APPENDICES

APPENDIX A

Media

MRS medium

Carbon source (Sugar or Starch)	10	g
Casein peptone	10	g
Beef or meat extract	10	g
Yeast extract	5	g
Di-ammonium hydrogen citrate	2	g
K_2HPO_2	2	g
$CH_3COONa \cdot 3H_2O$	5	g
$MgSO_4 \cdot 7H_2O$	0.2	g
$MnSO_4 \cdot H_2O$	0.2	g
Tween80	1	g

Mix all compositions and add 1000 mL of water. Add 15 g of agar in order to prepare MRS agar and add 125 ppm of Bromocersol purple if necessary. The final pH should be 6.4-6.6. No need to adjust the pH.

LB medium

Casein peptone	10	g

NaCl	10	g
Yeast extract	5	g

Mix all compositions and add 1000 mL of water. Add 15 g of agar in order to prepare LB agar. The final pH should be 6.4-6.6. No need to adjust the pH.

Stock of ampicilin solution (1 mg/mL)

Dissolve 100 mg of ampicilin powder in 100 mL of water and filter through 0.2 µm filter before storage at -20^{o}C.

Stock of Erythromycin (100 mg/mL)

Dissolve 100 mg of erythromycin powder in 100 mL of water and filter through 0.2 µm filter before storage at -20^{o}C.

Stock of D-alanine (100 mg/mL)

Dissolve 100 mg of D-alanine powder in 100 mL of water and filter through 0.2 µm filter before storage at -20^{o}C.

Stock of IPTG (100 mM)

Dissolve 0.595 g of IPTG in 50 mL water and filter through 0.2 µm filter before storage at -20^{o} C.

APPENDIX B

Assay of enzyme activity

1. Amylase activity assay

The amylase activity represents the capability of the enzyme on the hydrolysis of α, 1-4 glucosidic linkages on starch molecules to release mono-, di- or oligosaccharides containing reducing ends. The reducing sugar is detected by colorimetric method known as DNS method (Miller, 1959). 1 IU is defined as the amount of the enzyme catalyzes the hydrolysis of starch and releases 1 µmole of reducing sugar under the assayed condition.

Preparation of chemicals

DNS solution:

Dissolve 8 g of NaOH in 200 mL of water. Add 5 g of DNS powder, the solution becomes viscose. Gradually add 150 g of K-Na-tartrate while stirring. Water can be added more if necessary. Adjust to 500 mL with water.

Stock of glucose: Dissolve 2 g of glucose in 1 L of water

Preparation of calibration curve:

Dilute the stock solution to 0.1-2 mg/mL in water. Pipette 100 µL of each concentration and 100 µL of dinitrosalicylic acid solution in microtube. Mix well by vortex and heat at 100^{o}C for 10 min. Stand the tube until cool and add 800 µL of water. Mix well by vortex and measure at absorbance 540 nm (A_{540}).

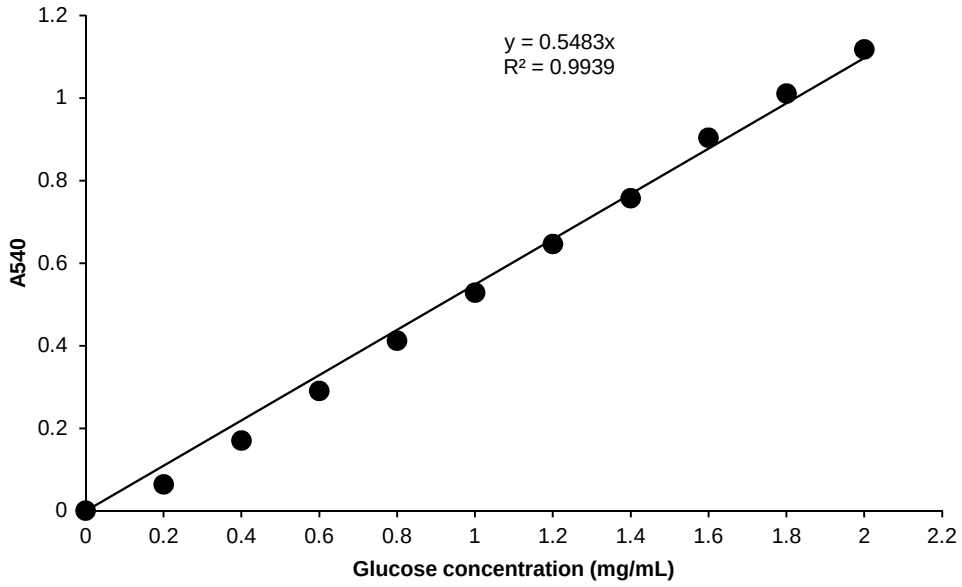

Figure A.1 Calibration curve of glucose concentration and absorbance at 540 nm

Reaction mixture for amylase activity assay

Buffer: 0.1 M Na-phosphate buffer pH 6.5

Substrate: 0.5%(w/v) of soluble starch – dissolve 0.5 g of soluble starch in 100 mL of buffer

Enzyme: Dilute enzyme in buffer to proper concentration

Table A.1 Reaction mixture for amylase activity assay

Reaction mixture	**ES**	**EB**	**SB**
Substrate (µL)	50	-	50
Enzyme (µL)	50	50	-
Buffer (µL)	-	50	50

Incubate the tubes containing reaction mixture at 37°C on shaking incubator for 10 min and terminate the reaction by adding 100 µL of DNS solution. Heat the tubes at 100°C for 10 min. Stand until cool and add 800 µL of water. Mix well by vortex and measure at absorbance 540 nm (A_{540}).

Calculation of amylase activity

Net absorbance (Abs) = ES-EB-SB

Reducing sugar (mg/mL) = $\frac{NetAbs}{slope}$

$$Amylase\ activity\ (\frac{U}{mL}) = \frac{Reducing\ sugar\ (mg/mL) \times 0.5(mL) \times 1000(\mu L/mL) \times 2(internal\ dilution\ factor)}{180(\frac{g}{mole}) \times 10(min) \times enzyme(\mu L)}$$

2. α-glucosidase activity assay

The α-glucosidase activity represents the capability of the enzyme on the hydrolysis of α,1-4 glucosidic linkage of p-nitrophenylα-D-glucopyranoside (pNPG). The p-nitrophenol and glucose is released but only p-nitrophenol is detected by absorbance at 310 nm as yellow soluction. 1 IU is defined as the amount of the enzyme catalyzes the hydrolysis of pNPG and releases 1 µmole of p-nitrophenol under the assayed condition

Chemical preparation

Stock of p-Nitrophenol: Dissolve 278.22 mg of p-Nitrophenol in 100 mL of water

1M Na_2CO_3: Dissolve 10.60 g of Na_2CO_3 in 100 mL of water

Preparation of calibration curve:

Dilute stock solution with water up to 500x to concentration of 0.4 mM. Dilute with water again to obtain final concentration of 0-0.4mM.Pipette 100 µL of solution in each concentration and 400 µL of 1 M Na_2CO_3. Mix well by vortex prior to measuring absorbance at 405 nm(A_{405}).

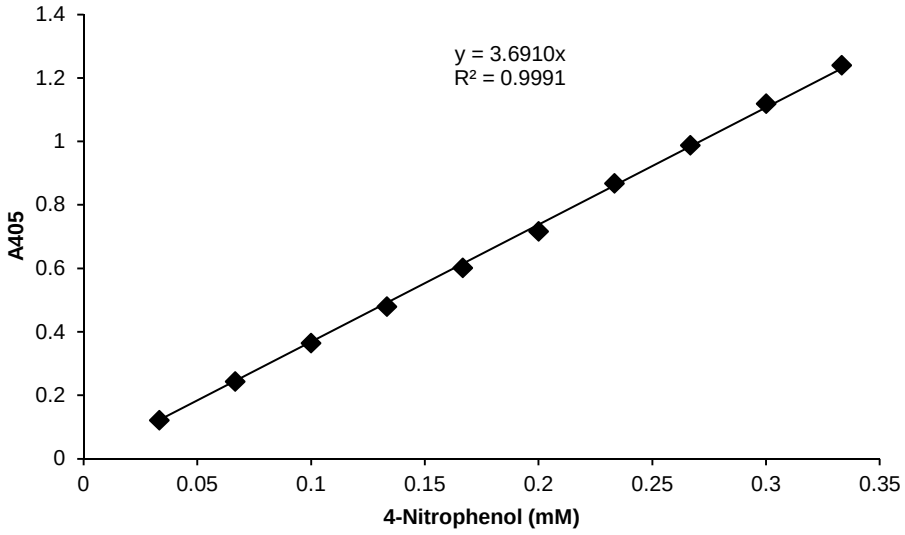

Figure A.2 Calibration curve of 4-Nitrophenol concentration and absorbance at 405 nm

Reaction mixture for α-glucosidase activity assay

Buffer: 0.1 M Na-phosphate buffer pH 6.5

Substrate: 2 mMpNPG in buffer – dissolve 60.25 mg of pNPG in 100 mL of buffer

Enzyme: Dilute enzyme in buffer to proper concentration

Table A.2 Reaction mixture for α-glucosidase activity assay

Reaction mixture	ES	EB	SB
Substrate (µL)	50	-	50
Enzyme (µL)	50	50	-
Buffer (µL)	-	50	50

Incubate the tubes containing reaction mixture at 37°C on shaking incubator for 10 min and terminate the reaction by adding 400 µL of 1M Na_2CO_3. Mix well by vortex and measure at absorbance 405 nm (A_{405}).

Calculation of α-glucosidase activity

Net absorbance (Abs) = ES-EB-SB

p-Nitrophenol (mmole/L or µmole/mL) = $\frac{NetAbs}{slope}$

$$Glucosidase\ activity\ (\frac{U}{mL}) = \frac{p-Nitrophenol\ (\mu mole/mL) \times 0.5(mL) \times 1000(\mu L/mL) \times 2(internal\ dilution\ factor)}{10(min) \times enzyme(mL)}$$

APPENDIX C

Total carbohydrate

Preparation of chemical

5% phenol solution: 5% Phenol in methanol: Dissolved 5 g phenol in 100 mL of methonol

Stock of glucose: Dissolve 2 g of glucose in 1 L of water

Preparation of calibration curve

Dilute stock of glucose solution to 20, 40, 60, 80 and 100 µg/mL with water. Pipette 0.25 mL of each concentration to test tubes. Carefully add 0.25 mL 5% phenol solution and mix thoroughly. Carefully add 1.25 mL concentrated H_2SO_4 and mix well and stand at room temperature for 30 min prior to measurement at absorbance 490 nm.

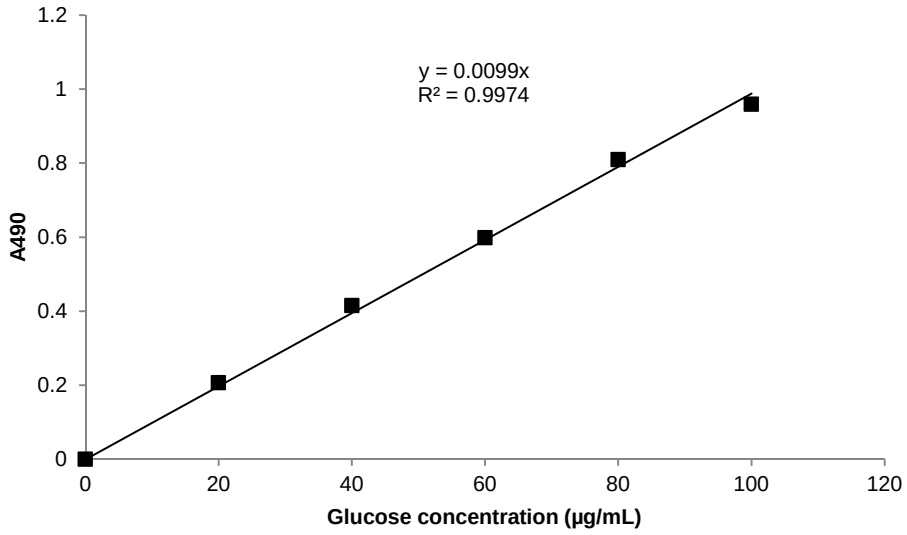

Figure A.3 Calibration curve of glucose concentration and absorbance at 490 nm

APPENDIX D

Electrophoresis

Preparation of polyacrylamide gel

SDS-PAGE

Chemical preparation

3.5x gel buffer: 1.25 M Bis-Tris pH 6.5 – Dissolve 26.15 g of Bis-Trisin water and adjust to pH 6.5 withHCl

10% APS – Dissolve 10 g of APS in 100 mL of water (fresh solution is necessary)

5x running buffer: Mix all components in 200 mL of water

250 mMBis-Tris - 10.46 g of Bis-Tris

250 mM MOPS - 10.45 g of MOPS

5 mM EDTA - 0.37 g of EDTA

0.5%(w/v) SDS - 1 g of SDS

Preparation of sample

Pipette 15-20 µL of sample in microtube with and equal volume of Laemmli buffer. Denature proteins by heating at 100^{o}C for 4 minutes and suddenly place it on ice.

Table A.3 Composition of gel for SDS-PAGE

Composition	Resolving gel	Stacking gel
4x gel buffer (mL)	1.5	0.8
Water (mL)	2	1.4
30% Acrylamide (mL)	1.75	0.6
10%APS (µL)	50	30
Temmed (µL)	5	4
Total volume (mL)	5.25	2.80

Running of gel electrophoresis

After assembling the SDS-PAGE equipment, fill 1x running buffer in SDS-PAGE chamber. The 30-40 µL of samples is loaded in each well of the gel. Set the voltage of 100 V and switch to 150 after the samples achieve the top of resolving gel until the blue border comes to the bottom of the gel.

Staining with Coomassie Brilliant Blue

Take out the gel from the glass plate and move it to a try containing water. Wash the gel on rotary shaker for 10 min. Remove the water and wash the gel twice with fresh water. Move the gel to a new tray containing Coomassie staining solution. Stain on rotary shaker for 60 min and destain by washing with buffer until protein bands are visualized.

Native PAGE

Chemical preparation

4x running gel buffer: Dissolve 12.11 g in 100 mL water and adjust to pH 8.8 with HCl

4x stacking gel buffer: Dissolve 12.11 g in 100 mL water and adjust to pH 6.8 with HCl

10x running buffer: Mix all components in 200 mL of water

250 mMTrizma Base	6.06	g
2 M Glycine	30	g

Sample buffer:

Glycerine	10	mL
10x running buffer	5	mL
Bromophenol Blue	0.25	g
Water	85	mL

Staining solution: Bio-Safe Coomassie, Bio-Rad

Destaining solution: Water

Table A.4 Composition of gel for native-PAGE

Composition	Resolving gel	Stacking gel
4x gel buffer (mL)	1.875	0.315
Water (mL)	3	1.725
30% Acrylamide (mL)	2.475	0.415
10%APS (µL)	62.1	25
Temmed (µL)	3	2.5
Total volume (mL)	7.5	2.5

APPENDIX E

Zymography (activity staining)

Wash the SDS or Native PAGE gel 3 times in cold solution of 20 mM Na-phosphate buffer pH 6.5 on rotary shaker for 10 min in order to remove SDS out of the gel. Immerse washed gel in 0.1%(w/v) soluble starch in the same buffer and incubate at 37^{o}C for 10 min. The active bands under dark purple background according to amylase activity are visualizedby flooding the gel with iodine solution. To stain α-glucosidase activity, the washed gel is immersed in 4 mM of 4-Methylelumberiferyl-α-D-glucopyranoside(MUGlu) in buffer and incubated at 37^{o}C for 10 min. The active bands are visualized under UV light in a few minute.

APPENDIX F

Thin layer chromotography (TLC)

Chemical preparation

TLC plate:

Mobile phase: n-Butanol: Ethanol: Water = 5:3:2 (v/v)

n-Butanol	50	mL
Ethanol	30	mL
Water	20	mL

Staining solution: 0.5%(w/v) thymol in 5%(v/v) sulfuric acid

Thymol	0.5	g
Sulfuric acid	5	mL
Water	95	mL

APPENDIX G

Colony PCR

Preparation of template

Pick single colony to microtube containing 30 µL of sterilized water and cook at 100^{o}C. After 10 min cooking, place the tube on ice. The cell suspension will be used as template in PCR reaction.

Preparation of master mix

Water	7.5	µL
PhusionHigh-Fidelity PCR master mix	12.5	µL
Forward primer	2	µL
Reverse primer	2	µL
Template	1	µL

APPENDIX H

Gene and plamid purification

The gene and plasmid are purified using illustraTM GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare Ltd., UK) and plasmids are isolated using PureYield^{TM} Plasmid Miniprep System (Promega Corp., Madison, WI) according to the instruction of manufacturer.

APPENDIX I

Digestion/restriction analysis

Single digestion

Vector/Gene (µL)	5	Vector/Gene	5
NdeI (µL)	0.5	XhoI (µL)	0.5
Buffer O (µL)	3	Buffer R (µL)	3
H_2O (µL)	21.5	H_2O (µL)	21.5
Total (µL)	30	Total (µL)	30

Vector/Gene (µL)	5	Vector/Gene	5
BsaI (µL)	0.5	NcoI (µL)	0.5
Buffer G (µL)	3	Buffer Tango (µL)	3
H_2O (µL)	21.5	H_2O (µL)	21.5
Total (µL)	30	Total (µL)	30

Vector/Gene (µL)	5	Vector/Gene	5
PstI (µL)	0.5	XmaI (µL)	0.5
Buffer O (µL)	3	Buffer XmaI (µL)	3
H_2O (µL)	21.5	H_2O (µL)	21.5
Total (µL)	30	Total (µL)	30

Double digestion

Vector/Gene (µL)	5	Vector/Gene	5
NdeI (µL)	0.5	NcoI (µL)	0.5
XhoI (µL)	1	XhoI (µL)	0.5
Buffer O (µL)	3	Buffer Tango (µL)	6
H_2O (µL)	20.5	H_2O (µL)	18
Total (µL)	30	Total (µL)	30

Vector/Gene (µL)	5	Vector/Gene	5
XmaI(µL)	0.5	BsaI (µL)	0.5
PstI (µL)	1	XhoI (µL)	1
Buffer XmaI (µL)	3	Buffer G (µL)	3
H_2O (µL)	20.5	H_2O (µL)	20.5
Total (µL)	30	Total (µL)	30

Incubate the reaction at 37^{o}C for 12-16 h and stop reaction by heating at 65^{o}C for 20 min.

APPENDIX J

Ligation

Ligation mixture

Insert (µL)	4
Vector (µL)	12
Buffer for T4 ligase (µL)	2
T4 ligase (µL)	2
Total (µL)	20

The amount of insert and vector can be calculated by the following equation;

$$\textbf{\textit{Amount of insert}}\ (\textbf{\textit{ng}}) = plasmid\ concentration\ \left(\frac{ng}{\mu L}\right) \times volume\ (\mu L) \times \frac{gene\ size\ (bp)}{plasmid\ size\ (bp)} \times 3$$

APPENDIX K

Preparation of electrocompetent cells

Preparation of electro competent *E. coli*

1. Culture *E. coli* in LB medium on 125 rpm rotary shaker at 37^{o}C overnight.

2. Transferred overnight culture in 2x300 mL LB medium and incubate on 125 rpm rotary shaker at 37^{o}C until OD_{600} ~0.6-0.7 (This is the mid log phase of *E. coli*).

3. Prepare ice-cold centrifuge tube and harvest the cell by centrifugation at 4000 rpm, 4^{o}C for 15 min.

4. Carefully pour off and discard the entire supernatant (to remove all salts in the medium)

5. Gently resuspend the pellet in 500 mL ice cold 10%glycerol (do not vortex) and centrifuge at 4000 rpm, 4^{o}C for 15 min. Carefully pour off and discard the entire supernatant

6. Gently resuspend the pellet in 250 mL ice cold 10%glycerol (do not vortex) and centrifuge at 4000 rpm, 4^{o}C for 15 min. Carefully pour off and discard the entire supernatant.

7. Gently resuspend the pellet in 20 mL ice cold 10%glycerol (do not vortex) and centrifuge at 4000 rpm, 4^{o}C for 15 min. Carefully pour off and discard the entire supernatant.

8. Gently resuspend the pellet in 1 mL ice cold 10%glycerol.

9. Aliquot 50 µL in ice-cold microtube and freeze it immediately by liquid N_2 and keep it at -80^{o}C (The cell concentration should be 1-3x10^{10} cells/mL)

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Preparation of electro competent *Lactobacillus plantarum*

1. Culture *L. plantarum* in MRS medium at 37^{o}C overnight.

2. Prepare serial 10 fold-dilutions from 10^{-2}-10^{-8} in MRS broth containing 1% glycine and incubate at 37^{o}C overnight (12-14 h).

3. Dilute 1:20 in fresh MRS medium containing 1% glycine and incubate at 37^{o}C until OD600~0.7.

4. Place the culture on ice for 10 min and carefully pour it in ice-cold centrifuge tube.

5. Harvest the cell by centrifugation at 4500 rpm, 4^{o}C for 10 min.

6. Resuspend the pellet in 0.5-1 culture volume of ice-cold 30% PEG-1450.

7. Keep the cell suspension on ice for 10 min before centrifuge at 4500 rpm, 4^{o}C for 10 min.

8. Resuspend the pellet in 1:50-1:100 culture volume of ice-cold 30% PEG-1450.

9. Aliquot 40 µL in ice-cold microtube and freeze it immediately by liquid N_2 and keep it at -80^{o}C.

APPENDIX L

Transformation

Electroporation for *E. coli* competent cells

1. Thaw frozen electro competent cell on ice for 15 min

2. Pipette 5 µL of purified ligation mixture into electro competent cell and gently stir

3. Incubate on ice for 10 min

4. Pipette the mixture in dry electroporation cuvette. Remove the air bubbles by knocking on the table. Place the cuvette on ice prior to electrotransformation.

5. Set electroporator as follow;

Voltage: 1.8 kV

Capitance: 25 µF

Resistance: 400 Ω

Remove all liquid on cuvette and put it on pulser

5. Give electric pulse.

6. Resuspend the cell with 500 µL of SOC medium

7. Incubate at 37^{o}C for 1 h.

8. Spread culture on MRS agar with suitable antibiotic.

9. Incubate at 37^{o}C for 24 h.

Electroporation for *L. plantarum* competent cells

1. Thaw frozen electro competent cell on ice for 15 min

2. Pipette 5 µL of purified ligation mixture into electro competent cell and gently stir

3. Incubate on ice for 10 min

4. Pipette the mixture in dry electroporation cuvette. Remove the air bubbles by knocking on the table. Place the cuvette on ice prior to electrotransformation.

5. Set electroporator as follow;

Voltage: 1.5 kV

Capitance: 25 µF

Resistance: 400 Ω

Remove all liquid on cuvette and put it on pulser

5. Give electric pulse

6. Resuspend the cell with 500 µL of MRS broth containing 0.5 M sucrose and 0.1 M $MgCl_2$

7. Incubate at 37^{o}C for 1-2 h

8. Spread culture on MRS agar with suitable antibiotic

9. Incubate at 37^{o}C for 1-2 days

APPENDIX M

Lineweaver burk and Michaelis-menten plots

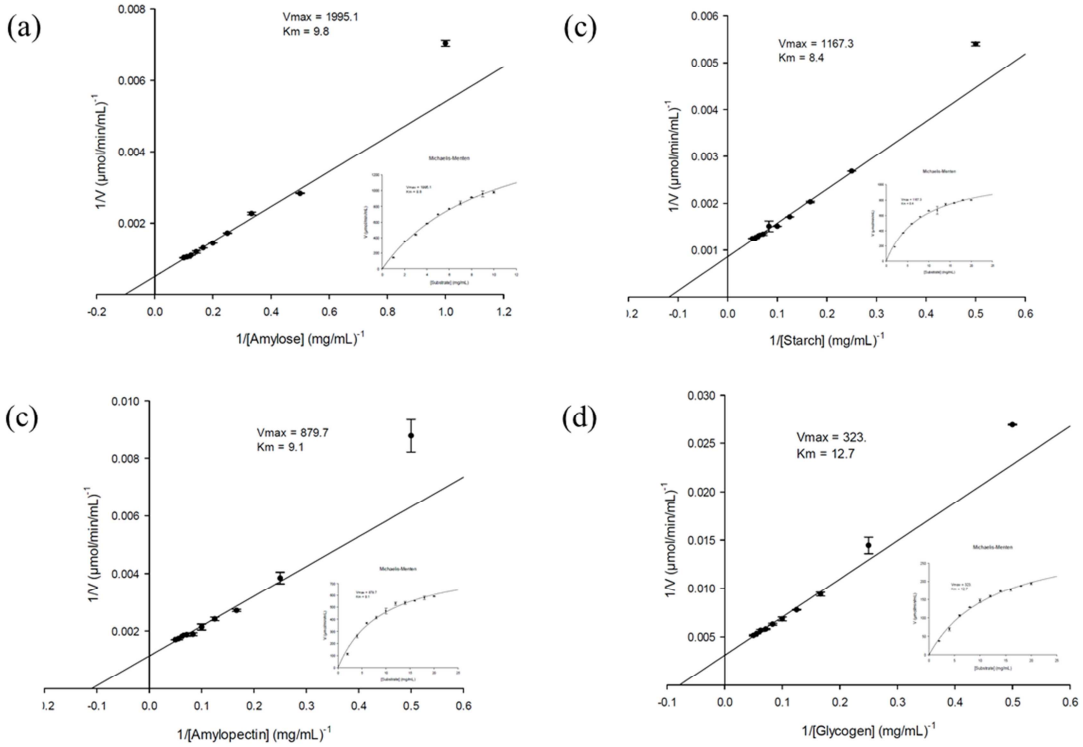

Figure A.4 Lineweaver-Burk and Michaelis-Menten plot of purified α-amylase from *L. plantarum* S21 towards starch (a), amylose (b), amylopectin (c), and glycogen (d)

APPENDIX N

Chromatogram of hydrolysis products

from *L. plantarum* S21 α-amylase

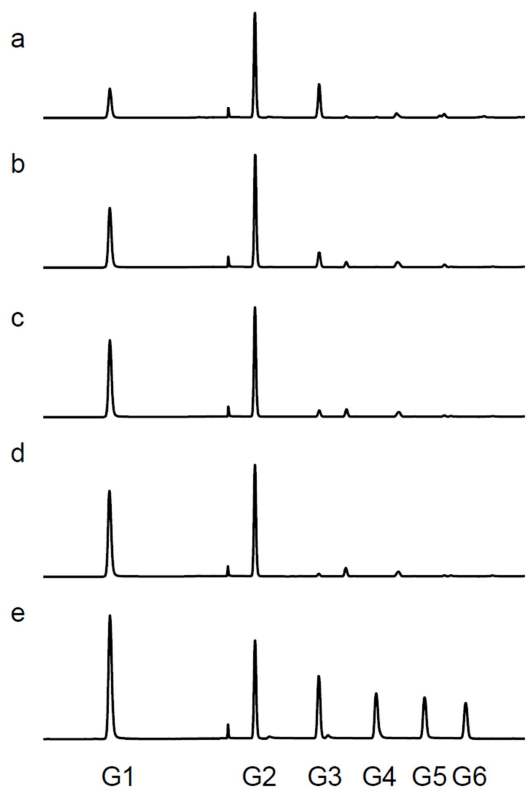

Figure A.5 Chromatogram of starch hydrolysis product at 12 h (a), 24 h (b), 48 h (c) and 72 h (d) compared to standard maltooligosaccharides (G1-G6)

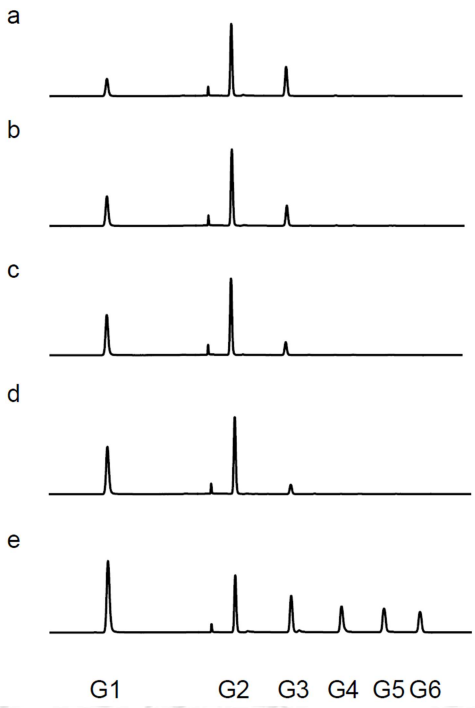

Figure A.6 Chromatogram of amylose hydrolysis product at 12 h (a), 24 h (b), 48 h (c) and 72 h (d) compared to standard maltooligosaccharides (G1-G6)

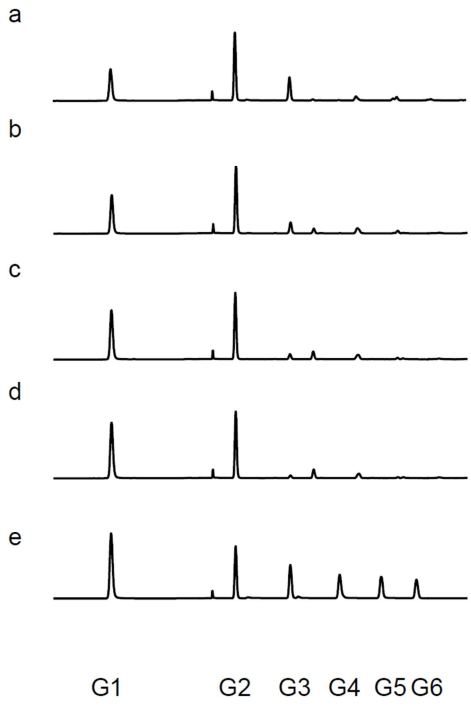

Figure A.7 Chromatogram of starch hydrolysis product at 12 h (a), 24 h (b), 48 h (c) and 72 h (d) compared to standard maltooligosaccharides (G1-G6)

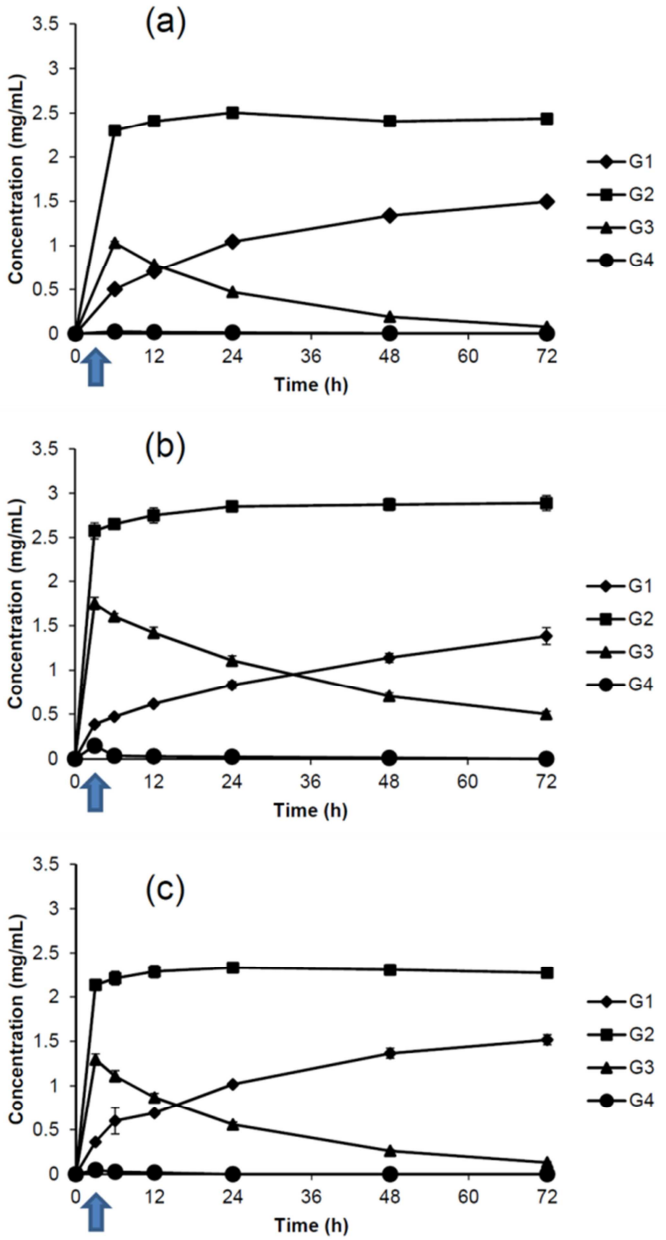

Figure A.8 Profiles of hydrolysis products from starch (a), amylose (b) and amylopectin (c)

CURRICULUM VITAE

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Conferences

Kanpiengjai, A., Khanongnuch, C. Proceeding and poster presentation on the topic of "Optimal conditions for phytase production from thermotolerant bacteria isolated from hot spring". The 2nd National Graduate Research Conference, Assumption University, Bangkok, Thailand, 12-14 June 2008.

Rientrakoonchai, W., **Kanpiengjai, A.,** Khanongnuch, C. Proceeding and oral presentation on the topic of "Screening of amylolytic lactic acid bacteria for lactic acid production from starch". The 6th International Naresuan Research Conference, Naresuan University, Pitsanulok, Thailand, 29-30 July 2010.

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Publications

Kanpiengjai, A., Unban, K., Khanongnuch, C. (2013). Optimal medium and conditions for phytase production by thermophilic bacterium, *Anoxybacillus* sp. MHW14. *Food and Applied Bioscience*. (In press).

Kanpiengjai A., Lumyong S., Pathom-aree W., Khanongnuch, C. (2014). Starchy effluent from rice noodle manufacturing process as feasible substrate for direct lactic acid production by *Lactobacillus plantarum* S21. *Journal of Korean Society for Applied Biological Chemistry*. doi 10.1007/s13765-013-4311-2.

Kanpiengjai A., Lumyong S., Pathom-aree W., Khanongnuch, C. (2014). High efficacy bioconversion of starch to lactic acid using amylolytic lactic acid bacterium isolated from Thai

indigenous fermented rice noodle. *Journal of Food Science and Biotechnology* (Inpress).