ABSTRACT

Title of Document: Effect of LYCH (*Lycium chinense* P. Mill.) leaf hydrolysates on the growth of *Pediococcus acidilactici* IMT101

Yi-Chun Yeh, Master of Science, 2006

Directed By: Associate Professor, Y. Martin Lo, Department of Nutrition and Food Science

Growth stimulating effects of LYCH leaf hydrolysates on *P. acidilactici* IMT101 cells were observed when MRS broth was supplemented with 20% (v/v) H1+H2, the mixture of hydrolysates prepared by a tea-making process. Cells grown on MRS containing H1+H2 showed a shortened lag phase while yielding a cell concentration (X_s) significantly higher than other conditions. The maximal specific growth rate (μ_{max}) was also the highest among all. Microwave-assisted extraction (MAE) at 80°C for 2 hrs ($M80_{2h}$) released more amino acids but less sugar (fructose, glucose, and sucrose) than in H1+H2. No correlations between amino acids and cell growth were found. In the absence of FOS, the high glucose concentration in the H1+H2 hydrolysates was found responsible for the stimulatory effects on *P. acidilactici* growth. These effects of LYCH leaf hydrolysates indicate the potential of developing new applications in promoting the growth of other probiotic cells using a simple process.

Keywords

P. acidilactici, *Lycium chinense*, cell yield, specific cell growth rate, probiotic

EFFECT OF CHINESE WOLFBERRY (*LYCIUM CHINENSE* P. MILL.) LEAVE HYDROLYSATES PREPARED BY TEA-MAKING PROCESS AND MICROWAVE EXTRACTION ON THE GROWTH OF *PEDIOCOCCUS ACIDILACTICI* IMT101

By

Yi-Chun Yeh

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Advisory Committee:
Associate Professor: Y. Martin Lo, Chair
Professor and chairman: Mickey Parish
Assistant professor: Brian Bequette

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Chapter 1: Introduction

The leaf of *Lycium chinense* P. Mill. (LYCH), a.k.a. Chinese desert-thorn (USDA NRCS, 2006) or Chinese wolfberry (Zhang and Fritz, 1989), which is a plant belonging to the family Solanaceae, is regarded in traditional Chinese medicine as a medical herb for eternal youth and long life (Soga, 1985), a nourishing ingredient, and a tonic to reduce the risk of arteriosclerosis and essential arterial hypertension (Mizobuchi *et al*., 1969). Used as tea in the Orient for more than 2,000 years due primarily to the stamina-improving, tranquillizing, and thirst-quenching activities, LYCH leaves are considered a healthful food (Kim *et al*., 1997). Besides abundant betaine (Hansel *et al*., 1992), a phytochemical used to abate the risk of fatty liver (Mehta *et al*., 2002) or as a digestive aid for persons with insufficient production of acid in the stomach, LYCH leaves contain anti-aging vitamins ascorbic acid and tocopherols (Park, 1995), a group of antioxidative compounds such as rutin (Duke, 1992) and chlorogenic acid (Terauchi *et al*., 1997a), and lyciumoside I, a methanol extract showing antimicrobial activities against gram positive rods (Terauchi *et al*., 1998). Moreover, LYCH leaves reportedly increased the amino acids content of broiler meat while improving its flavor, taste, and tenderness (Na *et al*., 1997), indicating their potential application as feed supplement.

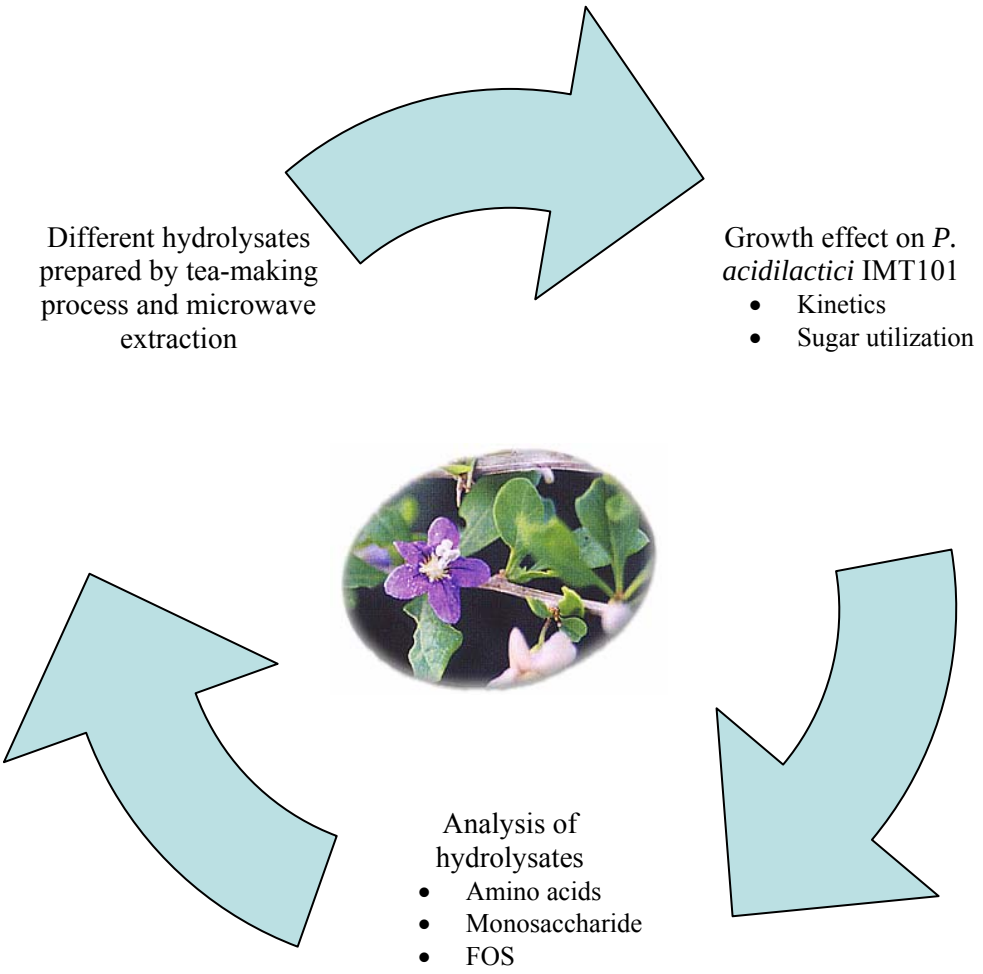
Nishiyama (1965) demonstrated the growth stimulating effects of LYCH leaves on *Lactobacillus acidophilus* cells, the most commonly used probiotic in today's food industry. His article (in Japanese) remained the only study on the subject in the literature until Bae and coworkers (2005) reported (in Korean) that addition of

methanol extract of LYCH leaves enhanced the antioxidative activity in yogurt. The lack of research activities in this area could be attributed in part to limited circulation of literature published in non-English languages or to the void in Western literature on applications of herbal ingredients. Nonetheless, such growth stimulating effects are intriguing and might be valuable for promoting the growth of other probiotic strains that are of commercial importance.

Pediococci, gram-positive, facultatively anaerobic cocci belonging to the group of lactic acid bacteria, carry a GRAS (generally recognized as safe) status (Simpson *et al.*, 2002). *Pediococcus acidilactici* has been widely used in the fermentation of dairy products (Bhowmik and Marth, 1990; Litopoulou-Tzanetaki *et al.*, 1989), meats (Luchansky *et al*., 1992; Mattila-Sandholm *et al*., 1993), vegetables (Knorr, 1998), dough (Nigatu *et al*., 1998), fruit juices (Knorr, 1998), and silage (Cai *et al*., 1999; Fitzgerald, 2000). Pediocins, inhibitory to a range of food pathogens, have been isolated from *P. acidilactici* (Nielsen *et al*., 1990; Nettles and Barefoot, 1993; Kang and Fung, 1999; Cheun *et al.*, 2000), which has been shown to present in the natural micro biota in the gastrointestinal tracts of animals, poultry, and duck (Juven *et al*., 1991; Kurzak *et al.*, 1998; Hudson *et al.*, 2000; Rekiel *et al*., 2005). Additionally, *P. acidilactici* showed preservative effects against yeast and mold spoilage when applied to alfalfa feed (Sindou and Szucs, 2005). To date, *P. acidilactici* has become a favorable ingredient in commercial probiotic feeds (Vanbelle *et al*., 1990; Tannock, 1997; Geary *et al*., 1999) and a promising probiotic for fish larvae as a growth promoter (Gatesoupe, 2002). There is a pressing need to identify a cost effective approach to produce sufficient cells in a timely manner in order to meet such a

demand. However, the growth kinetics of *P. acidilactici* available in the literature has been geared towards pediocin production, which often requires conditions less favorable for cell mass accumulation (Biswas *et al*., 1991; Cho *et al.*, 1996; Guerra and Pastrana, 2003; Vázquez *et al*., 2003), leaving considerable discrepancy with reference to producing *P. acidilactici* cells at the industrial scale.

In the present study, the feasibility of using LYCH leaves to promote the growth of *P. acidilactici* and the variations among different leaf preparation methods with respect to chemical constituents and growth-promoting effects were addressed. From a processing standpoint, if a simple operation could be established to release ingredients that stimulate the growth of probiotic cells, it would most likely be readily convertible for industrial applications and the process could be easily optimized to enhance cost effectiveness. In respect of biomass utilization and efficacy, it is highly desirable if the LYCH leaves were able to provide dual functionalities—both as a growth promoter for probiotics in feed and as a feed themselves to enhance the amino acids content and to improve the flavor, texture, and taste of the end products.


Different hydrolysates prepared by tea-making process and microwave extraction
Growth effect on *P. acidilactici* IMT101
• Kinetics
• Sugar utilization
Analysis of hydrolysates
• Amino acids
• Monosaccharide
• FOS

Chapter 2: Literature Review

2.1 LYCH leaves

2.1.1Traditional Chinese Medicine Perspective

Herbs in Traditional Chinese Medicine (TCM) formulation fall into four different categories: (1) Imperial herb—the chief herb (main ingredient) in a formula (2) Ministerial herb—ancillary to the imperial her, it augments and promotes the action of chief herb (3) Assistant herb—reduces the side effects of the imperial herb (4) Servant herb—harmonizes or coordinates the actions of other herbs. Five thousand years ago in China, Shen Nung (a famous herbalist) grouped 365 herbs into three classes: upper, middle, and lower based on herbal toxicities. The nontoxic and rejuvenating upper class herbs can be taken continuously for a long period and form the main components of "Yao Shan". Chinese people consume herb-based Chinese medicine dishes in their daily life for more than 5000 years. Termed "Yao Shan" in Chinese, herb-based Chinese medicinal dishes are popular in various forms, including herbal foods, teas, wines, congees, and pills. Unlike western medicine, Chinese medicine uses processed crude multi-component natural products, in various combinations and formulations aimed at multiple targets, to treat entirety of different symptoms. LYCH is categorized as an imperial herb. The major use of LYCH is for kidney disease (Table 2.1.1). LYCH leaves belong to the upper class herbs and can be consumed in daily life.

Table 2.1.1. Function of LYCH leaves described in traditional Chinese medicine books.

Book Name (Time)	Description
Chen Nan Pen Tsao (Anonymous, Warring States, 475 B.C. - 221 B.C.)	LYCH leaves can be cooked with egg and cure the leucorrhea problem in women.
Yin Shan Chen Yao (Hu Sihhusi, Yuan Dynasty, 1279-1368)	LYCH leaves make people strong, refresh the spirits and enhance sexuality.
Ben Tsao Kang Mu (Li Shizhen, Ming Dynasty, 1368-1644)	"Tien Chin Tso", the leaf of Lycim chinense, is consumed to improve human body health and prolong human life. The ripe fruit of this plant known as Lycii fructus and the leaves known as Lycii folium are used as foods, while the root, known as Lycii cortex radicis, is used as a Chinese herbal medicine. "Tien Chin Tso" nourishes the liver and kidney and is effective to treat people with yin and blood deficiency of vital essences manifested by aching of the loins and knees, nocturnal emission, impotence, dizziness and tinnitus. It can be decocted as tea for daily drinking.
Yao Yao Fen Ji (Shen Jinbie, Qing Dynasty, 1644-1911)	LYCH leaves taste bitter and sweet. The property of the leaf is cold. The medical uses for the leaf are mainly for relieving the depression, nourishing the heart, and releasing the tiredness of joints.
Herbal Pharmacology in the People's Republic of China (American Herbal Pharmacology Delegation, contemporary, 1975)	LYCH can be used for curing impotence and backache with decoction of *Rehmannia glutinosa* and *Viscum coloratum*. For dizziness, it can be decocted with *Chrysanthemum morifolium*, *Cornus officinalis*, *Dioscorea batatas*, and *Rehmannia glutinosa*. For weakness and fever, it can be decocted with *Anemarrhena asphodeloides*, *Angelica sinensis*, *Artemisia apiacea* and *Gentiana macrophylla*. *Lycium chinense* has been reported to give hypoglycemic effect in mice, antifungal effects and has been used as an herbal remedy in China for hypertension, nephritis and for cancer.

2.1.2 Morphology

Chinese wolfberries are also called Chinese matrimony vines which belong to Solanaceae. This plant is also known as Chinesischer Bocksdorn in Germane, Daun Koki in Indonesia, Gou Qi in China, Kaukichai in Malaysia, Kuko in Japan, Lyciet de Chine in France, Spina Santa Cinese in Italy, Box thorn in Korea (Duke, 1992). *Lycii folium* is its Latin name and it appears as "Tien Chin Tso" in traditional Chinese medicine formulation books. These plants grow in thickets along riverbanks in Japan, Korea, Manchuria, China, Ryukyus, Taiwan, and the northeastern part of the United States (Fig. 2.1.1). They are ornamental shrubs valued chiefly for their showy berries, but they also provide wildlife habitat, watershed protection, and shelter hedges. The shrubs mature when they grow to 3 to 7 feet high. The purplish flowers bloom from June to September and are followed by scarlet to orange-red berries which ripen from August to October. The leaves can be collected from May to November in north part of America (Rudolf, 1974). LYCH leaves have also been known for improvement of stamina, tranquillizing activity, thirst-quenching and anti-aging activity (Soga, 1985). In Indonesia, an infusion of the leaves with tea is gargled as a mouthwash to relieve toothache (Perry, 1980). LYCH leaves (Fig. 2.1.2) have been used as tea substitute in China, Korea and Japan for more than 2000 years. The traditional tea making process includes cleaning, cooking and then filtered. There is no caffeine detected in the leaves thus there is no limitation to drink LYCH tea as a daily drink.

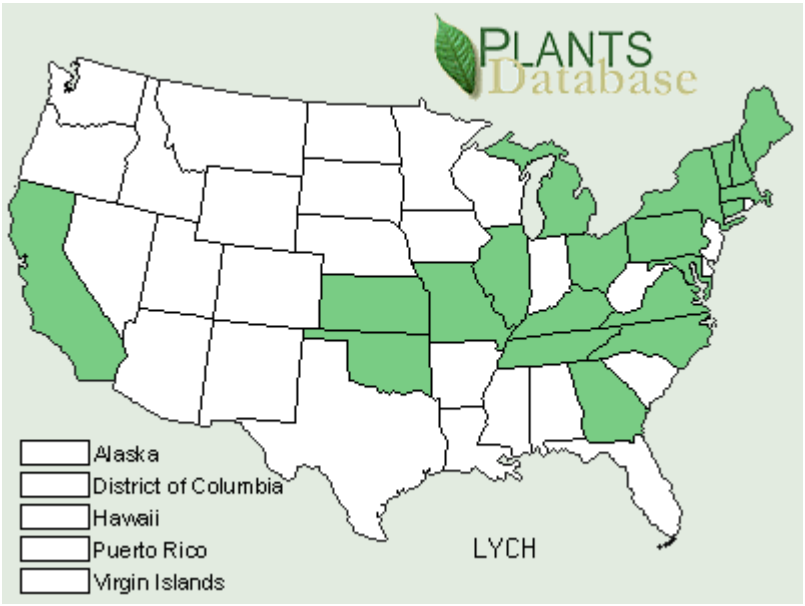


Fig 2.1.1. *Lycium chinense* P. Mill. distribution in USA.
(http://plants.usda.gov/java/profile?symbol=LYCH, URL accessed on May 19, 2006)

Fig 2.1.2. Specimen of *Lycium chinense* P. Mill.

(http://research.kahaku.go.jp/botany/wild_p100/autumn/x600_jpg/09kuko.jpg,

URL accessed on May 19, 2006)

2.1.3 Nutrition Values

Nutrition evaluation of LYCH were collected and categorized in Table 2.1.2. Among all of the drying methods, freeze-drying maintained the highest components of water soluble compounds in LYCH leaves (Terauchi *et al.*, 1997b). No *et al.* (1995) concluded that using water as solvent at 80℃ with four times immersion for eight hours each immersion to extract LYCH leaves achieved 30.27% yield of extractable solids compared with 25.14% yield using 30% ethanol solvent in the same condition. Long time immersion and 80℃ are good parameters to extract solids from LYCH leaves. Price *et al.* found that there are only small changes in either the overall level or the composition of quercetin glucosides during normal commercial storage. Boiling and frying did not result in gross changes in glucosides composition, although an overall loss of up to 25% was found for both processes, in the former by leaching into the cooking water and in the latter by thermal degradation into products.

Seasonal fluctuation could be observed on the total free sugars and other water soluble components. Total sugars reached the highest amount in May, 1997, but fructose was found to be highest in June, 1997 (Kim *et al.*, 1997). Amount of vitamin C and rutin were highest in May and November (Mizobuchi *et al.*, 1964). Flavonol glycosides (quercetin-3-O- sophoroside and kaempferol-3-O- sophoroside) increased from February to March and from November while the leaves were sprouting (Terauchi *et al.*, 1997b).

Table 2.1.2. Reported nutritional data on LYCH leaves.

Composition	Amount (mg/g)	Remarks	Reference
Kilocalories	29 Kcal/100 g	Dry basis.	Duke, 1992
Water	896		
Crude lipid	0.003		No *et al.*, 1995
Crude protein	0.00125		
Free Amino acids			
Alanine	19.8	Dry basis. Sampled in May, 1997, Korea.	Kim *et al.*,1997
Arginine	6.77		
Aspartic acid	11.1		
Cysteine	1.43		
Glutamine	1.06		
Glutamic acid	1.33		
Glycine	1.28		
Histidine	22.4		
Isoleucine	11.9		
Leucine	14.9		
Lysine	8.81		
Phenylalanine	8.55		
Proline	26.7		
Serine	2.04		
Threonine	1.83		
Tryptophan	3.89		
Tyrosine	8.58		
Valine	12.5		
Carbohydrates	385		Duke,1992
Fiber	125		
Total sugar	29.8		No *et al.*, 1995
Reducing sugar	0.00001		
Free sugars			
Fructose	0.00077	Dry basis. Sampled in May, 1997, Korea.	Kim *et al.*, 1997
Glucose	0.00133		
Maltose	0.00098		
Sucrose	0.00068		
Mineral			
P_2O_5	0.00036		No *et al.*, 1995
K_2O	0.00025		
CaO	0.00002		
MgO	0.00001		Duke, 1992
Iron	0.519		
Sodium	0.18365		

Table 2.1.2. Reported nutritional data on LYCH leaves. (Cont.)

Composition	Amount (mg/g)	Remarks	Reference
Vitamin		Dry basis. Sampled in April, 1964, Japan.	Mizobuchi *et al.*, 1964
Ascorbic acid	0.31		
Tocopherol	0.177		Duke, 1992
Beta carotene	0.428		
Thiamine	0.0077		Hansel, 1992
Total flavonoids* (Sweet, refreshing, apple-like, honey-like flavonoids)	0.6785	Dry basis.	Aubert and Kapetanidis, 1989
Quercetin	0.1315	Dry basis.	Miean and Mohamed, 2001
Apigenin	0.547		
Glycosides		Fresh leaves basis. New compounds were found in 1998**.	Kaznowski *et al.*, 2005
Lyciumoside Ⅰ	0.00014 - 0.00025		
Lyciumoside Ⅱ	0.00097 - 0.0015		
LyciumosideⅢ	0.00028 - 0.00053		
Rutin	0.000006 - 0.000015		
Antioxidants			
Chlorogenic acid	0.01-0.017		Na *et al.*, 1997
Quercetin-3-O-sophoroside	0.00095-0.0015		
Kaempferol-3-O-sophoroside	0.00012-0.00018		
Betaine	13.8		Hansel, 1992
Anticancer compounds			
Withanolide A	0.2		Duke, 2006
Withanolide B	0.3		
Withasteroids	1		

Table 2.1.2. Reported nutritional data on LYCH leaves. (Cont.)

Composition	Amount (mg/g)	Remarks	Reference
Other compounds: β-sitosetrol- β-D-glucoside, nicotianamine, scopoletin, vanillic acid, (+)-3-hydroxy-7, 8-dehydro-β-ionone, 9-hydroxy-10,12, β-ionone, 9-hydroxy-10,12,15-octadecatrienoic acid, α-dimorphecolic acid.			Shih, 1991

* New flavonoids are identified as: quercetin-7-O-glucoside-3-O-glucosyl [1-2]galactoside, quercetin -7-O-glucoside-3-O- sophoroside, kaempferol-7-O-glucoside-3-O-glucosyl [1-2]galactoside, and kaempferol-7-O-glucoside-3-O- sophoroside. (Aubert and Kapetanidis, 1989)

** Six new acyclic diterpene glycosides named lyciumosides Ⅳ-Ⅸ were isolated from LYCH leaves, and there structures were elucidated as

Lyciumoside Ⅳ: 3-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-17-hydroxygeranyllinalool-17-O- β-D-glucopyranoside

Lyciumoside Ⅴ: 3-O-β-D-glucopyranosyl-17-hydroxygeranyllinalool-17-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside

Lyciumoside Ⅵ: 3-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-17-hydroxygeranyllinalool-17-O- α-L-rhamnopyranosyl-(1→6)- β-D-glucopyranoside

Lyciumoside Ⅶ: 3-O-β-D-glucopyranosyl-17-hydroxygeranyllinalool-17-O- β-D-glucopyranosyl-(1→2)-(α-L-rhamnopyranosyl-(1→6))- β-D-glucopyranoside

Lyciumoside Ⅷ: 3-O-β-D-glucopyranosyl-12, 17-dihydroxygeranyllinalool-17-O- β-D-glucopyranoside

Lyciumoside Ⅸ: 3-O-(6-O-malonyl)-β-D-glucopyranosyl-17-hydroxygeranyllinalool-17-O- β-D – glucopyranoside. (Terauchi *et al.*, 1998)

2.1.4 Pharmacology and Other Uses

Major pharmacological effects of LYCH leaves were listed in Table 2.1.3. LYCH leaves extracts were also used in several animal fertility studies in 1971. Hojyo found that the extracts increased luteinizing hormone (LH) activity in rats and rabbits. The water extracts were found to induce the ovulation in adult female rabbits, but the mechanism and the active compounds still remained to be further researched (Suzuki *et al.*, 1972). Active substances were demonstrated in LYCH leaves upon extraction with water but not with organic solvent. LYCH leaves were tested and used as animal feed in Korea for a long history. When the dietary LYCH leaves levels were increased, the amino acids content of broiler meat also increased. A significant effect

was observed of the 3%, 6% and 9% leaf extract on glutamic acid and valine in the broiler meat products (Na *et al.*, 1997).

Table 2.1.3. Pharmacological effect of LYCH leaves.

Compounds	Pharmacologic effect	Ref.
Vitamin C and E	Abating or reducing the risk of certain diseases such as arteriosclerosis, essential arterial hypertension, diabetes, and night blindness	Soga, 1985
Betaine	Lipotropic and hepatic function-protecting effects, and work as preventive phytochemical for reducing or abating the risk of fatty liver	Nishiyama, 1963
Rutin	Preventive phytochemical for hypertension and stroke	Mizobuchi *et al.*, 1964
Vitamin C, vitamin E, rutin, chlorogenic acid, quercetin-3-O-sophoroside, and kaempferol-3-O-sophoroside	Antioxidants	Na *et al.*, 1997
Quercetin	Inhibit oxidation and cytotoxicity of low-density lipoprotein in vitro, reduce risk for coronary heart disease or cancer, work as strong antioxidant that can contribute to the prevention of atherosclerosis and also work as chemopreventive and chemotherapeutic agent that can relieve local pain caused by inflammation, headache, oral surgery, and stomach ulcer	Miean *et al.*, 2001
9-hydroxy-10, 12, 15-octadecatrienoic acid and α-dimorphecolic acid	Angiotension converting enzyme inhibitor which can lower the blood pressure	
Water extract of LYCH leaves	Inhibit the activity of angiotensin converting enzyme (ACE): ACE catalyzes the conversion of angiotensin I to angiotensin II and the breakdown of bradykinin. Angiotensin II and bradykinin are hypertensive and hypotensive agents, respectively	Shih, 1991
Withaferin A, withaphysalin A and withangulatin A	Anticancer reagents	
Lyciumoside Ⅰ	Antimicrobial function on *Helicobacter pylori* strains and *Micrococcus flavus* strain.	Terauchi *et al.*, 1998

The prebiotic effect of LYCH tea leaves was first observed in 1965 by Nishiyama. The 1% tea extracts stimulated the growth of *Lactobacillus acidophilus* cells to 8.0×10^{9} CFU/mL compared with control group 1.1×10^{9} CFU/mL. The tea leaves extracts also increased acidity in the growth of *Lactobacillus acidophilus* cells in Nishiyama's report.

LYCH leaves have been used as nutritional supplement in the form of tea in oriental area for thousands of years. The abundant amino acids, antioxidants, anticancer component in the leaves can be extracted with water into the drinking tea format. In 1998 Terauchi *et al.* concluded that the LYCH leaves of maybe beneficial as a health food.

2.2 Extraction Methods

2.2.1 Traditional Tea Making Process

Traditional tea making process includes sun drying for three to five days or mechanic drying overnight. Different tea leaves go throughout different fermentation process. Green tea as well as LYCH leaves are non-fermented tea leaves. The temperature to be used to cook non-fermented tea leaves cannot be over 80°C. Usually the cooking time depends on personal preference, but at least the leaves should be immersed with warm water for 3-5 min (Lin, 1985). To release higher soluble carbohydrates, longer time of extraction was recommended (No *et al.*, 1995).

2.2.2 Microwave Extraction Method

Microwave system is consisted of microwave generator, wave guide for transmission, resonant cavity and a power supply. The microwave generator is a magnetron which is a cylindrical diode with a ring of cavities which acts as the anode structure. The heating effect in microwave cavities is from dielectric polarization. The polarization is achieved by the reorientation of permanent dipoles by the applied electric field. There are two basic systems (open and closed) commonly appear on market. The one with closed system as the equipment used in this study was supplied by Ethos, Milestone Inc. The chassis of the Ethos oven (Fig. 2.2.1) is made of corrosion-resistant stainless steel. The large interior cavity and the inside of the door are plasma coated with 5 layers of polytetrafluoroethylene (PTFE) applied at 350°C to protect the interior of the unit from aggressive acids. The heat is evenly distributed with a rotating diffuser. The system can provide up to 1600 W of microwave installed power. The maximum temperature the oven can reach is 300°C. The system allows up to 12 extraction vessels to be irradiated simultaneously. Vessels are placed in a sample rotor and secured with a calibrated torque wrench to achieve uniform pressure. If the operating pressure exceeds the vessel limits, a patented spring device allows the vessel to open and close instantaneously; bringing the internal pressure down to a containable level thus they are inherently safe.

Fig 2.2.1. Ethos E Microwave extraction station. (Milestone Inc., Shelton, CT.) (http://www.milestonesrl.com/analytical/product/ex_ethose.html, URL accessed on May 19, 2006)

Several classes of compounds such as essential oils, aromas, pesticides, phenols, dioxins, and other organic compounds have been extracted efficiently from a variety of matrices (mainly soils, sediments, animal tissues, foods and plant material). All the reported applications showed that microwave assisted solvent extraction (MAE) is a viable alternative to conventional techniques for such matrices (Dean, 1998). According to Saoud *et al.*, the essential oil was obtained the highest at 800-1000W by using Ethos microwave lab station (Saoud *et al.*, 2005). Standard MAE method was described in Fig. 2.2.2.

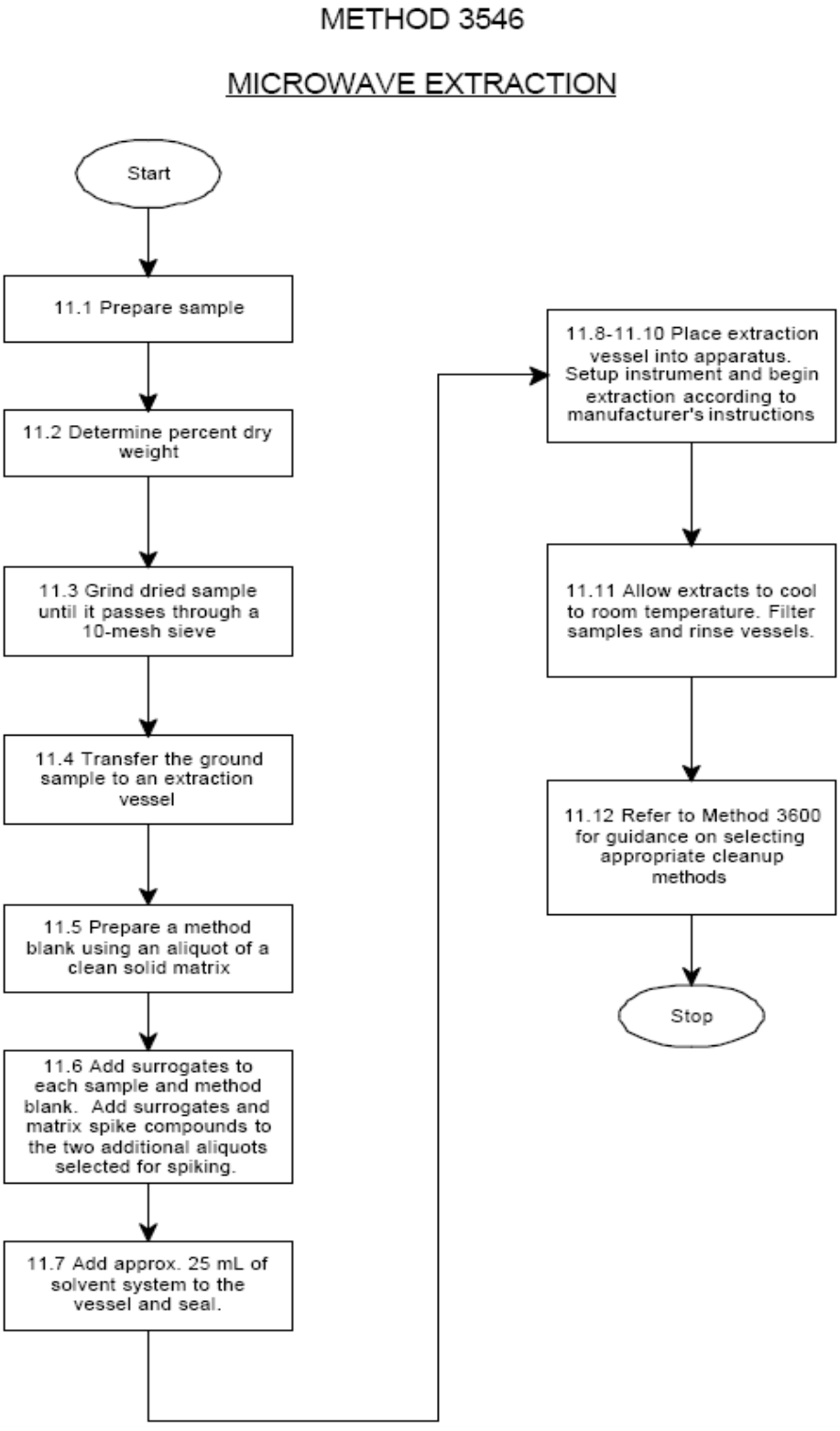


Fig 2.2.2. Patented microwave extraction method flow chart (Paré *et al.*,1998).

(Courtesy of Milestone Inc.)

2.3 Prebiotics and Fructooligosaccharides

2.3.1 Definition of Prebiotics

Prebiotic was first defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health"(Gibson and Roberfroid, 1995). Prebiotics are required with the ability to resist gastric acidity and hydrolysis by mammalian enzymes and gastrointestinal absorption, to be fermented by intestinal micro flora, and selectively stimulate the growth and activity of intestinal bacteria associated with health and well being. A newly modified definition of prebiotic is "a prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and activity in the gastrointestinal micro flora that confers benefits upon host well-being and health (Roberfroid,2005)."

Prebiotics in practice are short-chain carbohydrates (SCCs) that are not digestible by human enzymes and which have been named resistant SCCs. Short chain fructooligosaccharides (scFOS) have been isolated from onions, wheat, barley, bananas, tomatoes, garlic, and artichokes. scFOS are non-reducing sugars and will not undergo the Maillard reaction. More than 70% of the energy from carbohydrate fermentation is conserved as short chain fatty acids (SCFA) and other fermentation products such as methane, carbon dioxide, and hydrogen. The SCFA (acetate, propionate and butyrate) serve as a source of energy for the host. Acetate is primarily used as fuel for host tissues. Propionate is used primarily in the liver as a substrate for gluconeogenesis. Butyrate is preferentially oxidized by colonocytes. Nondigestible oligosaccharides are not strictly oligosaccharides and their nondigestibility is not

always proved. Table 2.3.1 shows some of the SCCs which were considered as prebiotics available for human consumption. They can be more properly defined as "carbohydrates with a degree of polymerization (DP) of two or more, which are soluble in 80% ethanol and are not susceptible to digestion by pancreatic and brush-border enzymes", but several of the prebiotics even have DP value >10 (Roberfroid, 2005). Until now, only three products meet the requirements for prebiotic classification (Table 2.3.1). They are inulin-type fructans, (trans)-galactooligosaccharides, and lactulose (Roberfroid, 2005).

Table 2.3.1. Classification of certain carbohydrates as colonic foods and prebiotics (adapted from Gibson and Roberfroid, 1995).

Carbohydrates		Colonic food	Prebiotics
Resistant starch		Yes	No
Non-starch polysaccharides	Plant cell wall polysaccharides	Yes	No
	Hemicelluloses	Yes	No
	Pectics	Yes	No
	Gums	Yes	No
Nondigestible oligosaccharides	Fructooligosaccharides	Yes	Yes
	Galactooligosaccharides	Yes	-
	Soybean oligosaccharides	Yes	-
	Glucooligosaccharides	-	No

2.3.2 Fructans

Fructans are generally defined as being a polymer of fructose having more than 10 fructose units. In plants, up to 200 fructose units can be linked in a single fructan molecule (Table 2.3.2). Regardless if the fructose ring has a furanose form, the oligomeric molecule is still considered to be fructans. In nature, the various fructans are broadly classified into three groups. The inulins, the levans (or phleins or phleans),

and mixtures or highly branched chain fructans referred to as the graminan type. The inulin type are linear fructans made up of fructosyl units linked by a β (2→1)-bond. The molecule is typically terminated by a glucosyl unit bound to one of the fructose moieties via an α1- β2 type linkage. Some of them from plants contain small degree of branching of a β (2→6)-linkage (Roberfroid, 2005).

Table 2.3.2. Inulin or oligofructose content of fresh or prepared vegetables, fruit and cereals (adapted from Van Loo *et al.*, 1995).

Foodstuff	Form	DP range
Onion	β (2→1) fructan; 75% 1-kestose and 25% neokestose	DP 2-12 1.1% to 7.5% on fresh weight
Jerusalem artichoke	β (2→1) fructan; 1-kestose	DP 2-50 16%-20% on fresh weight
Rye	β (2→1) fructan; 1-kestose and neokestose	0.5%-1% on fresh weight
Dandelion(leaf)	β (2→1) fructan	12%-15% on fresh weight
Garlic	β (2→1) fructan; 1-kestose and neokestose	DP 2-50 9% -16% on fresh weight
Banana	β (2→1) fructan	0.3%-0.7% on fresh weight
Barley		0.5-1.5%
Asparagus	β (2→1) fructan; 1-kestose And small amount of neokestose	
Chicory	β (2→1) fructan; 100% 1-kestose Roasted chicory still has >70% original fructan	DP 2-65 15%-20% on fresh weight

The degree of polymerization (DP) of inulin and the presence of branches are important properties that influence its functionality. Until recently, plant inulin was considered to be a linear molecule, but it has been possible to demonstrate that even native chicory inulin (DP_{av}=12) has a very small degree of branching (1-2%) (Roberfroid, 2005).

FOS are mixture of β-D-fructans containing between 2 and 4 β (2→1) linked fructosyl units displaying a terminal α-D-glucose residue, named 1-kestose (GF_2), 1-nystose (GF_3), and 1^F-fructosylnystose (GF_4), with average DP 3.7 (Fig. 2.3.1). Inulin is highly polymerized fructan of DP 10-60 whereas oligofructose with DP 2-9 (average DP 4.5) is produced during the process of chemical degradation of inulin (Tokunaga, 2004).

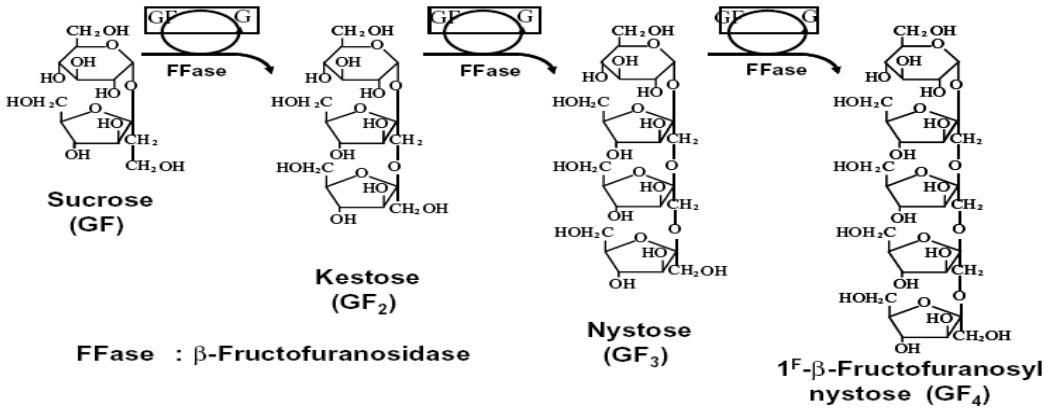


Fig 2.3.1. Chemical structure of fructooligosaccharides (FOS) and its enzymatic preparation from sucrose (adapted from Tokunaga, 2004).

The second group is the levans. They are linear and predominately made up of fructose moieties linked via β (2→6) bond. The levan type fructans might also contain small amount of β (2→1) branching. These types of fructans are found in many of the monocotyledons and in almost all bacterial fructans.

The third group is the mixed, graminan or grass type. This group is distinguished by having significant amount of both β (2→1) and β (2→6) linked fructose units and thus contain significant branching. General structures of fructans are:

α- D- glucopyranosyl-[β-D-fructofuranosyl]$_{n-1}$- β-D- fructofuranoside ($G_{py}F_n$)

β - D- glucopyranosyl-[β-D-fructofuranosyl]$_{n-1}$- β-D- fructofuranoside ($F_{py}F_n$)

Classification and chemistry of fructans were listed in Table 2.3.3.

Table 2.3.3. Chemistry of fructans.

Name	*Linkage(fructosyl-fructose)	Chemical structure	natural origins
Inulin	β (2→1)	linear, branched, cyclic	plant, bacteria, fungi
Levan	β (2→6)	linear, branched	plant, bacteria, fungi
Phlein	β (2→6)	linear, branched	plant
Graminan	β (2→1)and β (2→6)	linear, branched	plant
Kestoses	β (2→1)and β (2→6)	linear, branched	plant

*in such a representation, the numbers indicate the linkage's position on the C atoms of the fructose or glucose rings and the arrow points away from the reducing C atom (C_2 in fructose or C_1 in glucose)

2.3.3 FOS Detection Methods

In 1997, AOAC international adopted method 997.08, the fructan method that specifically allows the accurate quantitative determination of inulin and oligofructose in foods (Fig. 2.3.2). The concentration of total FOS and/or inulin was calculated according to the method of Hoebregs, 1997:

$$G = G_t - \frac{S}{1.9} - G_f \quad (1)$$

$$F = F_t - \frac{S}{1.9} - F_f \quad (2)$$

where G and F represent the glucose and fructose from FOS, and G_t, G_f, F_t, and F_f indicate the total glucose, initial free glucose, total fructose, and initial free fructose, respectively. $S/1.9$ is the amount of glucose or fructose from sucrose. The total FOS is the sum of G and F and corrected for the water loss during hydrolysis. Thus,

$$Total\ FOSs = k\,(G + F) \quad (3)$$

where $k = 0.925$ for FOS with an average degree of polymerization (DP) of 4 or $k = 0.91$ for the inulin-type (linear) FOS that has an average DP of 10 (Pedreschi *et al.*, 2003).

High-performance anion-exchange chromatographic (HPAEC) coupled with pulsed amperometric detection (PAD), enables complete, single step separation of neutral and charged oligosaccharides and polysaccharides differing by branch, linkage, and positional isomerism (Fig. 2.3.3). The sensitivity of PAD detector

decreases rapidly from DP = 2 to DP = 6; for longer oligomers (DP =7-17), the sensitivity of detector only decreases slightly. The HPAEC-PAD technique was more sensitive in terms of detection limit than Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (Wang *et al.*, 1999). The MALDI-MS results more accurately reflect the true amounts of FOS from food samples. Using linear oligosaccharide PAD response factors, one would overestimate FOS with branched forms present. MALDI-MS is a faster analysis method than HPAE-PAD, taking about 20 minutes rather than an hour for each analysis and MALDI-MS is more tolerant to impurities. MALDI-MS gives better assurance of correct molecular assignment since the isotopic mass of each peak is available. The High Proficiency Liquid Chromatography (HPLC) method was developed with a combination of enzymatic treatment and carbohydrate analysis before and after the treatment. After the quantification of fructose, glucose and sucrose, the FOS content was calculated by Hoebregs's method developed in 1997. A simple and convenient direct HPLC method was developed by Gan, 1999 using water as running solution and the FOS could be determined in 20 minutes. Available detection methods and equipments for FOS are compared in Table 2.3.4.

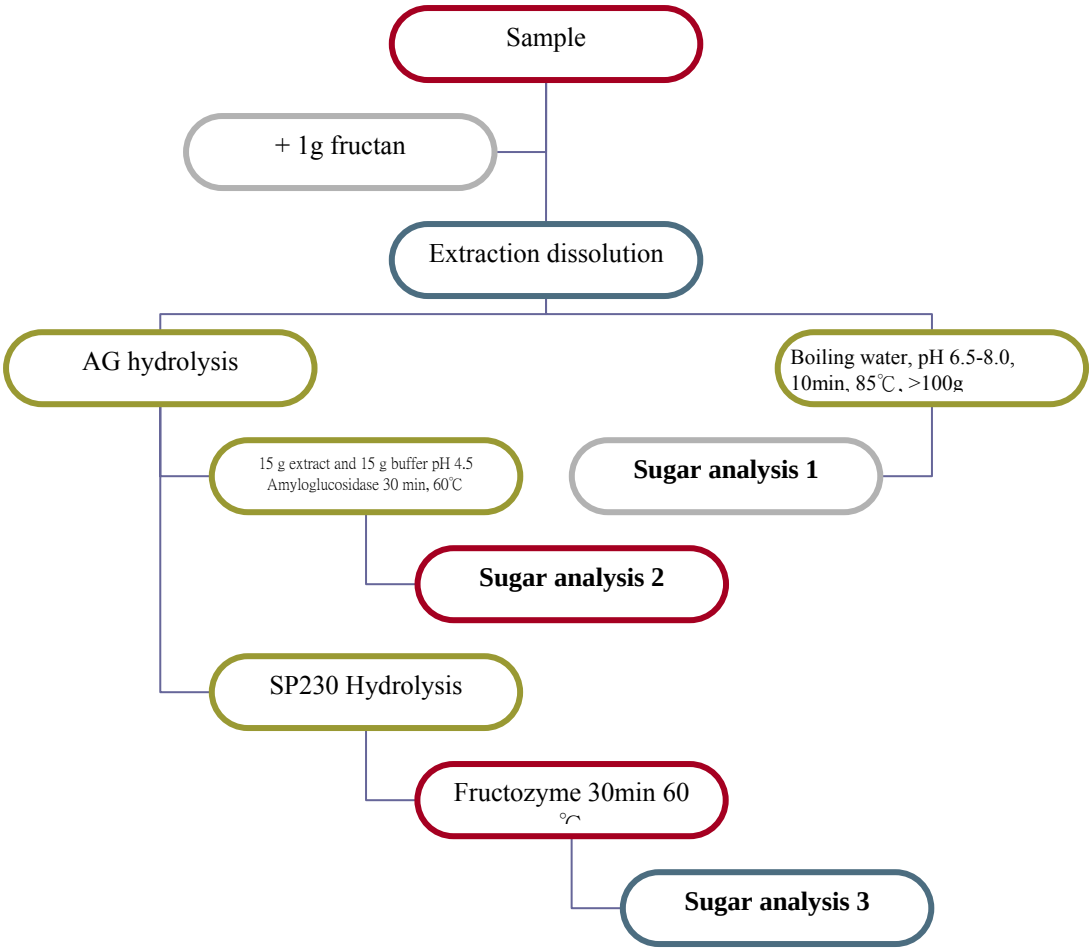


Fig 2.3.2. Flow diagram of enzymatic fructan determination method (adapted from Hoebregs, 1997).

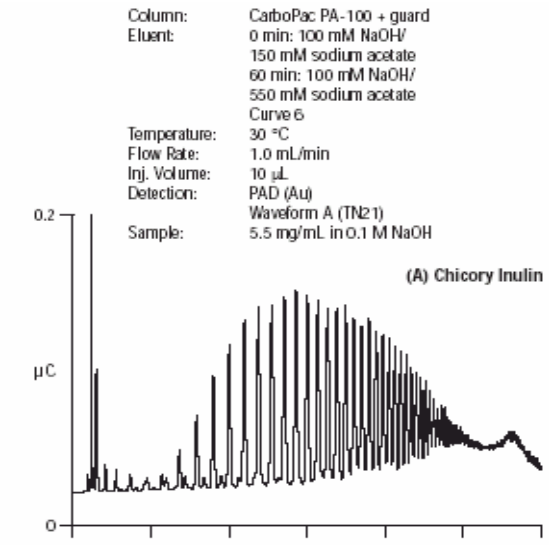


Fig 2.3.3. HPAEC-PAD data of chicory inulins (Roberfroid, 2005).

Table 2.3.4. Comparison of different FOS detection methods.

Methods	Sample Preparation	Equipment	Sample Injection Volume (μL)	Detector	Column	Temperature (℃)	Running Solution	Flow Rate (mL min^{-1})	Time (min)	Reference
Modified HPAEC-Pad method, 2004	Samples were injected hydrodynamically into the capillary in 5 s at 0.5 psi	HPAEC, Dionex Model 4000i gradient pump equipped with PED	10	UV detector at 214nm	Untreated fused-silica capillary of 75 μm i.d. × 60 cm	30	89% water, 10% 0.6M aqueous sodium hydroxide, 1% 0.5M aqueous sodium acetate solution	0.8	110	Corradini *et al.*, 2004
AOAC method, HPAEC-Pad method, 1997	Enzymatic treatment (Fructozyme SP 230)	HPAEC, Dionex Model 4000i gradient pump with Pad mode	50	Pulsed electrochemical detector in PAD mode	Carbopac PA1 4.0 mm i.d. ×25 cm	40±0.5	Mobile phase A, carbonate-free 10 mM NaOH; Mobile phase B, carbonare-free 1 M NaOH	1.0	83	Hoebregs, 1997
MALDI-MS method, 1999	Freeze dried samples were hydrolyzed with water, mixed with same volume 0.01 M potassium chloride solution	Proflex Ⅲ Bruker Analytical Systems Inc. MALDI-MS	50	Waters 464 pulsed amperometric detector (PAD) with a dual gold electrode and triple pulsed amperometry	Carbo Pac PA1 250 ×4 mm	-	A: 100 mM sodium hydroxide,B:100 mM sodium hydroxide/400 mM sodium acetate, C: 300 mM sodium hydroxide	0.7	60	Wang *et al.*, 1999

Table 2.3.4. Comparison of different FOS detection methods. (Cont.)

Methods	Sample Preparation	Equipment	Sample Injection Volume (μL)	Detector	Column	Temperature (℃)	Running Solution	Flow Rate (mL min^{-1})	Time (min)	Reference
HPLC method, 2000	Enzymatic treatment (Novozym 230)	HPLC	-	Refractive index detector	Aminex HPX-42C (0.78 cm × 30cm, Bio-Rad)	85	Deionised water	0.5	-	Jaimei *et al.*, 2000
Modified HPLC method, 2005	Samples were heated to 95 ℃ for 20 min	HPLC	-	-	Modified HPX 42A (7.8 ×300 mm, Bio-Rad) by passing 0.5 M NaNO3 at 2 mL mim $^{-1}$for 18 h with a cation and anion exchange guard column	-	HPLC-grade water	0.4	-	Livingston *et al.*, 2005
Direct HPLC method, 1999	-	HPLC, Waters 244	10	Refractive index detector R401, Waters	μ-Bondapak C_{18} column (3.9 mm i.d. × 300 mm, Waters, USA)	30 ℃	HPLC-grade water	0.8	20	Gan, 1999

2.3.4 Physiological Effects

Oligofructose and inulin are nondigestible oligosaccharides; they pass through the upper gastrointestinal system without significant hydrolysis and reach the colon as they have been ingested. This is an important characteristic of prebiotics.

The colon has a major role in digestion which is achieved by microbial fermentation through the salvage of energy. The colon also has important roles in absorption of minerals and vitamins, production and absorption of fermentation end products such as SCFAs and lactate, protection of the body against translocation of bacteria and against proliferation of pathogens, endocrines functions, regulation of intestinal epithelial cell growth and proliferation and immune function. The microflora colonizing the large bowel is the key to keep the colon healthy. A balanced microflora implies that the intestinal microflora must be composed predominantly of bacteria recognized as potentially health-promoting (like lactobacilli, bifidobacteria, and fusobacteria), to prevent, impair, or control the proliferation of the potentially pathogenic and harmful microorganisms (like *Escherichia coli*, clostridia, vellonellae, and candida) (Gibson and Fuller, 2000). A strategy to promote colon health is to consume prebiotics aimed toward the stimulation of the growth of beneficial bacteria to the ultimate goal of beneficial management of gut micro biota (Salminen and Wright, 1998).

Because of the ß configuration of the glucosyl linkages inside the FOS chain, all inulin-type fructans resist hydrolysis in the upper part of the gastrointestinal tract. During the passage through the upper part of the gastrointestinal tract, the inulin-type fructans may well influence transit time as well as digestion and adsorption of different macronutrients and micronutrients. Inulin-type fructans are classified as

"colonic foods" or foods that feed the large bowel and the microflora it contains (Gibson and Fuller, 2000). In the colon, fructans are hydrolyzed, most likely inside the bacterial cells and primarily inside the bifidobacteria, and rapidly ferment to produce short chain fatty acids, lactate and gases. Being nondigestible but highly fermentable, inulin-type fructans are dietary fiber. Being fermented in the large bowel, inulin-type fructans improve stool production, both quantitatively and qualitatively. Fermentation also produces SCFAs that are effectively absorbed and reach the systemic circulation where they may exert miscellaneous metabolic regulations. Moreover, this fermentation even induces changes in colonic epithelium stimulating proliferation in the crypts, increasing the concentration of polyamines and changing the profile of mucins (Roberfroid, 2005).

Inulin-type fructans are not only dietary fiber but also low calorie carbohydrates. The energy content is 1.5 kcal/g and is perfectly in line with recommended value for all nondigestible carbohydrates (Spiegel *et al.*, 1994).

Calcium and magnesium are specific nutrients most important for attaining peak bone mass, for reducing the risk of osteoporosis. Increasing bioavailability of an essential nutrient and mineral is recognized as a valid enhanced function claim. The claim "inulin-type fructans enhance calcium absorption" is scientifically substantiated. The most active product is a mixture of oilgofructose and long chain inulin (inulin HP) that is effective at a daily dose of 8 g. Regarding magnesium absorption, the human trials have demonstrated a beneficial effect of inulin-type fructans (Tokunaga, 2004).

Inulin-type fructans improve systemic health by their effects on modulation the expression of genes of hepatic lipogenic enzymes, on circulating levels of incretins

and other gastrointestinal peptides, systemic infections, systemic immunities and tumor growth and tumor metastasis (Fig. 2.3.4) (Tokunaga, 2004).

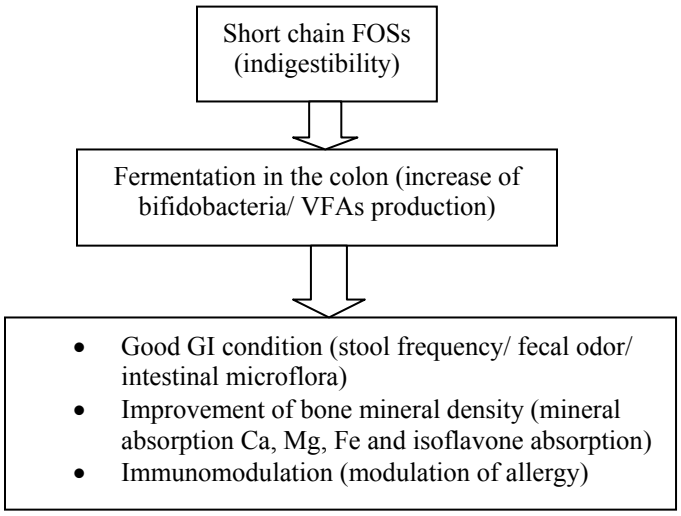


Fig 2.3.4. An overview of physiological functions of FOS and their key properties (adapted from Tokunaga, 2004).

In 1993, FOSHU, the Health Claim Approval System in Japan, approved the claim that FOS encourages a good gastrointestinal condition, inducing normal stool frequency, relief from constipation, and healthy intestinal microflora. The claim about the increase of mineral absorption and improvement of isoflavone bioavailability was approved in 2000. Japanese researches are making rapid progress on the studies of immunomodulation such as allergy prevention (Tokunaga, 2004).

Different *Bifidobacterium* strains (known probiotic bacteria) were capable of metabolizing L-(2, 6)-FOS if supplied as the sole carbon source. As already shown for inulin-type FOS, metabolization of L-(2, 6)-FOS is species-dependent. *B. adolescentis* showed the best growth and the highest degree of acidification and was

the only strain, of those tested, able to metabolize both short- and long-chain FOS (Gibson and Fuller, 2000; Kaplan and Hutkins, 2000; Durieux *et al.*, 2001; Kaznowski *et al.*, 2005; Rossi *et al.*, 2005). Human studies showed that with consumption of inulin-type fructans increased the total bifidobacteria sampled from the feces (Roberfroid, 2005).

2.3.5 Applications on Animals

Oligofructose reduces canine's small intestinal bacterial growth. It enhances small intestinal absorptive capacity, improves the balance between epithelial cell proliferation and differentiation in the colon, and tends to decrease fecal excretion of putrefactive compounds. In cats, oligofructose may improve colonic bacterial balance. Ideal digestibility of nutrients is improved in pigs, colonic concentrations of beneficial bacteria are increased in pigs and quails, fecal and colonic epithelial cell proliferation is stimulated in young pigs, fecal excretion of ammonia is reduced in pigs and rabbits, and contamination and colonization of poultry by pathogen is reduced. In swine it increases total digestibility of zinc. Oligofructose improves growth performance and meat production of broilers and is as effective as antibiotics in poultry data. Numerous feed-efficiency studies in male broiler chicks revealed no adverse effects related to feed supplementation with FOS. In addition, positive effect on the gut flora are shown in piglets, dogs and cats (Roberfroid, 2005).

2.3.6 Applications in Food Industry

Prebiotics have distinct advantages such as in situ stimulation of the growth of certain resident bacteria, activation of bacterial metabolism, and their own

physiological effects. FOS and inulin have strong bifodogenic activity as prebiotics. In addition to nutritional properties they also contribute to improve palatability of food products. Inulins can be incoperated into cream making to replace fat to reduce calorie in products such as spreads, margarines, and ice cream. High-molecular-mass levans have potential as food ingredients in various food products as emulsifying, thickening or stabilizing agents (Spiegel *et al.*, 1994).

Native inulin and FOS both can be used as effective binders and provide low calorie fiber sources in beverages, health bars, and confection applications either in combination with other non-sugar bulking agents such as polyols, or alone. The use of inulin and FOS has been shown to provide desirable sweetness and mask the aftertaste of several high intensity sweeteners. Unlike other fibers, inulin and FOS are unique by not contributing to objectionable flavor profiles or significant increasing the viscosity of a food system.

The commercial product of FOS is sold under the brand name Nutraflora™ and produced by Golden Technologies, Inc., Westminister, Colo. and is 0.4-0.6 times as sweet as sucrose. The commercial product is treated by β-fructofuranosidase from *Aspergillus niger* and is a mixture of GF_2, GF_3, GF_4, sucrose, glucose, ad fructose (Spiegel *et al.*, 1994).

In Japan, FOS is considered as food, not food ingredients. FOS is currently used as feed additive in poultry in the United States and Japan. Subchronic and chronic toxicity and carcinogenicity studies in rats revealed no significant adverse effects at dose up to 2170 mg/kg/day (Roberfroid, 2005). Hata and Nakajima (1985) found that the minimum dose of FOS required to induce diarrhea was 44 g for men and 49 g for

women when FOS are added to food. The daily intake of FOS from common food items has been estimated to be approximately 806 mg/day (Spiegel *et al.*, 1994).

2.4 Probiotics and Pediococcus acidilactici

2.4.1 Definition of Probiotics

Probiotics can be described as organisms and substances which contribute to intestinal microbial balance. In 1989, Fuller redefined a probiotic as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Gibson and Roberfroid, 1995).

2.4.2 *Pediococcus acidilactici*

2.4.2.1 *Introduction of* Pediococci

Pediococci are gram-positive lactic acid bacteria that are used as starters in the industrial fermentation of meat and vegetables. Gardner *et al.* (2001) studied various lactic acid bacteria for the fermentation of cabbage, carrot and beet-based vegetable products. It was found that a starter culture consisting of *P. acidilactici* AFERM772 accelerated the fermentation process and prevented deterioration of fermented products for up to 90 days (Gardner *et al.*, 2001).

In simulated gastrointestinal conditions, *P. acidilactici* had a strong capacity for surviving acidic conditions and 0.30% bile salts. At pH 3 and at pH 6 the number of this bacteria decrease approximately 1 log unit indicating that as many as 10% survived. This strain might be regarded as potentially probiotic (Erkkila and Petaja,

2000). The optimum growth temperature for *P. acidilactici* is over 40°C and it does not grow under 8°C.

2.4.2.2 Nutrition need and metabolism in *Pediococcus acidilactici*

According to Bergey's manual of systematic bacteriology (Butler, 1986), Pediococci are facultative anaerobes, but tolerant to oxygen, homofermentative, gram positive, nonmotile, and spherical cocci. Growth is dependent on fermentable carbohydrate and probably by the Embden-Meyerhof pathway (Fig. 2.4.1), to DL or L-(+) lactate. Pediococci is characterized by the splitting of fructose 1, 6-bisphosphate with aldolase into two triose phosphate moieties which are further converted to lactate. They ferment pentose via the same pathway with heterofermentative organism: pentoses are taken up by specific permeases and converted by appropriate enzymes to D-xylulose 5-phosphate which is fermented to lactate and acetate (Kandler, 1983).

P. acidilactici can grow between 35 to 50°C, pH 4.2 to 7.5. It grows rapidly on MRS agar and broth and requires the most amino acids for growth, but they can grow without the supply of methionine. This strain also requires riboflavin, pyridoxine, pantothenic acid, nicotinic acid and biotin, while purines, pyrimidines or especially leucovorin (folinic acid, an adjuvant used in cancer chemotherapy) was not needed (Sakaguchi, 1960). *P. acidilactici* can ferment glucose, fructose, maltose, galactose, lactose [wild-type cannot ferment lactose (Caldwell *et al.*, 1998)], sucrose, arabinose, ribose, and xylose, but the ability to use pentoses is limited (Table 2.4.1). Little information is available about the carbohydrate fermentation pathway, but it contains

lactate dehydrogenase in the reaction. In the Sant'Anna and Torres study, the highest biomass production was obtained when *P. acidilactici* was grown in MRS-5 (5% w/v sugar cane blackstrap molasses added in MRS base medium) broth at initial pH 6.5 (Sant'Anna and Torres, 1998). The influence of supplementation with nutrients on cell density (optical density at 600 nm) after growth of *P. acidilactici* H in TGE broth (TGE broth contains the following components: Trypticase, glucose, and yeast extract, each at 1%; Tween 80, 0.2%; Mn^{2+}, 0.033 mM; and Mg^{2+}, 0.02 mM, pH 6.5, used as a basal broth) for 16 h at 37°C was studied by Biswas *et al.* (Biswas *et al.*, 1991). Addition of sucrose 1% reached highest optical density (4.0) after 16 h incubation, galactose 1% reached 2.3, arabinose 1%, 0.6, xylose 1%, 2.0, trehalose 1%, 1.4 and raffinose 1%, 0.4 and glucose 1%, 3.5.

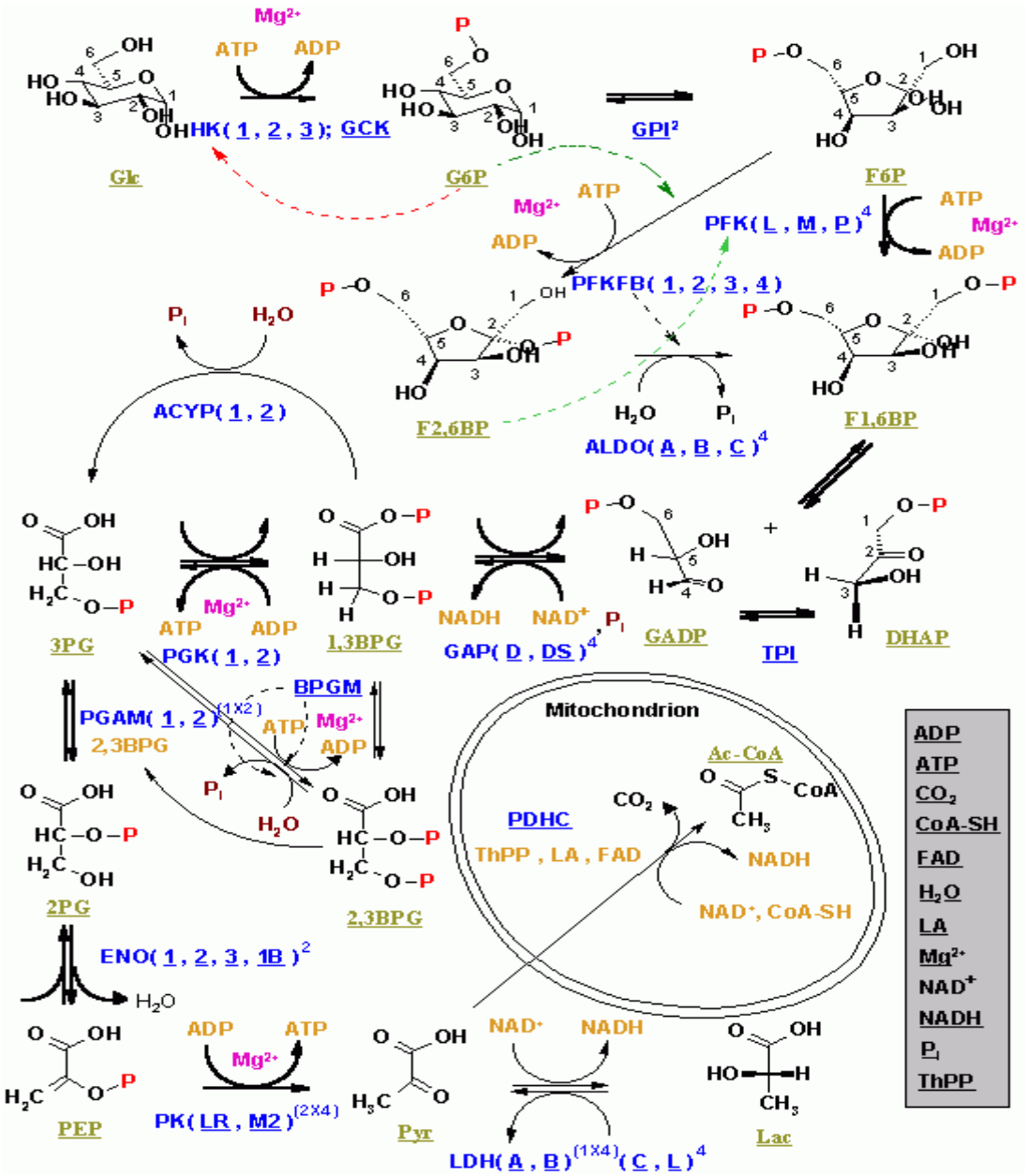


Fig 2.4.1.Glycolysis pathway.

The color scheme is as follows: **enzymes**, **coenzymes**, **substrate names**, **metal ions**, **inorganic molecules**, **inhibition**, **attached phosphate**, and **stimulation**

(**http://en.wikipedia.org/wiki/Glycolysis**, URL accessed on May 19, 2006)

Table 2.4.1. Sugar-utilizing ability of different *P. acidilactici* strains.

Carbohydrate	*Bergey's Manual*[1]	H[2]	G24[3]	IL01[4]	C20[5]
Fructose	NA	NA	+	NA	NA
Glucose	NA	+	+	+	NA
Sucrose	-	+	NA	+	+
Lactose	D	NA	NA	NA	+

Symbols: +, positive; -, 90% or more of strains are negative; D, 11-89% of strains are positive; NA, not available.

([1]Butler, 1986; [2]Halami *et al.*, 2000; [3]Sant'Anna and Torres, 1998; [4]Fitzsimons *et al.*, 1992; [5]Biswas *et al.*, 1991)

2.4.2.3 Application of *Pediococcus acidilactici*

P. acidilactici has been used as inoculants to control fermentation in human foods such as soda crackers, fermented milks and sausages. *P. acidilactici* can produce pediocin PA-1/AcH (Halami *et al.*, 2000). Pediocin has been shown to be more effective than nisin against some food-borne pathogens, such as *Listeria monocytogenes* and *Staphylococcus aureus*. Pediocin has not yet been legally approved by the regulatory agencies, nor is it available commercially. Pediocins PA is stable over a pH range of 3–8. The molecular weight of the partially purified pediocins from *P. acidilactici* is less than 5 kDa. In dry sausages fermented by

bacteriocin producing from *P. acidilactici* JD1-23, the numbers of *L. monocytogenes* per gram dry sausage (pH > 5.0) were 1-2 log units lower than in the control sausages (Erkkilä and Petaja, 2000).

Probiotics is an existed idea in the field of human and is extended to animals to promote the development of fortifying diets for the intestinal micro biota, which improve feeding yields and survival. Commercial probiotic products designed for land animals contain lactic acid bacteria, *P. acidilactici*, or yeast, *Saccharomyces cerevisiae*. *P. acidilactici* has been authorized for use as a feed additive in Europe and approved for use in piglets, sows and fattening pigs (Simon, 2005). In October 2005, Bactocell® which contains *P. acidilactici* MA 18/5M by Lallemand Company was approved by the European Commission for use as a feed additive in fattening pigs for its probiotic use. In the United States it is considered GRAS by FDA and complies with the AAFCO (Association of American Feed Control Officials) requirements. Research done with this commercial product did not provide significant increase in the weight of weaner pigs, but reducing the pH of the liquid diet to 4.00 by fermentation with *P. acidilactici* was a cost effective method of eliminating enteropathogens and spoilage organisms from the diet (Geary *et al.*, 1999). *P. acidilactici* also can be used in preservation of alfalfa for cow feed and to lower the pH value of alfalfa leaves and prevent yeast and molds spoilage (Sindou and Szucs, 2005). It is a promising probiotic for fish larvae in view of its effect as a growth promoter (Gatesoupe, 2002).The long-term dietary supplementation with *P. acidilactici* seemed promising as a preventive treatment against the vertebral column compression syndrome (VCCS) in rainbow trout (Aubin *et al.*, 2005). In eel (*Anguilla*

japonica) production, feeding with *P. acidilactici* can increase the body weight by 50% and improve the immune system (Yu *et al.*, 2005). Advantages of *P. acidilactici* supplementation include better bee survival and higher dry mass and crude fat level in comparison with bees fed with pollen substitute only (Kaznowski *et al.*, 2005).

In the present study, the feasibility of using LYCH leaves to promote the growth of *P. acidilactici* and the variations among different leaf preparation methods with respect to chemical constituents and growth-promoting effects were addressed. From a processing standpoint, if a simple operation could be established to release ingredients that stimulate the growth of probiotic cells, it would most likely be readily convertible for industrial applications and the process could be easily optimized to enhance cost effectiveness. In respect of biomass utilization and efficacy, it is highly desirable if the LYCH leaves were able to provide dual functionalities—both as a growth promoter for probiotics in feed and as a feed themselves to enhance the amino acids content and to improve the flavor, texture, and taste of the end products.

Chapter 3: Material and Methods

3.1 LYCH Leaf Samples

Leaves of *Lycium chinense* P. Mill. (Chinese wolfberry or desert-thorn), originated from Zhou Zheng Garden in Suzhou, China and now grown in California and many eastern states in the U.S. (USDA NRCS, 2006), were plucked every two weeks between June and November 2005 at the High Fall Garden in Philmont, NY. Freshly picked, chemical-free leaf samples were transported overnight via express mail to the University of Maryland, College Park. Upon arrival, the samples were cleaned by rinsing under running tap water for ca. 15 min, oven-dried at 60°C for three days, and sealed in air-tight plastic bags. The samples were stored at 4°C prior to treatments or analyses unless otherwise mentioned.

3.2 Sample Treatments

For LYCH leaf treatment, the traditional tea-making process was employed in comparison with microwave-assisted extraction (MAE), an effective method commonly used for extracting aromatic compounds from plants and as a pretreatment when analyzing soil minerals in GC or HPLC (Dean, 1998). In tea-making process, dried leaves (10.0 g) were placed in a 250 mL beaker containing 150 mL DI water and heated in a water bath at 80°C for 1 hr (Nishiyama, 1965). The hydrolysate (H1) was filtered through a Whatman No. 41 filter paper (Whatman Inc., Florham Park, NJ) and collected into three 50 mL centrifuge tubes. The leaves remaining in the beaker were added with 50 mL DI water and heated, following the same time and

temperature combination as aforementioned. The hydrolysate (H2) was filtered and collected. Both H1 and H2 hydrolysates were stored separately at -16°C before further uses. H1 and H2 combined at a 1:1 volumetric ratio H1+H2 were used in this study.

In MAE, dried LYCH leaves (5.0 g) were weighed, trimmed into small pieces, added with 100 mL DI water as a solvent, and placed into the chamber of the Ethos E Microwave Extraction Labstation (Milestone Inc., Monroe, CT) with two magnetrons (800 W ea.) installed. With the frequency set at 2,450 MHz and the processing time at 15 min, the samples were heated to 40, 80, and 120°C to produce hydrolysates M40, M80, and M120, respectively. Another hydrolysate ($M80_{2h}$) was obtained by heating the samples at 80°C up to 2 hrs. The hydrolysates were filtered, collected, and preserved at -16°C before use.

3.3 Bacteria Growth

3.3.1 Culture Preparation

Freeze-dried *Pediococcus acidilactici* IMT101, an osmotolerant starter strain used in the present study, was kindly provided by Imagilin Technology, LLC (Potomac, MD). Powdered cells (1.0 g) were hydrated with 9 mL autoclaved water in a 100 mL flask and shaken at 260 rpm for 30 min. The strain was propagated at 37°C in Man-Rogosa-Sharpe (MRS) (Fisher Scientific, Raleigh, NC) broth until the pH reached 4.6 (ca. 8 hrs). The cultures were placed on ice for 30 min to stop the acidification process and stored at 4°C until used (Champagne *et al*., 2003). Stock cultures were prepared by mixing 20 mL of freshly MRS-grown cultures with 50 mL of 20% skim milk and 50 mL of a 20% glycerol solution (glycerol and milk were sterilized

separately). The milk/glycerol/cell suspensions were divided into 1 mL fractions, added to sterile 2 mL cryovials (Nalgene, Rochester, NY), and stored at -70°C until used.

All cell growth experiments conducted in the present study were based on a 1% (v/v) inoculation of actively growing *P. acidilactici* cells into freshly prepared growth media unless specifically noted. The media compositions are discussed in the next section. The growth profiles of *P. acidilactici* cells were established by periodically removing 1% (v/v) samples (in duplicate) from the broth and centrifuging them at 10,000 rpm (9,159.4 x *g*) for 30 min under 4°C using a Beckman Coulter L7 Ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA) equipped with a Type 70.1 Ti rotor to precipitate the suspended cells. The supernatant was carefully removed and stored at 4°C for additional analyses when necessary. Cell pellets were washed twice with phosphate buffered saline (PBS) solution (Fisher Scientific Co., Raleigh, NC), vortexed, and recentrifuged to obtain media-free pellets. The cells were then resuspended in autoclaved water, reaching the concentration as in the sample, and the optical density at 600 nm (OD_{600}) was measured using a spectrophotometer (ThermoSpectronic, Rochester, NY). The OD_{600} readings (properly diluted to fall within the linear range of the calibration curve) were then compared to a calibration curve and the dilution factor to estimate the cell concentrations. The calibration curve was determined from the total cell dry weight in a concentrated solution and optical density values at various dilutions of this solution. A linear relationship between the optical density and cell density was obtained when the optical density was below 0.9.

3.3.2 Media Formulation

To establish the baseline growth profile of *P. acidilactici* cells, MRS broth prepared with purified water following the manufacturer's standard procedures was used as the control media. Growth kinetics of *P. acidilactici* grown on MRS broth containing H1+H2 at different levels (5%, 10%, 15%, and 20% v/v) was analyzed to determine the proper medium substitution level. The highest yield of *P. acidilactici* cells was reached in the medium containing 20% (v/v) H1+H2 (data not shown). Substitutions of MRS broth with hydrolysates H1+H2, M40, M80, $M80_{2h}$, and M120 (20% v/v) were conducted individually in comparison with MRS broth enriched with 2% (w/v) fructooligosaccharides (FOS) (Sigma-Aldrich, St. Louis, MO) as the growth media for *P. acidilactici* (1% inocula) (Rossi *et al.*, 2005). All media were autoclaved at 121°C for 30 min, cooled to room temperature, and kept sterile until use.

To evaluate the feasibility of using LYCH leaf hydrolysates as the carbon source for *P. acidilactici* cells, two different hydrolysates, namely H1+H2 and $M80_{2h}$, were incorporated (20% v/v) into M17 broth (Difco Laboratories Inc., Detroit, MI) in comparison with M17 broth supplemented separately by fructose, glucose, sucrose, or FOS (Sigma-Aldrich, St. Louis, MO) to the final concentration of 10 g/L (Rossi *et al.*, 2005). The growth profiles, as well as the viable cell counts of *P. acidilactici* after incubation at 35°C for 48 hrs on MRS agar, were analyzed.

3.4 Analytical Methods

3.4.1 Amino Acids Analysis

The hydrolysate samples were added with norvaline, an internal calibrator, before hydrolyzed with 6 M HCl containing 1% phenol at 110°C for 24 hrs, cooled, and dried. The samples were then dissolved in a sodium citrate buffer and properly diluted to accommodate the range of instrument sensitivity (1-16 nmol/injection) before analyzed by ion-exchange chromatography on a Hitachi L-8800 amino acid analyzer (Hitachi High Technologies America, Inc., Palo Alto, CA) to determine the amount of free amino acids in the hydrolysates.

3.4.2 FOS Analysis

The total concentration of FOS, which is composed of glucose-$(fructose)_n$ with β−2→1 linkage between the fructose monomer units, in the hydrolysates was first estimated using a calculation method (Hoebregs, 1997; Prosky and Hoebregs, 1999). Aliquots (0.9 mL) of LYCH leaf hydrolysates were mixed with 0.1 mL inulinase (2,259 U/g; density 1.2 g/mL) (Sigma-Aldrich, St. Louis, MO) and incubated at 60°C for 30 min. The amount of sucrose was measured, as well as the fructose and glucose contents before and after inulinase treatment, using enzymatic assays (Sigma-Aldrich, St. Louis, MO). The concentration of total FOS could be calculated based on the following equations:

$$G = G_t - \frac{S}{1.9} - G_f \quad (1)$$

$$F = F_t - \frac{S}{1.9} - F_f \quad (2)$$

where G and F represent the glucose and fructose from FOS, and G_t, G_f, F_t, and F_f indicate the total glucose, initial free glucose, total fructose, and initial free fructose, respectively. $S/1.9$ is the amount of glucose or fructose from sucrose. The total FOS is the sum of G and F and corrected for the water loss during hydrolysis. Thus,

$$Total\ FOSs = k\,(G + F) \quad (3)$$

where $k = 0.925$ for FOS with an average degree of polymerization (DP) of 4 or $k = 0.91$ for the inulin-type (linear) FOS that has an average DP of 10 (Pedreschi *et al.*, 2003).

To quantitatively determine the amount of FOS in the hydrolysates, reversed phase-high performance liquid chromatography (RP-HPLC) analysis (Gan, 1999) was performed on a Shimadzu LC 2010A system equipped with a RID-10A refractive index detector (Shimadzu Corp., Columbia, MD). The unit was interfaced to a computer through a Versa Comm+4 PCI data acquisition board (Sealevel Systems Inc., Liberty, SC) that integrated the data into the Class VP software (Shimadzu Corp., Columbia, MD). For all separations, a Waters reversed-phase μ-Bondapak C_{18} column (3.9 × 300 mm, 10 μm particle size) with a guard column (Waters Associates Inc., Milford, MA) was used. The mobile phase was HPLC-grade water (Fisher Scientific, Fair Lawn, NJ). The separation temperature was kept constant at 30°C,

flow rate and sample volume were set to 0.8 mL/min and 10 µL, respectively. To enhance sample separation, the hydrolysates were concentrated 10 times and filtered through a 0.45 µm filter before injected into the HPLC. The sampling frequency was set at 5.00032 Hz to achieve the optimal resolution. Peaks were assigned by spiking separately the samples with standard solutions of fructose, glucose, sucrose, and FOS (Sigma-Aldrich, St. Louis, MO), and comparison of the retention times on the chromatograms.

Fig 3.6.1. HPLC system: (left to right) Monitor, Computer with Class VP software and Versa Comm+4. PCI data acquisition board, Shimadzu RID 10A refractive index detector, and Shimadzu LC 2010A HPLC system.

3.4.3 Monosaccharides Analysis

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), a commonly used technique for the chain length analysis of amylopectin, was employed to profile the monosaccharides present in the

hydrolysates. Freshly prepared hydrolysates were treated with 2 M trifluroacetic acid at 100°C for 4 hrs to cleave all glycosidic linkages. After drying, samples were dissolved in water and analyzed by Dionex DX-500 HPLC (Dionex, Sunnyvale, CA) equipped with an LC20 chromatography enclosure, and an ED40 pulsed amperometric/conductivity detector (PAD) using a CarboPac PA-1 (4 × 250 mm) analytical column (Dionex, Sunnyvale, CA) eluted with 200 mM NaOH. Common monosaccharide standards (mannose, galactose, glucose, N-acetylglucosamine, N-acetylgalactosamine, fructose, and xylose) were treated in parallel and used for calibration.

3.5 Statistic Analysis

The cell density and total viable cell count were analyzed using the general linear model (GLM) of ANOVA using Statistical Analysis System version 6.02 (SAS Institute Inc., Cary, NC). Means of three replicates were reported. Cell counts were converted into logarithm values to determine the significance of differences at the 95% confidence limit ($P < 0.05$). Pairwise mean differences were evaluated using the Tukey's test.

Chapter 4: Results and Discussion

4.1 Bacteria Growth

4.1.1 Growth of *Pediococcus acidilactici* IMT101

Calibration curve (Fig. 4.1) was obtained by taking optical density and measuring dried bacteria weight. The growth curve was obtained by converting optical density to concentration of bacteria via the equation from the calibration curve: Concentration= (OD_{600}-0.0152) /3.9702.

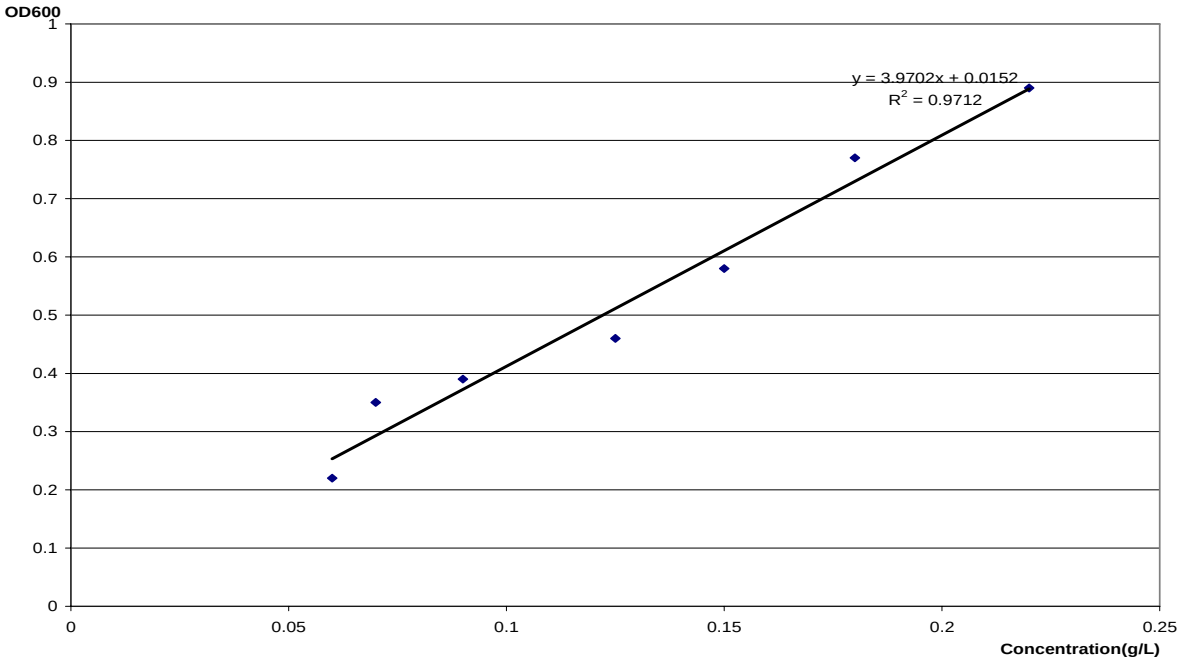


Fig 4.1. Calibration curve of *Pediococcus acidilactici* IMT101.

The typical growth profiles of *P. acidilactici* IMT101 cells in MRS broth (control), in comparison with MRS broth supplemented with 20% (v/v) H1+H2 or $M80_{2h}$ and MRS enriched with 2% (w/v) FOS, indicate that *P. acidilactici* cells

grown in MRS broth containing 20% H1+H2 had a shortened lag phase and entered exponential phase ca. 2 hrs earlier than in other media studied (Fig. 4.2). Determined in part by characteristics of the bacterial species and in part by conditions in the media (Black, 1996), the lag phase of bacteria can be shortened if they are supplied with metabolic intermediates, vitamins, amino acids, etc. *P. acidilactici* requires most amino acids for growth (Jensen and Seeley, 1954; Sakaguchi, 1960; Raccach, 1999), yet specific requirements remain unknown (Garvie 1984; Deguchi and Morishita, 1992).

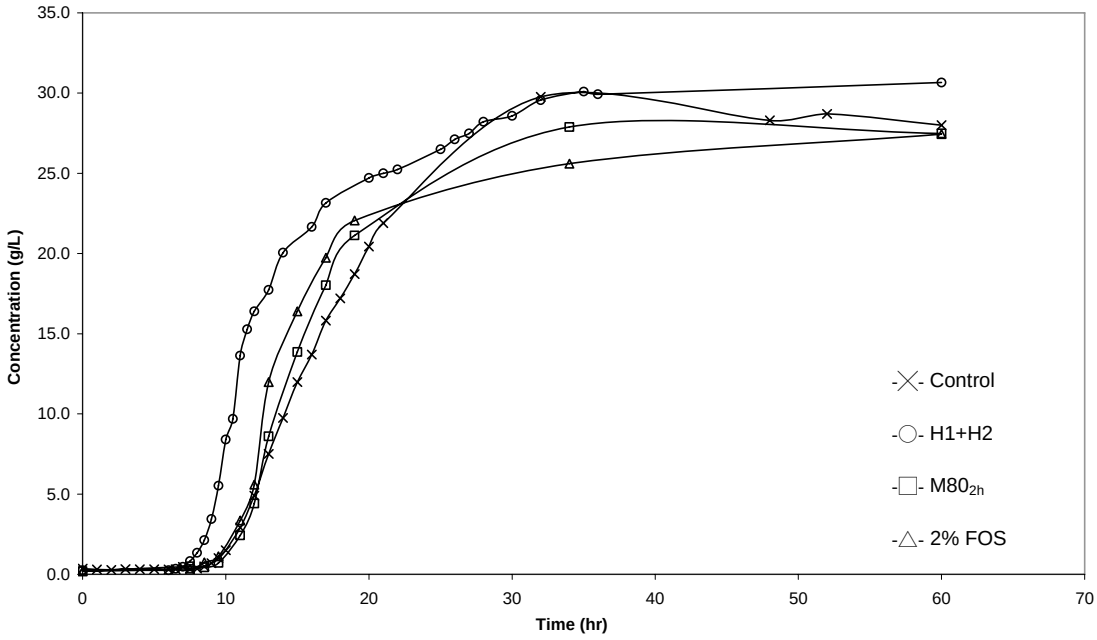
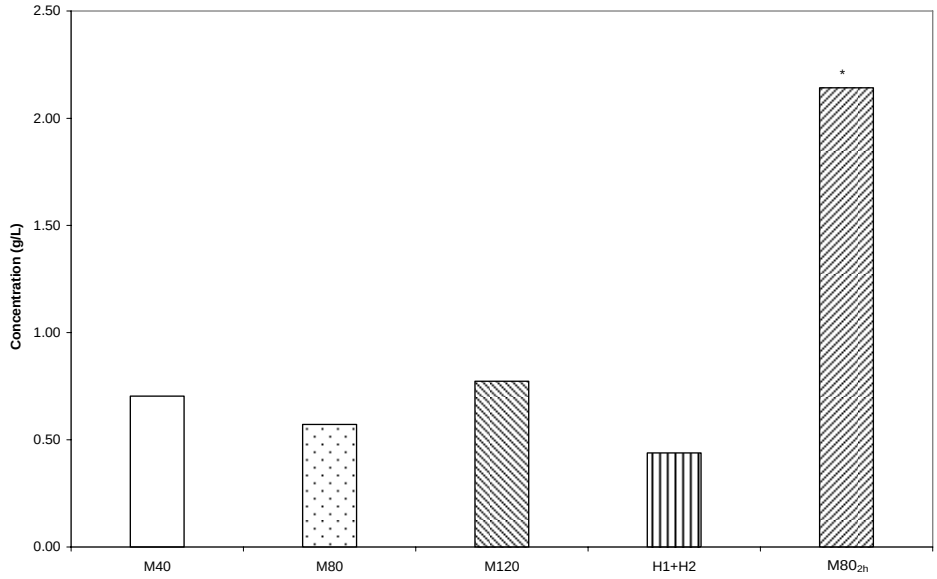


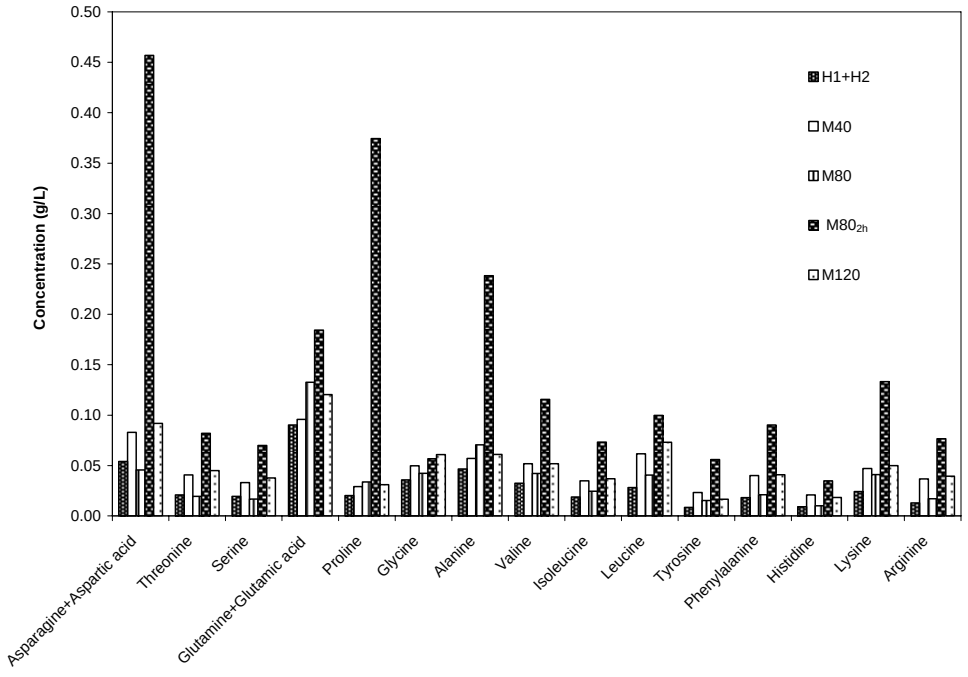
Fig. 4.2. Typical growth profiles of *P. acidilactici* IMT101 cells in MRS broth (×), MRS broth supplemented with 20% (v/v) H1+H2 (○) or M80$_{2h}$ (□), and MRS enriched with 2% (w/v) FOS (Δ).

Analysis of amino acids in H1+H2, M40, M80, M80$_{2h}$, and M120 showed that M80$_{2h}$ contained the highest amount of amino acids both in total, more than fourfold

of that in H1+H2 (Fig. 4.3a), and individually (Fig. 4.3b). Methionine and lysine have been found stimulatory to the growth of pediococci (Raccach and Tully, 1999); however, only small amount of lysine was detected in LYCH hydrolysate samples. Despite the slight variations found in samples collected in different months, in agreement with Terauchi *et al.* (1997), asparagine + aspartic acid, proline, and alanine were found the most abundant amino acids in $M80_{2h}$. However, no significant reduction in lag phase was observed with *P. acidilactici* cells grown in MRS broth supplemented with 20% $M80_{2h}$ when compared with the control. On the other hand, H1+H2 surprisingly contained the lowest total amino acid concentration and in the majority of individual amino acids, indicating that the growth stimulating effect (lag phase reduction) observed in medium supplemented with H1+H2 (Fig. 4.2) did not have direct correlation with the level of amino acids in the medium. The results were in agreement with Nishiyama (1969) who reported the growth stimulating effects of the aqueous extract of LYCH leaves on lactic acid bacteria and identified strong presence of a spectrum of amino acids in the extract. However, in a following study in which a mixture of 22 amino acids were added to the growth medium for lactic acid bacteria, no significant growth-stimulating effects were observed despite a notable increase in acid production (Nishiyama and Kaya, 1969ab).



(a)



(b)

Fig. 4.3. Comparison of amino acid concentrations (g/L) in different LYCH leaf hydrolysates investigated: (a) Total amino acids; (b) breakdown of individual amino acids. Column with * is significantly higher than the others ($P < 0.05$).

The growth of *P. acidilactici* IMT101 cells in various growth media was characterized by the total viable cell count entering stationary phase (X_s) and the maximum specific growth rate (μ_{max}), an empirical parameter obtained from the steepest slope of the semi-logarithmic plot of cell density vs. growth time as defined by the Monod equation (Gardner *et al.*, 2001) (Fig. 4.4). *P. acidilactici* grown in MRS supplemented hydrolysates obtained by MAE for 15 min, namely M40, M80, and M120, did not show any significant differences in X_s or μ_{max} when compared with the control (100% MRS). On the contrary, cells grown in MRS broth containing 20% H1+H2 showed the highest values in X_s (5.5×10^9 CFU/mL) and μ_{max} (3.5 h^{-1}), both significantly higher than those obtained in other media investigated ($P < 0.05$). Cells grown in MRS supplemented with 20% $M80_{2h}$, the same extraction conditions as in M80 but for an extended period of time (2 hrs), showed an increase in X_s compared with the control, similar to the effect of MRS enriched by 2% (w/v) FOS, recognized prebiotics with growth stimulating effects on probiotic cultures (Wang and Gibson, 1993; Tokunaga, 2004). Although the increases of X_s in $M80_{2h}$- and 2% FOS-enriched MRS broth were statistically insignificant in relation to the control, such positive effects remained relatively consistent in all replicates studied (n = 3).

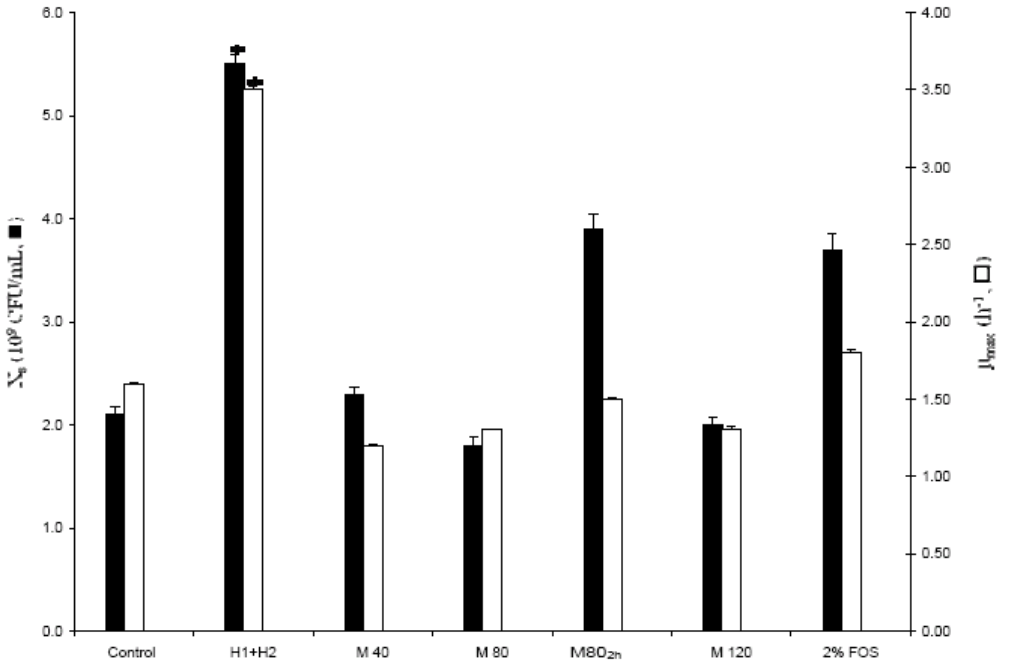


Fig. 4.4. Comparison of growth kinetics of *P. acidilactici* IMT101 cells grown in MRS broth supplemented with various LYCH leaf hydrolysates (20% v/v) or enriched with 2% (w/v) FOS. X_s: the total viable cell counts entering stationary phase; μ_{max}: the maximum specific cell growth rate. Columns with * are significantly higher than the others ($P < 0.05$).

4.1.2 Sugar Utilization by *P. acidilactici* IMT101

To assess how *P. acidilactici* IMT101 cells utilize fermentable sugars, M17 broth supplemented with H1+H2 and $M80_{2h}$ was employed in comparison with various carbon sources, including fructose, glucose, sucrose, and FOS (Fig. 4.5). While cells grown in fructose and glucose showed X_s at the level of 1-2 × 10^8 CFU/mL, *P. acidilactici* grown in $M80_{2h}$ reached a higher X_s, ca. 10^9 CFU/mL. Cells in M17 containing H1+H2 and sucrose both reached $X_s > 10^9$ CFU/mL. M17 broth plus H1+H2 yielded the highest cell concentration (2.1 × 10^9 CFU/mL), significantly higher than those achieved with fructose or glucose and even higher than when FOS was used as the sole carbon source (1.7 × 10^9 CFU/mL). The elevated level of X_s reached when M17 was supplemented with 20% (v/v) H1+H2 could be attributed to its higher level of fructose, glucose, and sucrose in comparison with M17 containing 20% (v/v) $M80_{2h}$ (Table 4.1).

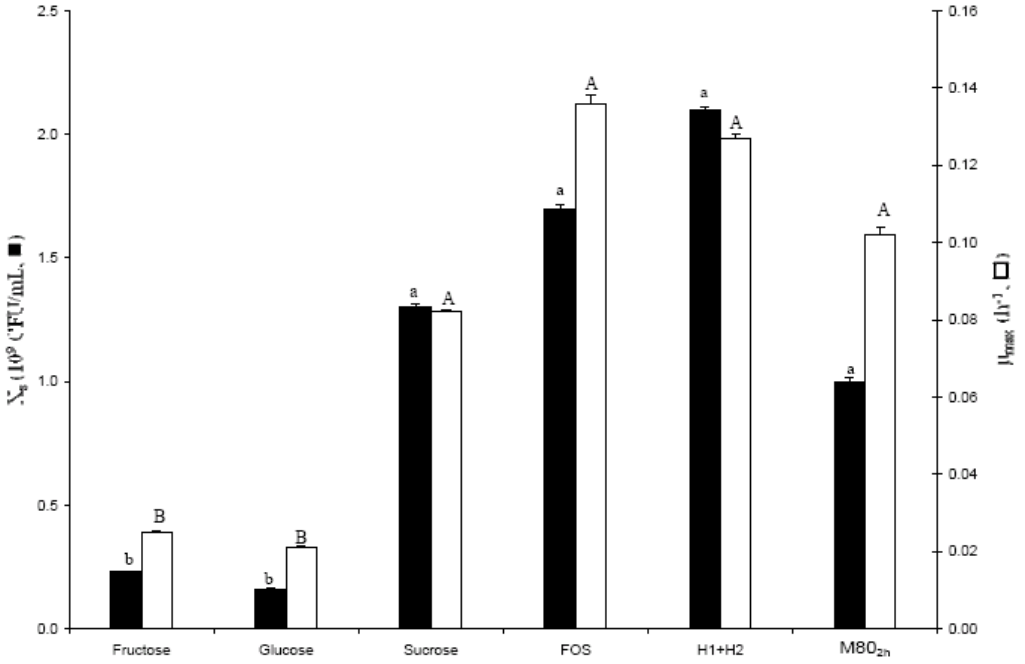


Fig. 4.5. Comparison of growth kinetics of *P. acidilactici* IMT101 cells grown in M17 broth supplemented with LYCH leaf hydrolysates (20% v/v) H1+H2 or M80$_{2h}$ and M17 broth enriched by fructose, glucose, sucrose, or FOS to the final concentration of 10 g/L. Columns bearing the same letter are not significantly different ($P < 0.05$).

Table 4.1. Comparison of sugar contents in growth media (M17) supplemented with 20% (v/v) LYCH leaf hydrolysates H1+H2 or M80$_{2h}$.

Sugar* (mg/mL)	80% (v/v) M17 + 20% (H1+H2)	80% (v/v) M17 + 20% M80$_{2h}$
Fructose	0.098 ± 0.011	0.025 ± 0.009
Glucose	0.26 ± 0.005	0.12 ± 0.012
Sucrose	0.98 ± 0.010	0.20 ± 0.002
Total	1.338	0.345

*Mean ± SD, n = 3.

The μ_{max} of *P. acidilactici* IMT101 cells grown in M17 supplemented with H1+H2 was in the same range as that in FOS-enriched M17, and was significantly

higher in relation to those grown in fructose- or glucose-supplemented M17 broth (Fig. 4.5). The μ_{max} of M17 supplemented with sucrose was significantly higher than M17 with fructose or glucose, but relatively lower than with FOS, H1+H2, or $M80_{2h}$. All values of μ_{max} reached in M17-based broth, as expected, were much lower than those achieved in MRS-based broth (Section 4.1.1). This could be attributed to the growth promoting effects of MRS medium, which because of the high consumption of carbohydrate resulted in almost an order of magnitude greater production of lactate in comparison with other basic growth media (Vázquez Alvarez *et al.*, 2003).

Although the metabolism of simple sugar in *P. acidilactici* IMT101 remains unclear to date, more than 90% of positive growth when lactose and trehalose were used as the sole carbon source has been reported in Bergey's Manual of Systematic Bacteriology (Butler, 1986). Undergoing homofermentative pathways that produce lactate exclusively, *Pediococus* is known to enter glycolysis by the splitting of fructose 1,6-bisphosphate with aldolase into two triose phosphate moieties that are further converted to lactate (Kandler, 1983). While glucose and most other monosaccharides are known to be fermented by *P. acidilactici,* the ability to use pentose remains inconclusive (Garvie, 1984; Riebel and Washington, 1990). Kandler (1983) suggested *Pediococcus* could ferment pentoses readily, yet Caldwell *et al.* (1998) reported that the ability of *P. acidilactici* to use pentoses is limited. Nonetheless, the necessity of phosphoketolase for pentose fermentation is recognized (Kandler, 1983).

Moreover, Biswas and coworkers (1991) reported that the yield of *P. acidilactici* H cells was higher in sucrose and glucose than in other carbon sources (arabinose,

xylose, trehalose, and raffinose), whereas *P. acidilactici* C20 showed 90% positive growth in sucrose, lactose, maltose, raffinose, and trehalose. *P. acidilactici* G24, when used as a silage inoculant, was found to grow on glucose and fructose with a short lag phase, a rapid acid production rate, and was able to grow within a broad range of pH and temperature (Fitzsimons *et al.*, 1992). The ability of *P. acidilactici* IL01 to grow on MRS broth substituting glucose with sugar cane molasses (Sant'Anna and Torres, 1998) also suggests that the efficiency of sugar utilization is strain-specific for *P. acidilactici*.

4.2 Determination of FOS and Other Monosaccharides

As discussed, addition of FOS (10 g/L) in M17 broth showed significant increases in *P. acidilactici* cell yield (X_s) as well as the maximal specific growth rate (μ_{max}) in comparison with fructose and glucose (Fig. 4.5). Such an increase was also observed in M17 broth containing 20% (v/v) of H1+H2, suggesting possible presence of FOS in H1+H2. By using inulinase treatment, which enables endohydrolysis of 2,1-β-D-fructosidic linkages in inulin, followed by the calculation method of Hoebregs (1997), the amounts of FOS in LYCH leaf hydrolysates could be estimated. It was found that in M120 and M80 the estimated FOS contents were significantly higher than those in H1+H2 and M40 ($P < 0.05$) (Table 4.2). This approach, which relies on the enzymatic treatment of samples with an inulinase, followed by determination of the released sugars, is appropriate for mixtures of molecules consisting of fructose moieties linked to each other by β (2→1) bonds with glucose molecules linked to the end of the chain by an α (1→2) bond as occurred in sucrose (Prosky and Hoebregs, 1999).

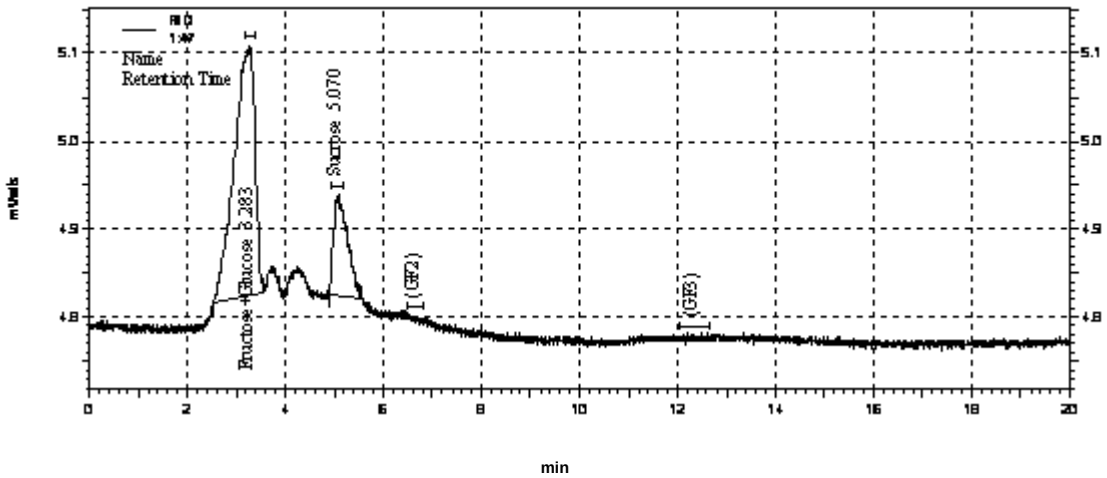
Table 4.2. Estimated FOS concentrations in different LYCH leaf hydrolysates based on Hoebregs (1997) using the concentration difference of fructose and glucose before/after inulinase treatment and the sucrose content.

Content* (mg/g dried leaf)	H1+H2	M40	M80	$M80_{2h}$	M120
Fructose	1.92/2.25	0.00/0.00	0.68/0.72	0.50/2.20	0.39/0.44
Glucose	5.22/5.91	0.21/0.45	0.00/1.21	2.40/10.60	1.49/3.59
Sucrose	0.63	0.00	0.00	4.00	1.57
Estimated FOS	0.33^{a}	0.22^{a}	1.16^{b}	5.27^{c}	1.90^{b}

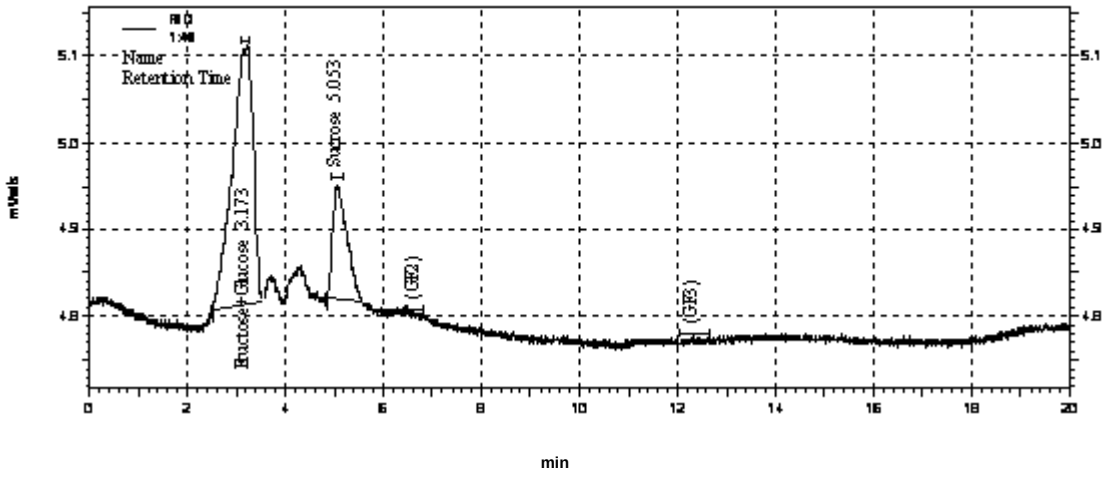
*Only mean values are shown (n = 3); all measurements with SD < 0.02.
Values bearing the same superscript in the same row are not significantly different ($P < 0.05$).

Direct measurement of FOS contents is thus desirable and could be achieved by using RP-HPLC with an RI detector (Gan, 1999). While fructose and glucose both eluted chromatographically in one combined peak at retention time around 3.2 min, short-chain FOS, including GF (sucrose), GF_2 (kestose), and GF_3 (nystose) could be separated at 5.0, 6.7, and 12.4 min, respectively (Fig. 4.6). Based on the aforementioned estimated FOS contents (Table 4.2), peaks representing GF_2 and GF_3 were supposed to show up in the RP-HPLC chromatogram of H1+H2, with even higher peaks expected in the $M80_{2h}$ chromatogram, since the estimated FOS content in $M80_{2h}$ was significantly higher than that in H1+H2. Surprisingly, however, no detectable amounts of GF_2 or GF_3 were present in LYCH leaf hydrolysate H1+H2 or $M80_{2h}$ (Fig. 4.6). This could be attributed to the inherent inaccuracies of the estimation method. The FOS concentration in this case is calculated by the difference from glucose and fructose determinations before and after the hydrolysis with

inulinase, small inaccuracies in the determination of high glucose or sucrose values from samples containing high levels of carbohydrates could significantly influence the small glucose content resulting from the FOS (Prosky and Hoebregs, 1999). Such discrepancies could also be due to the hydrolysis of long-chain oligofructose or the presence of fructan-metabolizing enzymes that cleavage branched fructans (Pavis *et al.*, 2001). However, further investigations are needed to identify the presence of these enzymes.



(a)



(b)

Fig. 4.6. RP-HPLC chromatograms showing the separation of monosaccharides (fructose and glucose), GF (sucrose), GF_2 (kestose), and GF_3 (nystose) in (a) H1+H2; and (b) $M80_{2h}$.

Measurements of monosaccharides using the HPAEC-PAD method revealed that glucose was the most abundant monosaccharide in the H1+H2 hydrolysate (Fig. 4.7). It is recognized that glucose could be readily transported into the pediococcal cell via the phosphoenolpyruvate:phosphotransferase system (PEP:PTS) and undergoes glycolysis utilizing the Embden-Meyerhof-Parnas (EMP) pathway yielding pyruvate. The pyruvate is then reduced to lactic acid with the coupled reoxidation of NADH to NAD^+ (Kandler, 1983). Therefore, based on the results gathered in the present study, the LYCH hydrolysate H1+H2, when added to the growth medium for *P. acidilactici* IMT101, elevated the level of glucose in the medium, consequently shortened the lag phase, increased the cell yields, and accelerated the specific cell growth rate.

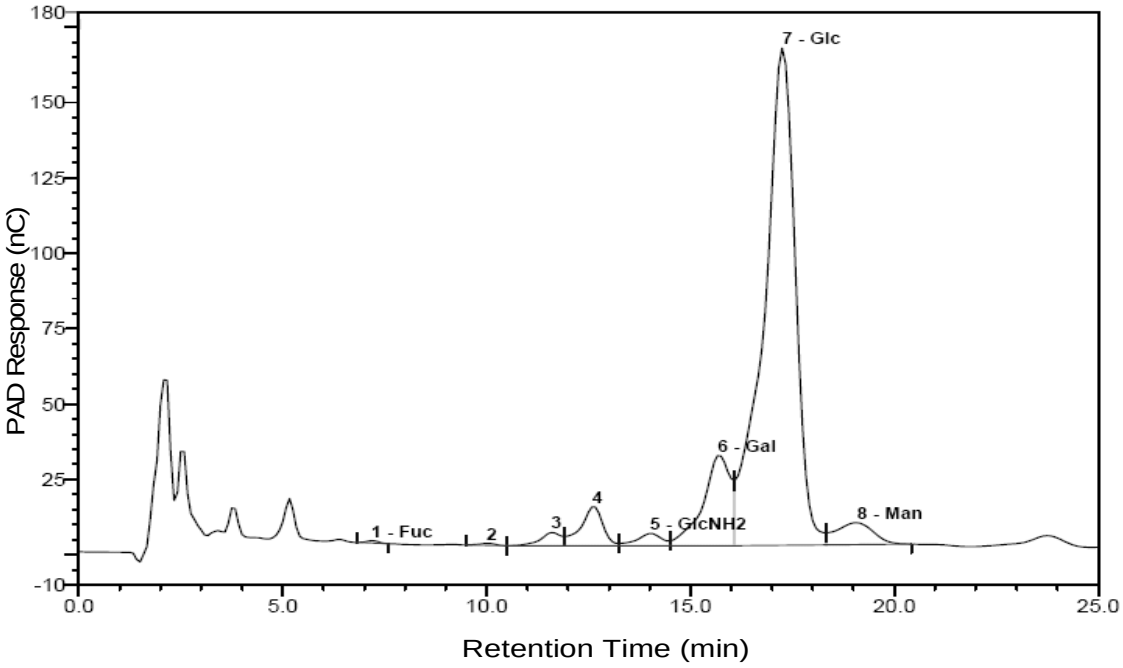


Fig. 4.7. HPAEC-PAD chromatograms of monosaccharides in H1+H2. Peaks shown include 1—Fuc; 2 & 3—unidentified; 4—GalNH2; 5—GlcNH2; 6—Gal; 7—Glc; and 8—Man.

Also seen in Fig. 4.7, small quantities of galactose were present in H1+H2. Although it is unclear whether *P. acidilactici* IMT101 is capable of utilizing galactose, existence of intracellular β-galactosidase has been shown in some pediococci strains, with the synthesis of β-galactosidase inducible by galactose (Raccach, 1999). Investigations into the activities of β-galactosidase in *P. acidilactici* IMT101 cells are recommended in order to provide additional evidence correlating the utilization of galactose with cell growth kinetics. Detailed analysis of LYCH leaf hydrolysate H1+H2, which is prepared by a simple, traditional tea-making process, is also required to elucidate the spectrum of compounds present in the hydrolysate that could be responsible for the growth stimulating effects observed in the present study. After all, the same as other chemoorganotrophs, *P. acidilactici* requires an array of vitamins and metals (e.g. potassium and magnesium) for growth besides carbohydrates and amino acids (Jensen and Seeley, 1954; Cho *et al.*, 1996).

Chapter 5: Conclusions

By using a simple, traditional tea-making process, LYCH leaf hydrolysates H1+H2 provided notable growth-stimulating effects on *P. acidilactici* IMT101 cells grown in partially substituted MRS broth with a shortened lag phase, an elevated cell concentration (X_s) entering stationary phase, and the highest maximal specific growth rate (μ_{max}). In the absence of FOS, the high glucose concentration in the H1+H2 hydrolysates was found responsible for the enhanced growth kinetics of *P. acidilactici* cells. Further studies are required to fully elucidate the spectrum of compounds in H1+H2 stimulatory to the growth of *P. acidilactici* IMT101.

Appendices

Correspondent Experimental Data

Cell Density (g/L)	O.D. 600
0.06	0.22
0.07	0.35
0.09	0.39
0.125	0.46
0.15	0.58
0.18	0.77
0.22	0.89

Fig 4.1. Calibration curve of *Pediococcus acidilactici* IMT101.

	Control		H1+H2		$M80_{2h}$		2% FOS
Hr	Cell Density (g/L)	Hr	Cell Density (g/L)	Hr	Cell Density (g/L)	Hr	Cell Density (g/L)
0	0.350648	0	0.25687	0	0.191633	0	0.191633
1	0.30172	6	0.25687	7.5	0.497431	7.5	0.354725
2	0.273179	6.5	0.338416	8.5	0.428117	8.5	0.737991
3	0.322107	7	0.472967	9.5	0.701296	9.5	1.11718
4	0.318029	7.5	0.81546	11	2.425994	11	3.355618
5	0.318029	8	1.337354	12	4.411639	12	5.581824
6	0.30172	8.5	2.136505	13	8.603103	13	11.98726
6.5	0.318029	9	3.445319	15	13.86282	15	16.39075
7	0.472967	9.5	5.520664	17	18.02167	17	19.73413
7.5	0.322107	10	8.399238	19	21.12041	19	22.05819
8	0.358802	10.5	9.683588	34	27.88873	34	25.60544
8.5	0.489276	11	13.63857	60	27.481	60	27.44023
9	0.693141	11.5	15.26949				
9.5	1.015248	12	16.39075				
10	1.496369	13	17.73626				
11	2.91527	14	20.06032				
12	4.909069	16	21.65046				
13	7.502232	17	23.15906				
14	9.744747	20	24.70844				
15	11.98726	21	24.99385				
16	13.69973	22	25.23849				
17	15.81992	25	26.50245				
18	17.20621	26	27.11405				
19	18.71481	27	27.481				
20	20.42727	28	28.21492				
21	21.8951	30	28.58187				
32	29.76429	32	29.56043				
48	28.29646	35	30.09047				
60	28.70419	36	29.92738				
		60	30.6613				

Fig. 4.2 Typical growth profiles of *P. acidilactici* IMT101 cells in MRS broth (×), MRS broth supplemented with 20% (v/v) H1+H2 (○) or $M80_{2h}$ (□), and MRS enriched with 2% (w/v) FOS (Δ).

	H1+H2	$M80_{2h}$	M40	M80	M120
Asparagine+Aspartic acid	0.054	0.457	0.083	0.046	0.092
Threonine	0.021	0.082	0.041	0.019	0.045
Serine	0.019	0.070	0.033	0.017	0.038
Glutamine+Glutamic acid	0.090	0.184	0.096	0.132	0.120
Proline	0.020	0.374	0.029	0.034	0.031
Glycine	0.036	0.057	0.050	0.042	0.061
Alanine	0.047	0.238	0.057	0.071	0.061
Valine	0.032	0.116	0.052	0.042	0.052
Isoleucine	0.019	0.073	0.035	0.024	0.037
Leucine	0.028	0.100	0.062	0.041	0.073
Tyrosine	0.008	0.056	0.023	0.015	0.017
Phenylalanine	0.018	0.090	0.040	0.021	0.041
Histidine	0.009	0.035	0.021	0.010	0.018
Lysine	0.024	0.133	0.047	0.041	0.050
Arginine	0.013	0.077	0.037	0.017	0.039
Total AA (g/L)	0.440	2.142	0.704	0.572	0.774

Fig. 4.3. Comparison of amino acid concentrations (g/L) in different LYCH leaf hydrolysates investigated: (a) Total amino acids; (b) breakdown of individual amino acids.

	X_s	SD	μ_{max}	SD
Control	1.6	0.01	2.1	0.07
H1+H2	3.5	0.02	5.5	0.09
M 40	1.2	0.01	2.3	0.07
M 80	1.3	0.01	1.8	0.09
$M\ 80_{2h}$	1.5	0.01	3.9	0.14
M 120	1.3	0.02	2.0	0.08
2% FOS	1.8	0.02	3.7	0.15

Fig. 4.4. Comparison of growth kinetics of *P. acidilactici* cells grown in MRS broth supplemented with various LYCH leaf hydrolysates (20% v/v) or enriched with 2% (w/v) FOS. X_s: the total viable cell counts entering stationary phase; μ_{max}: the maximum specific cell growth rate.

	X_s	SD	μ_{max}	SD
Fructose	0.23	0.003	0.025	0.0002
Glucose	0.16	0.007	0.021	0.0003
Sucrose	1.3	0.013	0.082	0.0004
FOS	1.7	0.016	0.136	0.002
H1+H2	2.1	0.009	0.127	0.001
M 80_{2h}	1.0	0.014	0.102	0.002

Fig. 4.5. Comparison of growth kinetics of *P. acidilactici* cells grown in M17 broth supplemented with LYCH leaf hydrolysates (20% v/v) H1+H2 or $M80_{2h}$ and M17 broth enriched by fructose, glucose, sucrose, or FOS to the final concentration of 10 g/L.

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