The Study of Naringin Reduction by *Lactobacillus Plantarum* ATCC 8014

By

Ms. Supiriya Sriwong
ID: 441-8982

A special project submitted to the Faculty of Biotechnology,
Assumption University in part of fulfillment of the requirement
for the degree of Bachelor of Science

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By	: Ms. Supiriya Sriwong
Advisor	: A. Chotirote Seeanukun
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Level of study	: Bachelor of Science
Department	: Agro-Industry
Faculty	: Biotechnology
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Abstract

Naringin is the main bitter component of citrus juices. It tastes threshold in water is approximately 20 ppm, but 1.5 ppm levels may be detected. In this experiment, were designed by using Lactobacillus plantarum ATCC 8014. Lactobacillus plantarum ATCC 8014 was lactic acid bacteria, contained α-l-rhamnosidase protein that hydrolyzes naringin. The level of naringin concentration was detected by Davis test at 420 nm. The first part was varying concentration of narigin to 80, 120, 160 mg/ml which study the suitable concentration for Lactobacillus plantarum ATCC 8014 to reduced naringin. The result shows that 160mg/ml naringin, the naringin was reduced at 20, and 24 hours by *Lactobacillus plantarum* ATCC 8014. The second part was control which studies the effect of media, naringin and Lactobacillus plantarum ATCC 8014 with Davis test. The result shows that nothing interfered to Davis test when detected.

Acknowledgement

I would like to express my sincere thanks to my advisor, A.Chotirote and co-advisor A.Waniya, in the faculty of Biotechnology, Assumption University. For her kind and constant help, suggestion, encouragement, valuable guidance and help me to solve many problem.
I also would like to thank the officers and technician of the Faculty for their supports and general assistance in the completion of this project. Finally, I would like to thank my friends and student member for help and kindness.

Ms. Supiriya S.

May 4, 2004

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INTRODUCTION

The processing of citrus juice has faced formidable problems in terms of bitterness and delayed bitterness, thereby affecting its consumer acceptability. All the processed citrus fruit juice contains naringin which attributes bitterness to the juices. Because of the various drawbacks, the capabilities of non-enzymatic debittering technologies are limited. A suitable debittering procedure is enzymatic reaction.

Naringin is the main bitter component of several citrus juices; the use of rhamnosidase to hydrolyze the naringin content of these juices, with a concomitant decrease in the bitterness, is a common industrial practice. Since prunin bitterness is less than one third of that of naringin, only the first hydrolyzing activity of α-L-rhamnosidase is, in fact, essential for bitterness removal. Thus, the specific measurement of the α-L-rhamnosidase activity of naringinase is of crucial importance to assay the "debitterizing power". The most common method used to measure the naringin level is the Davis method. And it is the direct method to measure the naringin in product after α-L-rhamnosidase activity.

Therefore, the study of naringin reduction is useful on many sides especially for the citrus juice industry. Thailand has a lot of oranges so, if we can reduce the bitter taste, it will become commercially useful.

OBJECTIVES

1. To reduce the bitter compound, naringin, by *Lactobacillus plantarum* ATCC 8014.
2. To study the naringin reduction by *Lactobacillus plantarum* ATCC 8014 in various phase of growth.
3. To determine the condition to reduce naringin by *Lactobacillus plantarum* ATCC 8014.

LITERATURE REVIEW

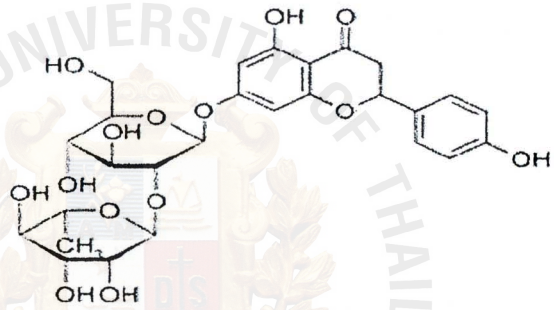
1. Naringin

Synonyms : 4',5,7-Trihydroxyflavanone 7-rhamnoglucoside / Naringenine-7-rhamnosidoglucoside

Molecular Formula : $C_{27}H_{32}O_{14}$

Molecular Weight : 580.53

Naringin structure :



Melting point : 166 °C

Solubility : dissolves in hot water

Structural class : flavanone glucoside

Appearance : white powder

Naringin is the main bitter component of several citrus juices and are found in all parts of citrus juice and bitter fruits. It tastes threshold in water is approximately 20 ppm, but 1.5 ppm levels may be detected. The presence of this bitter taste has been a major limitation in commercial acceptance of citrus juice. To improve the sensory properties and to stabilize finished fruit juice, the naringin level can be decreased by industrial juices treatment.

1.1 Technology for reducing the bittering compounds

The naringin level can be reduced by different technologies such as adsorptive debittering (Griffith, 1969; Johnson and Chandler, 1988), treatment with polystyrene divinyl benzene styrene (DVB) resin (Kimball, 1991; Puri 1984), and β-cyclodextrin treatment (Shaw and Wilson, 1983; Wagner et al., 1988). These technologies have the following limitation: (1) the juice must be previously deoiled, dewaxed, depulped, and then reblended with the clarified debittered juice; (2) adsorption columns are usually regenerated with dilute alkali solution and this may affect organoleptic properties and final quality of juice; (3) the methods may alter the composition of the juice either through chemical reaction(s) or removal of nutrients, flavor, color, etc.; (4) the methods are nonspecific, inherently inefficient, and they introduce batch-to-batch variation because of non-monitorable changes; (5) the methods of extraction and finishing affect the yield, quality, and characteristics of citrus juice produced. Methods such as the acid hydrolysis of naringin produce not only rhamnose and glucose but also the bitter aglycon and naringenin. Therefore, acid hydrolysis is not suited to commercial processing. Similarly, under selected conditions of pH and temperature, activated charcoal may almost completely remove naringin from a solution; however, many of the desirable flavoring components are simultaneously removed. Because of different drawbacks, nonenzymatic debittering technologies are limited and an alternative procedure could be the hydrolysis of naringin by naringinase enzymes.

1.2 Determination of naringin content

The procedures are based on the spectrophotometric determination of flavonones according to the alkaline diethylene glycol method of Davis (1947). The naringin reacts with the reagent to produce a yellow color that is measured at a wavelength of 420 nm. A high performance liquid chromatographic (HPLC) determination has also been used for naringinase activity (Horuichi et al., 1985). The HPLC method determines activity of the enzyme by measuring changes in concentration of α-rhamnoside. Romero et al. (1985) used p-nitrophenyl-L-rhamnopyranoside for the measurement of L-rhamnosidase activity of naringinase, by colorimetrically following the appearance of p-nitrophenol. The use of a synthetic substrate did not affect the pH, temperature, and ionic strength optima of the enzyme. Using naringin as substrate, the naringinase activity was determined by HPLC on C18μ Bondapak reverse-phase column and detection of absorbance at 280 nm.

1.3. Rhamnosidase

α-L–rhamnosidase is an enzyme that catalyzes the hydrolysis of rhamnosidases and the release of L-rhamnose from substrates. Enzymes specifically acting on plant glycosides have been isolated from animals, plants, fungi, and bacteria and characterized.

$$\text{Naringin} \xrightarrow{\alpha\text{- L – rhamnosidase}} \text{prunin} + \text{rhamnose}$$

The rhamnosidase activity of naringinase is considered suitable for aroma enhancement in wine making. In one study, the enzymes were

immobilized to a solid carrier with the aim of developing a continuous process for wine aroma development.

2. *Lactobacillus plantarum*

2.1 Characteristics

- Gram-positive
- Facultative
- Non-spore-forming rod
- Occurring singly, in pairs or in short chains.
- Catalase – negative
- Produces lactic acid as a major fermentation product from glucose.
- It is microaerophylic, when cultivated in liquid media (MRS), this strain forms turbidity and sediments.
- Straight rods with rounded ends, 0.9-1.2 by 3.0-8.0 μm
- Microscopically, cells consist of short to long rods that appear as single cells, in pairs and in short chains.
- Surface colonies on agar plates are 0.5 to 2.0 mm in diameter, circular, lenticular, creamy-white.
- It grows at temperatures ranging from 37°C to 42°C. Acid is produced without gas formation from arabinose, ribose, sorbitol, galactose, dextrin, dextran, mannose, glucose, maltose, sucrose, fructose, mannitol and lactose.
- Optimum pH 5.0-7.0
- No acid formation from: sorbose, raffinose, xylose, starch was detected.
- Acid production from rhamnose is variable.

- Strains are tolerant up to 8.0% NaCl and present multiple resistance to antibiotics.

2.2 Occurrences

- Strains were isolated from dairy products and environments, silage, sauerkraut, pickled vegetables, sourdough, cowdung, and the in human mouth, intestinal tract and stools, and from sewage.
- *Lactobacillus plantarum* is used as starter organism some fermented sausage and cereal product; it is a part of adventitious Lactic Acid Bacteria growing in fermented vegetable and meat products and it is a spoilage organism in citrus juice, wine and some cheese.

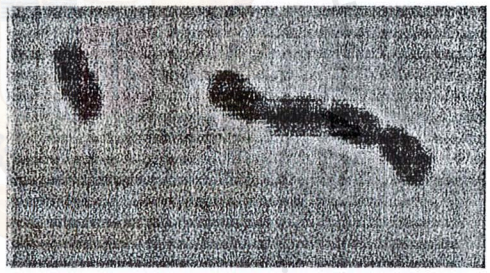


Figure 1: straight rods of *Lactobacillus Plantarum*

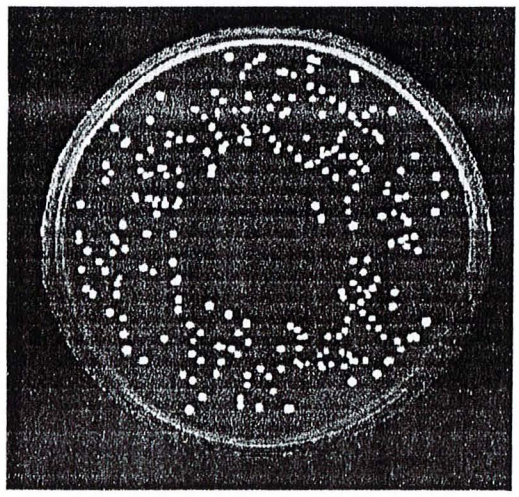


Figure 2: *Lactobacillus plantarum* on MRS agar

2.3 Rhamnosidase and *Lactobacillus plantarum*

- According to National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov), The Protein sequences of *Lactobacillus plantarum* which encoded with alpha -L-rhamnosidase are revealed below:

CDS /gene="ram1"
/product="alpha-L-rhamnosidase (putative)"
/EC_number="3.2.1.40"
/protein_id="CAD65558.1"

/translation="MLMSKEAVWLWYPGDFEIHQGMLQNFKREERGMG
WPAYWYIDDCNRNVNFKRHYDLKESTQFTVLAKGTGYVDVNGT
KYRLNHAINCDAGATDIQVFVGNVQGLPTIYIVGEIIKSDSGWLAS
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TATTKVRKRAFRYVFVPHCQLGDIELTAMHEYIPKNNPSSFTSDN
KLINQIWNVATETLNLCSDLFFIDGIKRDRWIWAGDAYQANFINQ
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/product="alpha-L-rhamnosidase (putative)"

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/protein_id="CAD65560.1"

/translation="MEEMAFTFQINNDVQFQHNQALLDKSASYRPILKET
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GDHQVGQFSININAVGSPMDAPLCFKIKFAEMPAELARKSEDYDG
WLSKSWIQEETVHLDVLPTTLTLPRRYSFRYAEITVVDTSPKWRA
VFSNPVVTATSAVDTATVHQPELADVQLQRIYEVGLKTLADCMQ
DVFEDGPKRDRRLWIGDLRLQALANYATFKDTDLVKRCLYLFGA
MPTTAGRIPANVFTKPTAVPDDTFLFDYSLFFISILADYEAFSSDKT
VLNDLYRVAKKQMDLALEQVTSEGKLKLTEENPVFIDWSNDFDK
ETAGQAIIIYTLKQFITLAELVNDTSLETYTAILRKLNQYAKTQLFD
SQSGLFVSGDQREVNVASQVWMTLAHVLDPEQTTVLMQTTVTK
LFPITGIATPYMYHHITEALFEAGLKQEAVQLMKDYWGKMVTLG
ADTYWEAFDPNQPDYSPYGSPILNSYCHAWSCTPVYLINKYLV"

RESEARCH METHODOLOGY

I. Equipments:

1. Incubator (Jouan.EB 280)
2. Autoclave (Hirayama Hicitve^{TM})
3. Top-load balance (Ohause, Precision Advance, GT2100)
4. Analytical balance (Ohause, Analytical Plus AP 210S)
5. Micropipette
6. Spectrophotometer (Spectronic Genesys 5 Milton Roy company made in USA)
7. Refrigerator
8. Centrifuge (Hermle Z 230A)
9. Glassware and lab utensils

II. Reagents:

1. Naringin (from SM Company)
2. Diethylene glycol
3. Sodium hydroxide
4. Man-Rogosa-Sharpe Agar (MRS Agar)
5. Man-Rogosa-Sharpe Broth (MRS Broth)

III. Experimental Procedures

1. Preparation of stock culture

- Pipette 1 ml of cell suspension into a sterile microfuge tube.
- Add 500 µl of 45%glycerol.
- Keep it in the refrigerator freezer.

2. Preparation of naringin stock

- Dissolve 1000 mg of naringin in 10 ml of distilled water. (10mg/ml concentration)

3. Standard curve of naringin

- Prepare a stock solution by dissolving 200 mg of naringin in 100 ml of distilled water.
- Make five standards by pipetting, or by using a graduated cylinder to transfer, 5.0, 12.5, 25.0, 37.5, and 50.0 ml of the stock solution into each of five 100 ml volumetric flasks, filling them to the mark to obtain 10, 25, 50, 75, and 100 mg/ml standard solutions.
- To each of five test tubes add 10 ml of the diethylene glycol, and then add 0.1 ml of each standard to separate glycol-containing test tubes.
- Add 0.1 ml of the NaOH solution to each standard-containing test tube, and after 15 minutes measure the absorbance at 420 nm using the spectrophotometer.

- Plots the absorbance versus mg naringin/100 ml (10mg/100ml) naringin.

4. Culture condition

- *Lactobacillus plantarum* is added into MRS broth and incubate overnight at 37 °C.
- 2% of overnight cultures and 2, 3, and 4 ml of naringin stocks are adding into each 250 ml MRS broth incubate at 37 °C and collect the sample at 0, 4, 8, 16, 20, 24 hour(s).
- Centrifuge the samples at 5,000 rpm and collect the supernatant.
- Repeat the lab four times.

5. Use Davis Test to measure the level of naringin

- Add 10 ml of diethylene glycol to a test tube along with 0.1 ml of naringin in distilled water.
- Measure the sample's absorbance on the spectrophotometer at 420 nm, comparing it to distilled water blank.
- The naringin content can be found by comparing the absorbance to a graph of absorbance versus naringin concentration values, C, or by calculation using Bear's law:

$$\text{Absorbance} = kC$$

- The standard graph or the factor k can be found from the standard curve of naringin.

RESULT AND DISCUSSION

To reduce the naringin, which was the main bitter component in several citrus juices, the experiments were designed by varying the addition of naringin concentrations in MRS broth, which contained *Lactobacillus plantarum* ATCC8014. *Lactobacillus plantarum* ATCC 8014 was lactic acid bacteria found in acid-fermented product. It contained α-L–rhamnosidase protein that hydrolyzes naringin. The level of naringin concentration was detected by Davis test at 420 nm. If the α-L–rhamnosidase reduced naringin content, the level of naringin after inoculate *Lactobacillus plantarum* ATCC 8014 would be reduced.

1. Standard curve

The standard curve of Absorbance Vs naringin concentration obtained from Davis test was shown in figure 3

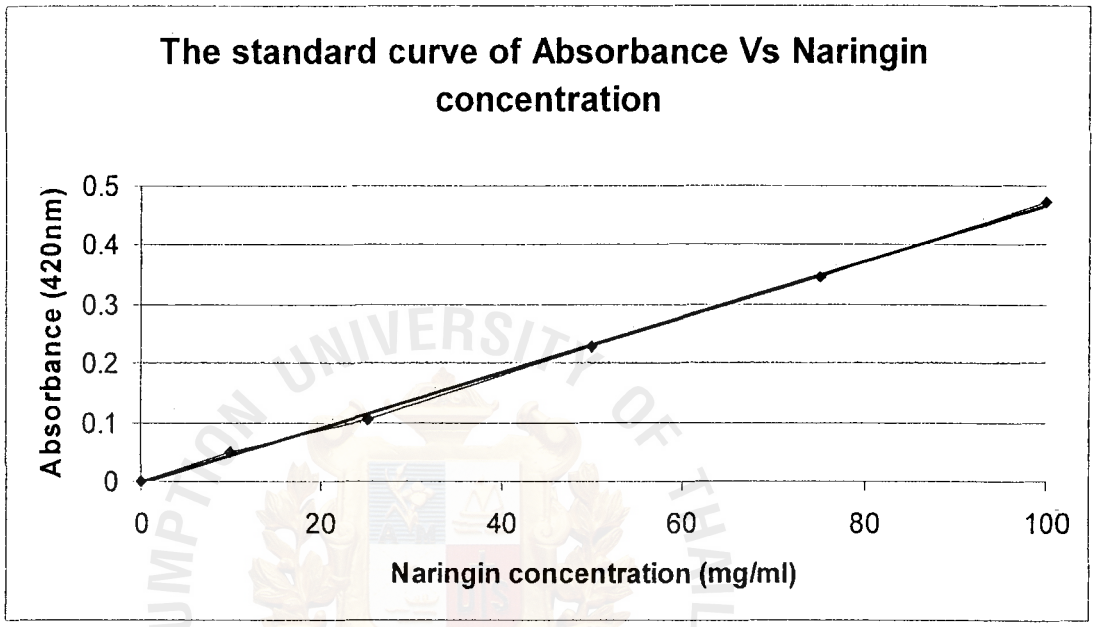

Figure 3: The standard curve of Absorbance 420 nm Vs naringin concentration.

The linear of regression (R^2) was 0.9988. The data and the conversion method from Absorbance at 420 nm to naringin concentration (mg/ml) were shown in appendix A.

2. Naringin reduction by various additional naringin concentrations in different growth phases.

2.1 The level of naringin in various growth phases when added 80 mg/ml Naringin in MRS broth

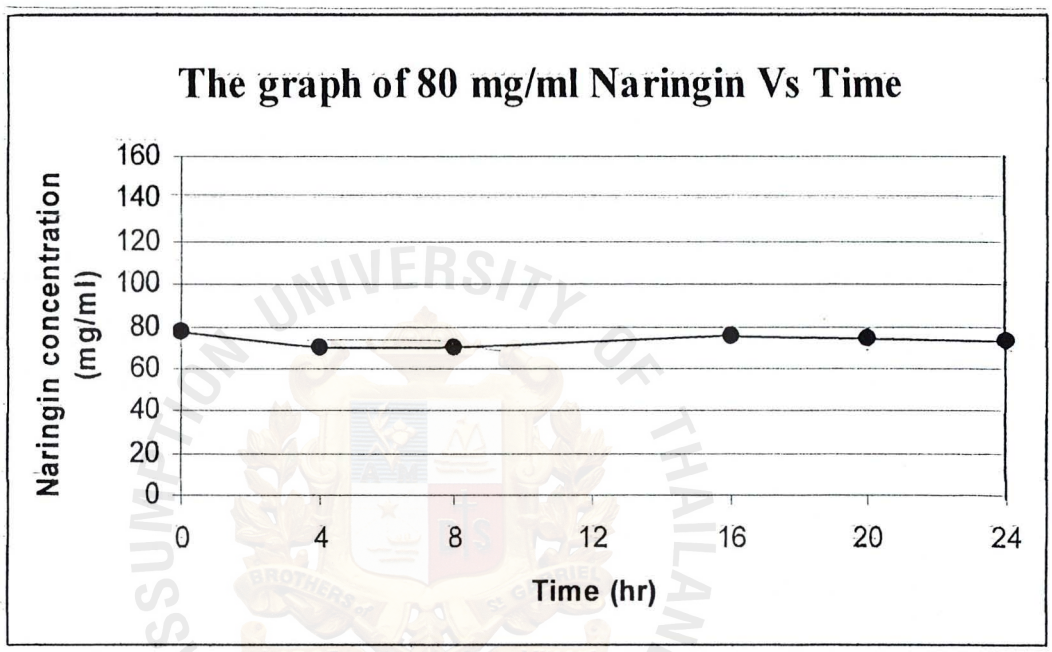

Figure 4: The graph of 80 mg/ml naringin concentration in MRS broth with *Lactobacillus plantarum* ATCC 8014.

In this experiment, the 80mg/ml naringin was added in MRS broth containing *Lactobacillus plantarum* ATCC 8014 to study the reduction of naringin in 0, 4, 8, 12, 16, 20, and 24 hour(s). The experiments were conducted in four replications.

From scheffe's test (The table was shown in Appendix A.), there was no significant difference among treatments ($p > 0.05$) that meant there was no difference between naringin in each treatment time.

The naringin was not reduced during 0 to 24 hours. The reason might be the 80mg/ml naringin was not inducing more α-L–rhamnosidase to degrade naringin in the culture during various phases of growth.

2.2 The level of Naringin in various growth phase when added 120 mg/ml Naringin in MRS broth.

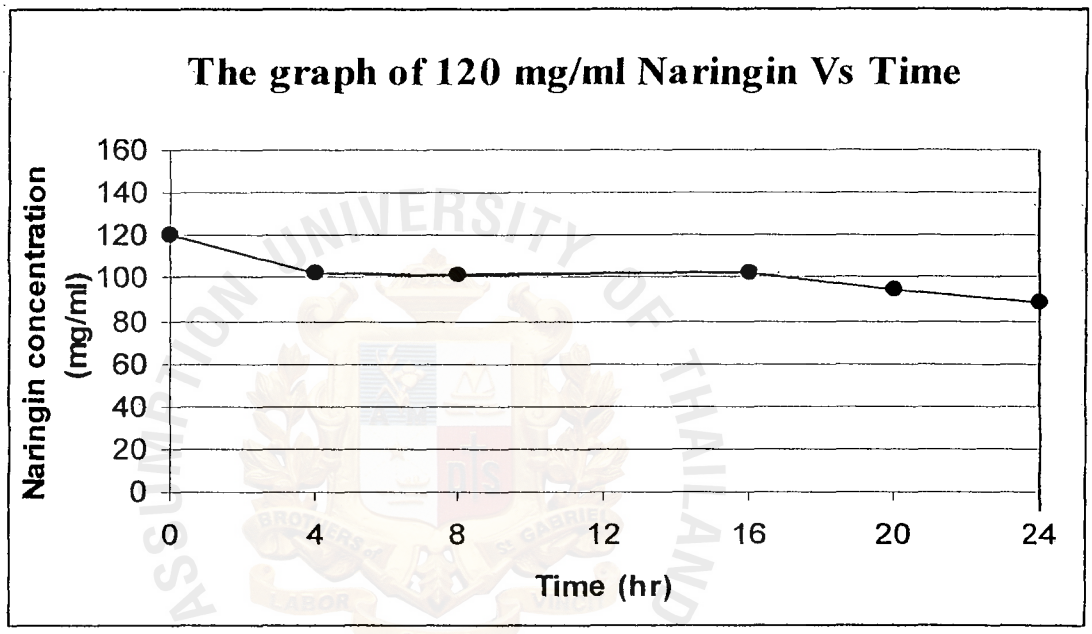

Figure 5: The graph of 120 mg/ml naringin concentration in MRS broth with *Lactobacillus plantarum* ATCC 8014 Vs Time.

In this experiment, the 120 mg/ml naringin was added in MRS broth containing *Lactobacillus plantarum* ATCC 8014 to study the reduction of naringin in 0, 4, 8, 16, 20, and 24 hour(s). The experiments were conducted in four replications.

From scheffe' 's test (was shown in Appendix A), there was no significant difference among treatments ($p > 0.05$) that meant there was

no different between naringin in each treatment time. The naringin was not reduced during 0 to 24 hours. The 120mg/ml naringin was not inducing more α-L–rhamnosidase to degrade naringin in the culture during various phases of growth.

2.3 The level of Naringin in various growth phases when added 160mg/ml Naringin in MRS broth.

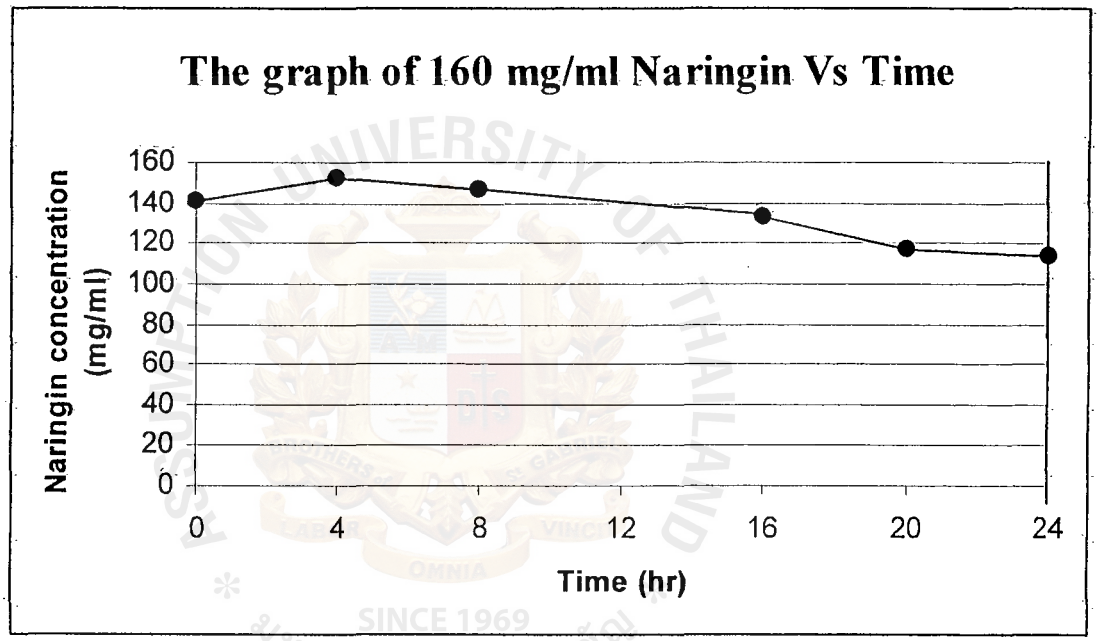

Figure 6: The graph of 160 mg/ml Naringin concentration in MRS broth with *Lactobacillus plantarum* ATCC 8014 Vs Time.

In this experiment, the 160 mg/ml naringin was added in MRS broth containing *Lactobacillus plantarum* ATCC 8014 to study the naringin reduction in 0, 4, 8, 16, 20, and 24 hour(s). The experiments were obtained in four replications.

From Duncan's New Multiple's Range Test (DMRT) (The tables were shown in Appendix A), there was significant difference among

treatments ($p<0.05$). It indicated that there were different between naringin concentrations in each treatment time.

The table in Appendix A from the DMRT test also revealed the 0 hour and less than 4 hours were different. It might be error from the practical laboratory. The solution samples were from the solid particles and they needed to completely melt before Davis test assay. The 4 hours and 8 hours were similar. The 8 hours and 16 hours were not different.

However, the 20 hours and 24 hours were different from 0, 4, 8, 16 hour(s). And both of them were not different.

2.4 Comparison of the different naringin concentrations by using SPSS programme version 12.0.

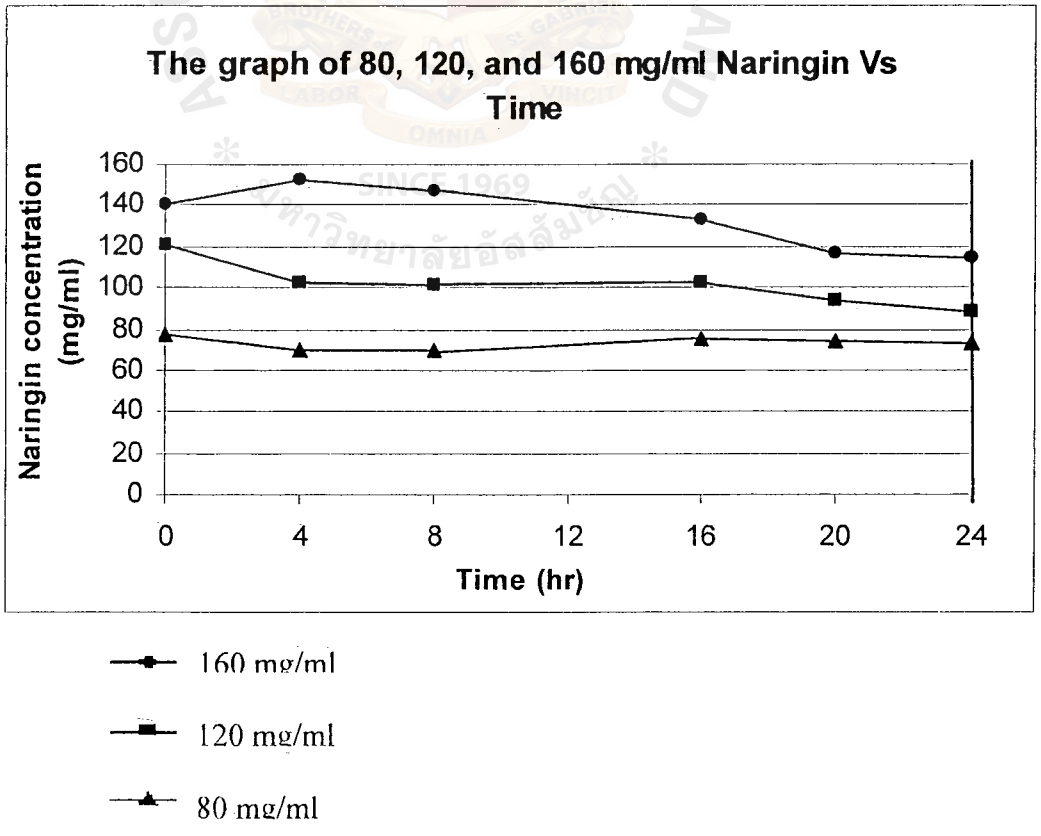

Figure 7: The graph of 80, 120, 160 mg/ml naringin concentration in MRS broth with *Lactobacillus plantarum* ATCC 8014 Vs Time.

To compare various concentrations, the two factors of time and concentration were analyzed by SPSS programmed version 12.0. From Duncan's New Multiple's Range Test (DMRT) (the tables were shown in Appendix A), there were significant difference in each concentration ($p<0.05$) and time ($p<0.05$) it meant 80, 120 and 160 mg/ml and at 0, 4, 8, 16, 20, 24 hours were different. Furthermore, the table in Appendix A revealed that there was no significant difference ($p>0.05$) in each replication.

From the statistics analysis, the 160 mg/ml was reduced 19.07% the naringin whereas the 80 and 120mg/ml were not reduced naringin. So, the conditions suitable for α-L–rhamnosidase induction were 160 mg/ml for 20 hours. Therefore, no need to grow *Lactobacillus plantarum* ATCC 8014 until 24 hours because there was no significant difference between times at 20 hours and 24 hours (From the result of 2.3 and Appendix A).

3. The factors affecting the naringin reduction

3.1 The naringin was added in MRS broth without containing *Lactobacillus plantarum* ATCC 8014.

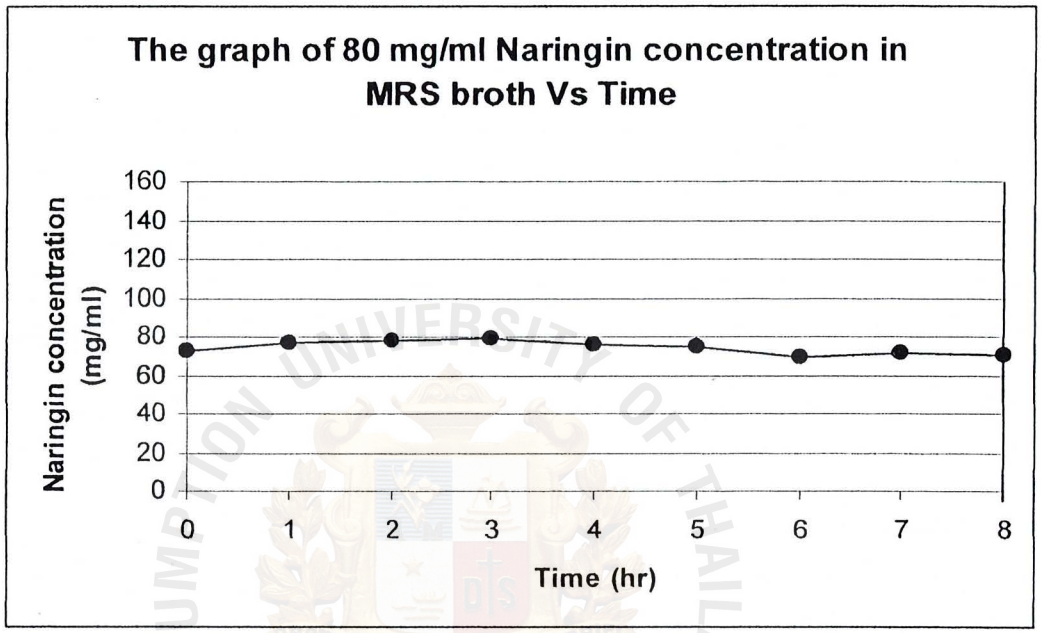

Figure 8: The graph of 80mg/ml naringin concentration in MRS broth without *Lactobacillus plantarum* ATCC 8014 Vs Time.

To study the naringin reduction in the 160 mg/ml did not result from the media reacted with naringin. The experiment was designed to add 80 mg/ml naringin in MRS broth without inoculating *Lactobacillus plantarum* ATCC 8014.

The results were analyzed by scheffe' 's test, and revealed that there was no significant difference among treatments ($p > 0.05$) that meant there was no different between naringin concentrations in each hour. The results were obtained from three replications. Naringin and MRS broth

was neither reduces nor increases when the time changed. Thus, the naringin reduction of 160 mg/ml did not result from MRS broth react to naringin. However, this experiment must be tested in longer time. Furthermore, the method of naringin assay should be changed to HPLC. The Davis test was not much accurately compared to HPLC.

3.2 MRS broth containing *Lactobacillus plantarum* ATCC 8014 without naringin addition.

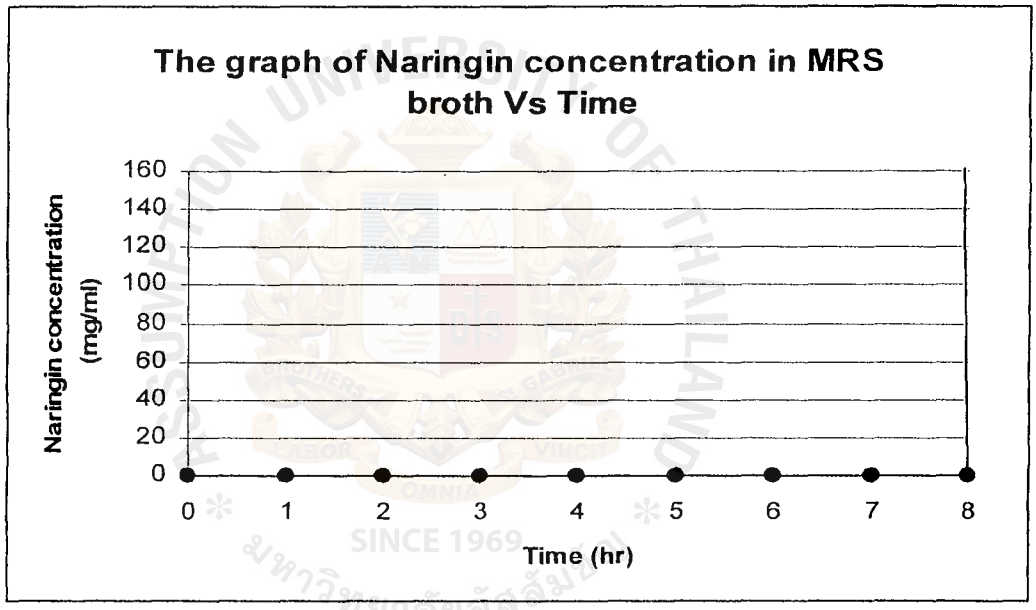

Figure 9: The graph of naringin concentration in MRS broth Vs Time.

To study the naringin reduction in the 160 mg/ml did not result from *Lactobacillus plantarum* ATCC 8014 and MRS broth. The experiment was designed to inoculate *Lactobacillus plantarum* ATCC 8014 into MRS broth without naringin addition.

The results were analyzed by scheffe' 's test, and revealed that there was no significant different among treatments ($p > 0.05$) that meant there was no different between naringin concentrations in each hour. The results were conducted from three replications. Naringin and MRS broth was neither reduce nor increase when the time changed. Thus, the naringin reduction of 160 mg/ml did not result from MRS broth and the culture. There was no effect from MRS broth and culture interfere Davis test.

CONCLUSION

1. At 80mg/ml and 120mg/ml naringin, the naringin was not changed by *Lactobacillus plantarum* ATCC 8014 during 0 to 24 hour(s).
2. At 160mg/ml naringin, the naringin was reduced at 20, and 24 hours by *Lactobacillus plantarum* ATCC 8014.
3. To produce α-L–rhamnosidase at 160mg/ml naringin, the 20 hours was better than 24 hours due to there was no significant different in both.
4. The naringin reduction did not result from the media reacted with naringin.
5. The naringin reduction did not result from *Lactobacillus plantarum* ATCC 8014 and MRS broth.
6. MRS was not interfered to Davis test when detected. Because of the amount of naringin that can detect was not change.

SUGGESTION

1. *Lactobacillus plantarum* ATCC 8014 should be used in high concentration of naringin in order to induce more α-L–rhamnosidase production to be used in other applications.
2. The method should be accurately analyzed by HPLC.

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APPENDIX A

1. Standard curve

Table 1 Naringin concentration (mg/ml) and Absorbance (420 nm)

Naringin concentration (mg/ml)	Absorbance (420 nm)
0	0
10	0.048
25	0.104
50	0.227
75	0.345
100	0.472

Conversion:

$Y = 0.0047X - 0.0045$ when X = Absorbance

2. Reduction of naringin level by varying the concentrations

2.1 The level of naringin and pH in various growth phases when added 80 mg/ml naringin in MRS

Table 2 Scheffe's test of 80 mg/ml naringin

Source	df	SS	MS	F	Sig.
Treatment	5	194.857	38.971	2.242 ^{ns}	0.095
Error	18	312.936	17.385		
Total	24	128080.608			

Table 3 Data of Time and 80 mg/ml naringin

Time (hr)	Average naringin concentration (mg/ml)
0	77.234
4	69.415
8	69.521
16	75.266
20	73.617
24	72.394

2.2 The level of naringin and pH in various growth phases when added 120 mg/ml naringin in MRS broth.

Table 4 Scheffe's test of 120 mg/ml naringin

Source	Df	SS	MS	F	Sig
Treatment	5	2379.512	475.902	1.352 ^{ns}	0.288
Error	18	6336.747	352.041		
Total	24	256308.167			

Table 5 Data of Time and 120 mg/ml naringin

Time (hr)	Average naringin concentration (mg/ml)
0	120.320
4	102.610
8	101.760
16	102.710
20	93.990
24	88.030

2.3 The level of naringin in various growth phases when added 160 mg/ml naringin in MRS broth.

Table 6 Duncan's New Multiple's Range Test (DMRT) of 160 mg/ml naringin.

Source	df	SS	MS	F	Sig
Treatment	5	5009.462	1001.892	8.384 *	.000
Error	18	2151.105	119.506		
Total	24	437051.702			

Table 7 Data of Time and 160 mg/ml naringin

Time (hr)	Average Naringin concentration (mg/ml)
0	140.851^{ab}
4	152.287^{b}
8	146.596^{ab}
16	132.925^{a}
20	116.367^{c}
24	113.989^{c}

2.4 The level of naringin in various growth phases of 80, 120, and 160 mg/ml naringin in MRS broth.

Table 8 Duncan's New Multiple's Range Test (DMRT) of 80, 120 and 160 mg/ml naringin

Source	df	SS	MS	F	Sig.
Concentration	3	44718.083	14906.028	101.419^{*}	0.000
Replication	3	1182.511	394.170	2.682^{ns}	0.057
Time	5	4035.999	807.200	5.492^{*}	0.000
Conc*Time	**10**	**3612.488**	**361.249**	$\mathbf{2.458^{*}}$	**0.018**
Error	50	7348.709	146.974		
Total	72	821438.075			

Remark: Conc = Concentration

Table 9 Data of 80, 120, and 160mg/ml naringin.

Time (hr)	80 (mg/ml) naringin	120(mg/ml) naringin	160(mg/ml) naringin
0	77.234	120.320	140.851
4	69.415	102.610	152.287
8	69.521	101.760	146.596
16	75.266	102.710	132.925
20	73.617	93.990	116.367
24	72.394	88.030	113.989

3. Control

3.1 The level of naringin in various times when added 80 mg/ml naringin in MRS broth.

Table 10 Data of Time and 80 mg/ml naringin

Time (hr)	Average naringin concentration (mg/ml)
0	72.908
1	76.312
2	78.014
3	78.652
4	75.745
5	90.567
6	69.078
7	71.064
8	70.142

3.2 MRS broth with cultures and pH Vs Time

Table 11: Time and naringin concentration (mg/ml)

Time (hr)	Average naringin concentration (mg/ml)
0	0
1	0
2	0
3	0
4	0
5	0
6	0
7	0
8	0

APPENDIX B

The Media formulation:

1. Man-Rogosa-Shape media (MRS)

- peptone	10.0	g/l
- meat extract	8.0	g/l
- yeast extract	4.0	g/l
- glucose	20.0	g/l
- di-potassium hydrogen phosphate	2.0	g/l
- tween 90	1.0	g/l
- di-ammonium hydrogen citrate	2.0	g/l
- sodium acetate	5.0	g/l
- magnesium sulphate	0.2	g/l
- manganese sulphate	0.04	g/l

2. Man-Rogosa-Shape media (MRS) agar

- peptone	10.0	g/l
- meat extract	8.0	g/l
- yeast extract	4.0	g/l
- glucose	20.0	g/l
- di-potassium hydrogen phosphate	2.0	g/l
- tween 90	1.0	g/l
- di-ammonium hydrogen citrate	2.0	g/l
- sodium acetate	5.0	g/l
- magnesium sulphate	0.2	g/l
- manganese sulphate	0.04	g/l
- agar	7.0	g/l