTAGCTTGCCTCGGTAAGTATTATAGCCTGTGGGA 479
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  444  GGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAGTATTATAGCCTGTGGGA 503

Query  480  ATACTGCCAGCTGGGACTGAGGACTGCGACG-AAGTC  515
            |||||||||||||||||||||||||||||| |||||
Sbjct  504  ATACTGCCAGCTGGGACTGAGGACTGCGACGTAAGTC  540
```

Figure 48. The similarity of DNA sequence between the strain DKF and *S. cerevisiae* clone N224

Lactobacillus plantarum 26S ribosomal RNA gene, partial sequence,

strain WCFS1

Length=269050

Score = 1101 bits (596), Expect = 0.0

Identities = 599/600 (99%), Gaps = 1/600 (0%)

Strand=Plus/Plus

```
Query  1    TTTAGACTTTAATGATTTGCCTGGCGGTGGATTATAAGTTAAGTCAGTGCAAGCTACTGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TTTAGACTTTAATGATTTGCCTGGCGGTGGATTATAAGTTAAGTCAGTGCAAGCTACTGG  60

Query  61   CTAAAAATATTTTCAGAAAGTGGCAGGGAACCATTTTAATATCTGTAAAATGATTTGACA 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CTAAAAATATTTTCAGAAAGTGGCAGGGAACCATTTTAATATCTGTAAAATGATTTGACA 120

Query  121  CGCAAACTTGCTGATTTACCAGTATTTATTAGCAGTTAGCGAGGATGATGACGACTAGCA 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CGCAAACTTGCTGATTTACCAGTATTTATTAGCAGTTAGCGAGGATGATGACGACTAGCA 180

Query  181  AATCGAGGGTGGACCAACATGTTTTTGTTGATTAATAGAAAAAGTTTGCAAAAACCATTT 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AATCGAGGGTGGACCAACATGTTTTTGTTGATTAATAGAAAAAGTTTGCAAAAACCATTT 240

Query  241  GACAATTCTATTGATTAGCCGTATATTTTAAGTCATCAAATACACATTATAAATTAAGGA 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  GACAATTCTATTGATTAGCCGTATATTTTAAGTCATCAAATACACATTATAAATTAAGGA 300

Query  301  GATTTTAATCATGACGATTAATACCAATTTACAATTGAATTCGTATTATTATTACTGGTT 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GATTTTAATCATGACGATTAATACCAATTTACAATTGAATTCGTATTATTATTACTGGTT 360

Query  361  TACTTAGTGCCTTGGCATCCACTGCGAGGCACGCCCTCGCAACGGATGTGACCAGTAAAT 420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TACTTAGTGCCTTGGCATCCACTGCGAGGCACGCCCTCGCAACGGATGTGACCAGTAAAT 420

Query  421  TTTGGAATGATTAAAATTTATGGAAAAGTCATATCAGTGTTGTGAACAACCACTGTGTGA 480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  TTTGGAATGATTAAAATTTATGGAAAAGTCATATCAGTGTTGTGAACAACCACTGTGTGA 480

Query  481  CTTTTCTACTTCTGAGTAGCGGTGTTTTTCGGGCACCCTGCGAAGAATTAGTGGACTCAC 540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  CTTTTCTACTTCTGAGTAGCGGTGTTTTTCGGGCACCCTGCGAAGAATTAGTGGACTCAC 540

Query  541  AGTTGGTGTAACTGCGAGt-ttttttATTAGCCAAAATGAGAGGGAGCGAAGTTAATGCA 599
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  541  AGTTGGTGTAACTGCGAGTCTTTTTTATTAGCCAAAATGAGAGGGAGCGAAGTTAATGCA 600
```

Figure 49. The similarity of DNA sequence between the strain DW3 and *L. plantarum* strain WCFS1

APPENDIX C

Chemical Analysis

1. **Gas chromatography condition for detections ethanol and methanol**

   (Yang and Choong, 2001)

   HP 6850 Gas Chromatograph with Flame Ionization Detector (FID)

   Inlet temperature: 240°C

   Oven initial temperature: 35°C 5 min

   Ramp to: 100°C at 4°C/min

   Ramp to: 230°C, hold 5 min, at 15°C/min

   Column: Innowax, length 30 m., 250 μm I.D, 0.25 μm film thickness

2. **Gas chromatography condition for detections lactic and acetic acid**s

   (Yang and Choong, 2001)

   HP 6850 Gas Chromatograph with FID

   Inlet temperature: 240°C

   Oven initial temperature: 75°C 1 min

   Ramp to: 180°C at 5°C / min

   Ramp to: 230°C, hold 5 min, at 15°C / min

   Column: Innowax, length 30 m., 250 μm I.D, 0.25 μm film thickness

3. **Total acidity (AOAC, 1990)**

   - Prepare CO_2 free water by boiling distilled water for 20 min
   - Prepare acid potassium phthalate ($KHC_8H_4O_4$) solution by dissolving 0.30 g $KHC_8H_4O_4$ with CO_2 free water 100 ml
   - Prepare phenolphalein solution by dissolving 1 g phenolphalein with 100 ml of 95% ethanol
   - Standardize NaOH solution by titration with $KHC_8H_4O_4$ solution

   (Add 3 drops of phenolphalein before titration) and calculate accuracy of normality of NaOH solution by using the equation below

$$\text{Normality of NaOH} = \frac{KHC_8H_4O_4\ (g) \times 1{,}000}{\text{NaOH (ml)} \times 204.229}$$

-Dilute 1 ml of sample with the CO_2 free water and add 3 drops phenolpthalein before titration with the standardized NaOH solution until obtain the endpoint (pink solution)

Calculate total acidity in a form of lactic acid by the equation as follows:

$$\text{Total acidity (g/100 ml)} = \frac{N \times V \times 90 \times 100}{1000 \times 1}$$

N = Accurate normality of NaOH solution

V = Volume of NaOH solution (ml)

4. Total sugar (Phenol-sulfuric total sugar) Dubois et al., 1956

- Prepare glucose stock solution 0.5 mg/ml and dilute to 20, 40, 60, 80 and 100 µg/ml with distilled water
- Prepare 5% phenol solution and concentrated sulfuric acid (H_2SO_4)
- Pipette 2 ml of standard glucose solutions into test tubes and keep in an ice box
- Add 1 ml of 5% phenol solution, mix well and place in ice for 2-3 min and then set test tubes to room temperature
- Pipette 5 ml of sulfuric acid in the glucose solutions mix well and leave them for 10 min. Shake them well again and leave for 15-20 min
- Determine the standard solutions by observing at OD 490 nm
- Plot standard curve (Y axis is OD_{490} value and X axis is glucose concentrations)

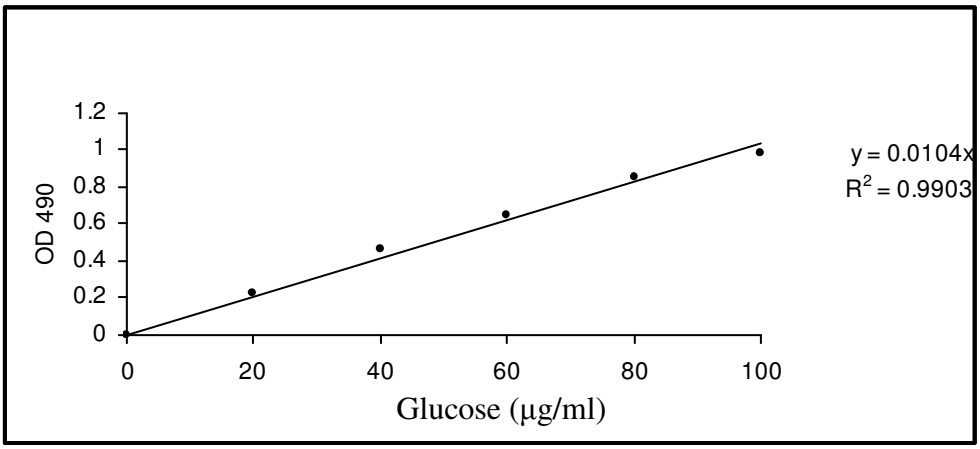


Figure 50. Standard curve of glucose concentrations

5. Calculation concentration of FDS, EA layer and Water layer

5.1. Freeze dried solution (FDS)

Concentrated culture filtrate supernatant (20 fold) were prepared by dissolving 7 g of freeze dried supernatant (FDS) in 10 ml water (7 g of FDS obtained from 200 ml of MRS broth) which corresponded to 700 mg FDS/ml and then diluted to 15, 10, 5, 2.5, 1 and 0.5 fold by distilled water which corresponded to 700, 525, 350, 175, 87.5, 35 and 17.5 mg/ml, respectively.

Concentration fold (ml of supernatant/ml H_2O)	mg of freeze dried sample/ml
20	700
15	525
10	350
5	175
2.5	87.5
1	35
0.5	17.5

5.2. EA layer (Ethyl acetate layer)

EA layer was prepared by dissolving 10 g of FDS in 125 ml water and extract three times by 60 ml EtOAc after adjustment to pH 2.0. The EA layer (150 ml) was

separated from water layer with a separating funnel. For this step, 150 ml of EA layer corresponds to10000 mg of FDS (1000 mg/15 ml). An aliquot (31.5 mL) of EA layer (corresponding to 700 mg of FDS) was concentrated to dryness and resuspended in 3 ml water. The concentrated sample was diluted to 525, 350, 175, 87.5, 35 and 17.5 mg/ml, respectively by distilled water and adjusted pH to 4 by 2 N NaOH before filtration though 0.22 μm filter.

5.3. Water layer

After removing the EA layer, 120 ml of the water layer was obtained. In order to prepare concentration of water layer equal to EA layer, 25.2 ml of the water layer was evaporated and dissolved in 3 ml of distilled water and adjusted pH to 4 by 2 N NaOH before filtration though 0.22 μm filter.

6. Calculation of dose and concentration of samples used in the microtiter plate well method

According to the microtiter plate well method, 100 μl of each sample (FDS, EA layer and water layer) was mixed with 100 μl of ME broth contained microbial indicator. Therefore dose and concentration of the sample solutions were corresponded to the list below. Concentration is calculated from each dose divided by each volume of the assay.

Dose = mg of sample per well

$$\text{Concentration} = \frac{\text{dose (mg)}}{\text{volume of assay (ml)}}$$

Sample solution (mg/ml)	Dose (mg)	Concentration (mg/ml)
700	70.0	350
525	53.0	265
350	35.0	175
175	18.0	90
87.5	8.80	44
35	3.50	17.5
17.5	1.80	9.0

7. Standard curve of PLA

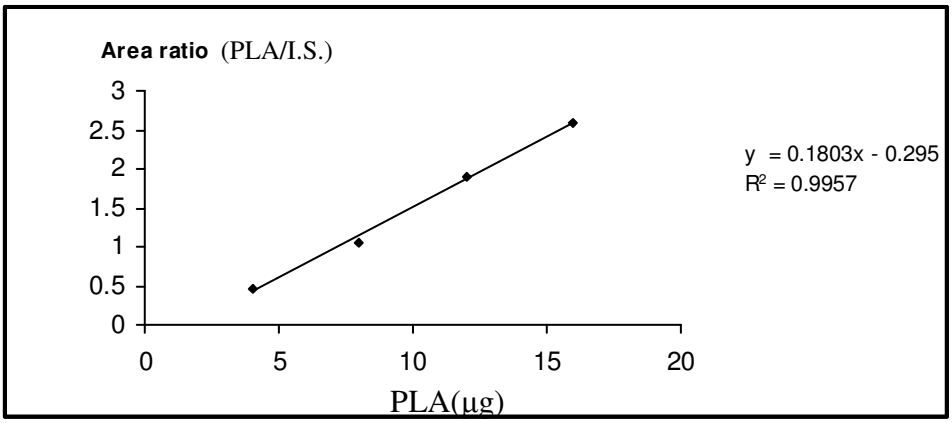


Figure 51. Standard curve of authentic phenyllactic acid (PLA)

Preparation of standard PLA solution and analysis by GC-MS

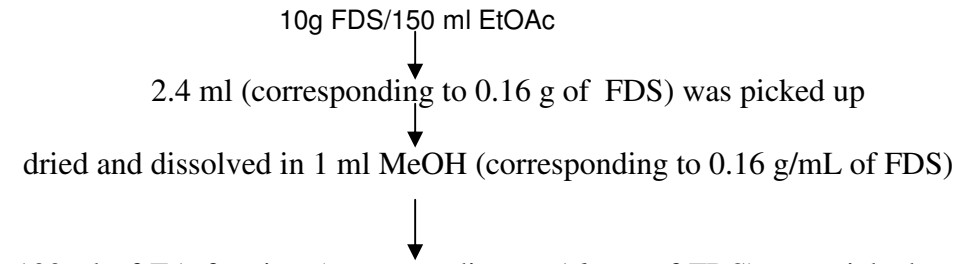
1. Prepare stock PLA solution (1 µg/µl)
2. Aliquot of stock solutions (4, 8, 12, and 16 µl) are mixed with 40 µg of the internal standard (*p*-hydroxybenzoic acid)
3. Dry the mixture by vacuum desiccator for 15 min and vacuum pump for 2 h.
4. Derivertize by adding 50 µl of BSA (*N*, *O* – bis (trimethylsilyl) acetamide) and heat at 70°C for 15 min
5. Inject 2 µl of the mixture in the GC-MS
6. Plot standard curve of the area ratio of PLA versus the internal standard (*p*-hydroxybenzoic acid) while X axis indicates amount of PLA)

Calculation of phenyllactic acid (PLA) in culture supernatant

1. 100 µl of EA layer is mixed well with 40 µl of the internal standard and performed as described above
2. The area ratios are used to calculate the amount of PLA by using the equation of the standard curve (raw data is shown in Table please add number below)

Replicate	Area ratio	PLA (μg)
1	2.21	13.89
2	2.31	14.45
3	2.28	14.28
Average	2.27	14.21±0.29

EA layer was prepared by dissolving 10 g of freeze dried sample in 125 ml water and extract three times by 60 ml EtOAc. The EA layer was separated from water layer with a separating funnel to yield 150 mL. An aliquot (2.4 mL) of the EA layer was dried up and dissolved in 1 ml of MeOH, and 100 μl was used for the GC analysis. The calculation and diagram is showed below.



Therefore, as shown in Table number above, PLA 14.21 μg as an average from 100 μl of EA fraction corresponded to 14.21 μg /16 mg FDS

= 0.89 μg /mg FDS

= 0.89 mg/g FDS

= 0.89 mg/28.57 ml (due to 7 g of FDS obtained from 200ml of MRS broth)

= 31.15 mg/liter

แบบทดสอบคุณภาพทางประสาทสัมผัส

Hedonic-5-scale

ผลิตภัณฑ์.....น้ำหมักสาหร่ายผมนาง (Fermented seaweed beverages)……

ชื่อ(Name) ……..……วันที่ (Date) ……..…เวลา (Time) ……....

คำแนะนำ กรุณาทดสอบตัวอย่างที่เสนอให้จากซ้ายไปขวาแล้วให้คะแนนความชอบตัวอย่างในแต่ละปัจจัยที่ใกล้เคียงกับความรู้สึกของท่านมากที่สุด โดยกำหนดให้

Please examine the purpose samples from left to right and then score each factor according to your sensory. The ranking scores are as follows:

1 = ไม่ชอบมาก (dislike extremely)

2 = ไม่ชอบ (dislike)

3 = เฉยๆ (neither like or dislike, moderately acceptable)

4 = ชอบ (like)

5 = ชอบมาก (like extremely)

	ตัวอย่าง (samples)			
	.....	.....	.....	.....
ความใส (clearness)	.....	.....	.....	.....
สี (color)	.....	.....	.....	.....
กลิ่น (odor)	.....	.....	.....	.....
กลิ่นรส (flavor)	.....	.....	.....	.....
ความชอบรวม (overall acceptable)	.....	.....	.....	.....

ข้อเสนอแนะ (comments).....

.....ขอบคุณครับ (Thank you)

VITAE

Name Pakorn Prachyakij

Student ID 4823003

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Degree	Name of Institution	Year of Graduation
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M. Sc.	(Food Technology) Faculty of Agroindustry, Prince of Songkla University 2004	2004

Scholarship Awards during Enrolment

Graduate School Songklanakarin Scholarship 2005-2007

Teacher assistant (TA) fund from Faculty of Science (2005)

Research scholarship from The National Science and Technology Development Agency (NSTDA) in the program year of 2006

List of Publication and Proceeding

1. Prachyakij, P., Schnürer, J., Charernjiratrakul, W., and Kantachote, D 2007. Selection and identification of lactic acid bacteria with ability to inhibit contaminated yeast in fermented plant beverages. Songklanakarin J. Sci. Technol, 29 (Suppl. 2): 211-218

2. Prachyakij, P., Charernjiratrakul, W., and Kantachote, D. 2008. Improvement in the quality of a fermented seaweed beverage using an antiyeast starter of *Lactobacillus plantarum* DW3 and partial sterilization. World Journal of Microbiology and Biotechnology. DOI 10.1007/s11274-008-9662-1. (in press)

3. Prachyakij, P., Kantachote, D .,Charernjiratrakul, W .and Schnürer,J . 2005. Inhibition of yeast contaminated in fermented plant beverages by lactic acid bacteria The 2nd PSU Symposium on Graduate Research 12 March, 2006 at Faculty of Science, Prince of Songkla University, Thailand. (oral presentation)

4. Prachyakij, P., Charernjiratrakul, W., and Kantachote, D. 2007. Yeast contamination in Fermented plant beverages**.** Proceeding of Fermented plant, Technology for sufficient to permanent healthy innovation for communication, 25th May 2007 at Grand Mercure Fortune Hotel, Bangkok (oral presentation)

5. Kantachote, D., Prachyakij, P., and Charernjiratrakul, W. 2007. Selection of lactic acid bacteria used as inoculants for improving the quality of fermented plant beverages**,** Proceeding of Fermented plant, Technology for sufficient to permanent healthy innovation for communication, 25th May 2007 at Grand Mercure Fortune Hotel, Bangkok (oral presentation)