Studies on Thermotolerant Acetic Acid Bacteria from Sri Lanka: Taxonomic Characterization and Analyses of Membrane-bound Enzyme Activities and Pellicle Polysaccharides

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List of Abbreviations

°C	degree Celsius
µl	microliter (s)
5KGA	5-keto-D-gluconic acid
AAB	acetic acid bacteria
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
AO	acetate oxidation
AR	acetate resistance
C	Carbon
CO_2	Carbon dioxide
CPS	capsular polysaccharide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EO	ethanol oxidation
EPS	exopolysaccharides
FAO	food and agriculture organization
FDA	food and drug administration
g	gram (s)
ITS	internal transcribed spacer
kDa	kilodalton(s)
KPB	Potassium phosphate buffer

LAB	lactic acid bacteria
M	molar
MDH	methanol dehydrogenase
mg	milligram (s)
ml	milliliter
mM	millimolar
N	normality
NaCl	Sodium chloride
NADH	nicotinamide adenine dineucleotide
NaOH	Sodium hydroxide
NJ	neighbor joining
nm	nanometer (s)
OD	Optical Density
PCR	Polymerase chain reaction
PQQ	Pyrroloquinoline quinone
Q	ubiquinone
R	rough colony
rDNA	ribosomal Deoxyribonucleic acid
rpm	rounds per minute
S	smooth colony
TCA	Tricarboxylic acid
v/v	volume/volume
w/v	weight/volume
WHO	World Health Organization

Abstract

Acetic acid bacteria (AABs) are of obligate aerobic Gram-negative bacteria with the ability to oxidize ethanol to acetic acid. Among them, *Acetobacter* and *Gluconacetobacter* are used in industrial vinegar production. Increasing temperature in the recent years poses a serious challenge to fermentation industries since a large cooling system is required for maintaining the optimum temperature, which increases the production cost. Thus, development of thermotolerant strains would be beneficial. In this thesis, isolation, characterization, and analysis of membrane bound enzymes and pellicle polysaccharides of thermotolerant AAB from Sri Lankan coconut water vinegar has been done.

From the pellicle formed on the top of brewing coconut water vinegar, three *Acetobacter* strains (SL13E-2, SL13E-3, and SL13E-4) which can grow at 42^{o}C and four *Gluconobacter* strains (SL13-5, SL13-6, SL13-7, and SL13-8) which grow at 37°C were isolated. The three *Acetobacter* strains could tolerate up to 8% and 3% ethanol concentration at 41°C and 42°C, respectively. Phenotypic and biochemical characterizations, and 16S rDNA sequence analysis, showed that the three *Acetobacter* strains belonged to *Acetobacter pasteurianus*, although their 16S rDNA sequences differed only by 1-3 bp. The 16S-23S internal transcribed spacer (ITS) sequences of SL13E-2 and SL13E-3 were identical; the ITS sequence of SL13E-4 differed by 6 bp. In contrast, all four *Gluconobacter*-type strains had identical 16S rDNA sequences; these sequences, together with biochemical characterization, allowed these strains to be identified as *Gluconobacter frateurii*. The ITS sequence data of SL13-5 and SL13-7 were identical, as were those of SL13-6 and SL13-8, and there was only 1 bp difference between SL13-5/SL13-7 and SL13-6/SL13-8. The ITS sequence data indicate that SL13-5 and SL13-7 are probably identical and SL13-6 and SL13-8 are likely identical. Acetic acid production by the isolated *Acetobacter* strains was examined: all three strains gave 4% acetic acid at 37°C and 6% initial ethanol, and at 40°C provided 2.5% acetic

acid with 4% initial ethanol. Compared with the two other strains, SL13E-4 showed both slower growth and slower acetic acid production rate. As well as thermotolerant SKU1108 strain, ADH and ALDH activities of SL13E-2 and SL13E-4 were more stable than those of the mesophilic strain. The isolated strains were used to produce coconut water vinegar at higher temperatures than typically used for vinegar production.

Acetobacter species are well known to have an ability to grow floating on the surface of the medium by producing pellicle, which consists of cells and a self-produced matrix of cell-attached polysaccharides. Thus, characterization of pellicle polysaccharides of *A. pasteurianus* SL13E-2, SL13E-3, and SL13E-4 from Sri Lankan coconut vinegar and *A. pasteurianus* SKU1108, which was originally isolated from Thailand, was done. The monosaccharide composition of pellicle polysaccharides of *A. pasteurianus* strains found to be varied among strains. The pellicle polysaccharides of SL13E-2 composed of rhamnose and glucose in the ratio of 1:8 and that of SL13E-4 and mesophilic *A. pasteurianus* NBRC3191 consisted of rhamnose, glucose, and xylose in the ratio of 1:5:2 and 1:4:2, respectively. On the other hand, the pellicle polysaccharides of SL13E-3 and SKU1108 strains were composed of rhamnose, glucose, and galactose in the ratio of 2:2:1 and 2:10:5 respectively. The pellicle formation of thermotolerant SL13E-2, SL13E-3 and SL13E-4 strains was significantly induced by the addition of ethanol, while a poor induction was observed in SKU1108 strain. The molecular size and sugar composition of the polysaccharides obtained from the ethanol induced cells were unchanged from those of the uninduced cells, indicating that number of molecules of the polysaccharides increased and not changed the individual molecule of polysaccharides. On the other hand, addition of sugar sources, such as glucose, sucrose, and fructose, showed a slight induction of the pellicle formation in SKU1108 strain, especially at 40°C.

Abstract (Japanese)

酢酸菌はグラム陰性の絶対好気性菌で、エタノールを酢酸へと酸化する能力を持つ。酢酸菌のうち、 *Acetobacter* と *Gluconacetobacter* が工業的酢酸生産に使用されている。またビタミンCの工業的合成過程で*Gluconobacter* が使用されている。酢酸生産過程は発熱を伴うので温度上昇は避けられない。温度が上昇しすぎると酢酸生産能力が損なわれるので、最適な温度になるように冷却装置で冷却しなければならず、特に熱帯や亜熱帯での酢酸生産のコストを上昇させている一因となっている。高い温度でも酢酸生産能力が損なわれない耐熱性酢酸菌を開発することは有益であると考えられる。

この学位論文では、まず熱帯のスリランカのココナッツ酢から耐熱性酢酸菌を単離し、その生化学的及び生理学的特徴付けを行い、細胞膜結合型酵素の耐熱性を調べ、更にそれぞれの菌株の菌膜から多糖を精製し、その解析を行った。 スリランカの食酢生産工場でココナツ酢発酵槽の上層に形成された菌膜を入手し、そこから42℃で生育する *Acetobacter pasteurianus* 酢酸菌3株（SL13E-2, SL13E-3, SL13E-4）と37℃ で生育する *Gluconobacter frateurii* 酢酸菌4株（SL13-5, SL13-6, SL13-7とSL13-8）を単離し同定した。単離した *Acetobacter pasteurianus* 酢酸菌3株は、37℃で6％エタノールから4％酢酸を、40℃では4％エタノールから2.5％酢酸を生産することができた。SL13E-4株は他の2株に比べ酢酸生産速度が遅かった。以前の研究でタイから単離されている耐熱性の *Acetobacter pasteurianus* SKU1108株と同様に、SL13E-2株と SL13E-4株のアルコール脱水素酵素及びアルデヒド脱水素酵素活性は、常温菌の酵素に比べ熱に対して安定であった。単離した *Acetobacter pasteurianus* 酢酸菌を使って、高い温度でのココナツ水からのココナツ酢の発酵生産が可能であった。

Acetobacter 酢酸菌は菌膜を形成して、培地の表層に浮かんだ状態で生育する能力を持つことが知られている。菌膜は、菌体が生産し細胞に結合したままの多糖のマトリックスと菌から形成されている。スリランカのココナツ酢から分離された *Acetobacter pasteurianus* の 3 株（SL13E-2, SL13E-3, SL13E-4）とタイから単離された耐熱性菌 SKU1108 株及び中温菌 NBRC3191 株の

菌膜からそれぞれ多糖を精製し、菌膜形成多糖の特徴付けを行った。菌膜多糖の構成単糖の組成は同じ *Acetobacter pasteurianus* であっても菌株ごとで異なっていた。SL13E-2 株の菌膜多糖は、ラムノースとグルコースのみから成る多糖で、ラムノース：グルコース＝1：8 であった。SL13E-4 株と NBRC3191 株では、ラムノースとグルコースとキシロースを成分として、組成比はそれぞれ 2：2：1 及び 1：5：2.5 であった。SL13E-3 株と SKU1108 株はラムノースとグルコースとガラクトースを含み、組成比はそれぞれ 2：2：1 及び 1：5：2.5 であった。SL13E-2, SL13E-3, SL13E-4 の３株の菌膜形成は培地にエタノールを添加することで増加したが、SKU1108 株ではあまり増加しなかった。エタノールで誘導された菌体から精製された多糖の分子サイズと構成単糖の組成は、エタノールを加えなかった菌体からの多糖と違いがなく、このことはエタノールによる誘導で多糖の分子数が増えたのであって、別の多糖が生産されたのでもなく、それぞれの多糖の分子の大きさや組成にも変化がなかったことを示している。一方、グルコースやスクロースやフルクトースの様な糖源を加えると、SKU1108 株では菌膜形成が促進され、40℃で特に顕著であった。他の株ではそのような糖源での誘導は起こらなかった。

Chapter 1

General Introduction

Acetic acid bacteria (AAB) are Gram-negative aerobic bacteria, and used for the commercial production of vinegar and other industrially important materials, such as L-sorbose for vitamin C production. Among ten genera of AABs, *Acetobacter* and *Gluconacetobacter* have been used in vinegar production due to their strong ability to oxidize ethanol and to tolerate high concentration of acetic acid accumulated by their own. Strains belonging to genus *Gluconobacter* oxidize a broad range of compounds such as sugars, sugar alcohols, and sugar acids, and accumulate high amounts of oxidized products outside of the cells. Such 'incomplete' oxidation is carried out by membrane-bound dehydrogenases uniquely found in AABs.

In vinegar fermentation, membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) successively oxidize ethanol to acetic acid via acetaldehyde, and the obtained reducing equivalents are passed to molecular oxygen through ubiquinone and terminal ubiquinol oxidase, generating proton-motive force across the cytoplasmic membrane. ADH is a quinoprotein-cytochrome *c* complex (quinohemoprotein), consisting of three subunit proteins. Subunit I includes the catalytic site for the substrate oxidation with pyrroloquinoline quinone (PQQ) and heme *c* as the prosthetic groups. Subunit II has membrane-attaching and ubiquinone-reacting sites with three heme *c* as the prosthetic groups. Subunit III is believed to be a kind of chaperon to assemble the ADH complex, however it is not found in the purified ADH from some strains of AAB. Three dimensional structure of ADH from *G. oxydans* IFO12528 was recently solved. Also ALDH comprise cytochrome *c* complex with three subunits components; subunit I is the catalytic subunit including molybdenum-molybdopterin as the prosthetic group. Subunit I is the catalytic

subunit with molybdenum-molybdopterin as the prosthetic group, the exact structure of which is not determined yet. Subunit II is similar to the one in ADH. Subunit III contains iron-sulfur cluster. The gene cluster for ALDH is already reported from several AAB strains; however, three dimensional structure of ALDH is not obtained yet.

Currently industrial oxidative fermentation is carried out by the use of mesophilic AAB strains which work well around 30°C, but, both growth and oxidation activity are impaired at 37˚C or higher temperature. During fermentation process, due to generate heat, a cooling system is required to maintain the oxidative fermentation in proper manner, which increases the production costs. Thus, development of thermotolerant AAB which can grow and oxidize substrates at higher temperature will be beneficial for fermentation industry, especially tropical and sub-tropical area such as Sri Lanka and Okinawa, Japan.

Sri Lanka is a tropical country. Thus, there should be AAB adapted to tropical climate, which can grow and perform oxidative fermentation at higher temperature. Under master course study, many AAB strains were isolated from Sri Lanka: *Acetobacter*-type and *Gluconobacter*-type strains, which shows high acid production from ethanol and D-sorbitol/D-mannitol, respectively.

In this thesis, thermotolerant *Acetobacter*-type and *Gluconobacter*-type strains were isolated from the pellicle formed on the top of the brewing vinegar fermentation tanks at a coconut vinegar factory in Panadura, Sri Lanka. Furthermore, studies on identification and characterization of the isolated thermotolerant AABs, studies on membrane-bound ADH and ALDH enzymes of *Acetobacter*-type strains, and characterization of the pellicle polysaccharides of thermotolerant *Acetobacter*-type strains have been done.

Chapter 2

Literature Review

2.1 Acetic Acid Bacteria (AAB)

The exploitation of acetic acid bacteria (AAB) has a long history in fermentation process and now represents an emerging field in biotechnological applications, especially with regard to the biosynthesis of useful chemicals with a potentially high economic value and, in food science. Historically, AAB were recognized as 'vinegar bacteria' because the first studies were done on vinegar, and later on wine and beer spoilage. In fact, vinegar AAB are a subset of larger AAB group, which includes bacteria that interacts with flowers, fruits, the rhizosphere of plants, fermented foods and rarely found in soils and insect guts.

AAB comprise a group of gram-negative obligate aerobic α-*Proteobacteria* which use oxygen as the terminal electron acceptor. It is well known that they are able to oxidize sugars and alcohols, resulting in an accumulation of organic acids as final products. Its metabolic uniqueness was also traditionally utilized to generate fermented food, especially vinegar, and also for industrial production of sorbose and dihydroxyacetone. They play a crucial role in the quantity, taste, nutrition, and hygienic quality of the productions.

Since its discovery, the AAB group has been rearranged several times, with division, renaming, restoration, and emendation of genera and species. Nowadays, ten genera of the *Acetobacteraceae* family are grouped under the collective name 'acetic acid bacteria' (class α-*Proteobacteria*): *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconoacetobacter*, *Asaia*, *Kozakia*, *Swaminathania*, *Sacchribacter*, *Neoasaia*, and *Granulibacter* (Cleenwerk et al. 2002; Greenberg et al. 2006; Yamada and Yukphan, 2008; Kanchanarach et al. 2010). A

phylogenetic tree showing relationships of type strains of the family *Acetobacteraceae* is shown in Figure 2-1.

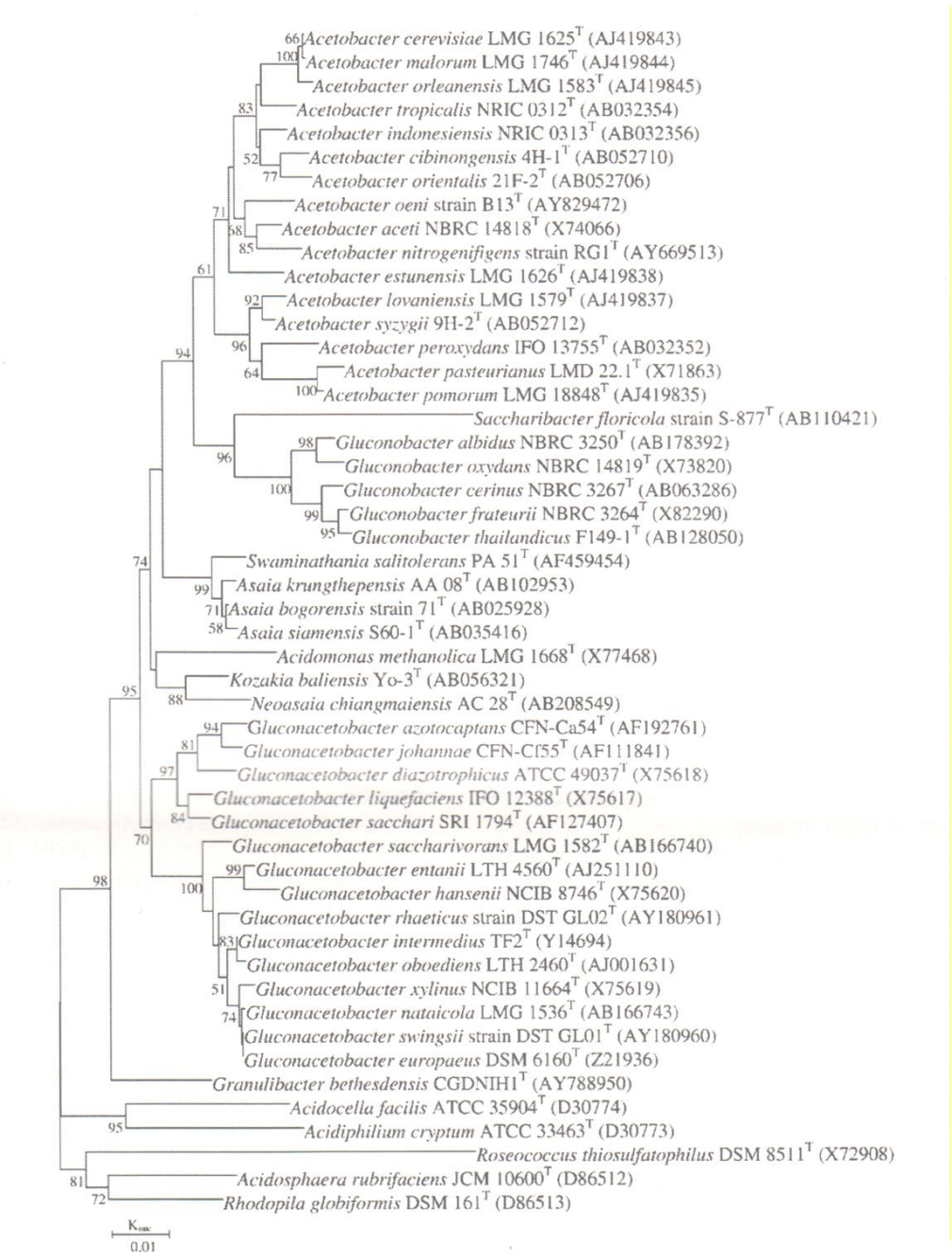

Fig. 2-1 Phylogenetic tree based on 16S rDNA sequences constructed by neighbor-joining (NJ) method.

Source: Yamada and Yukphan, (2008) International Journal of Food Microbiology, 125. 15-24

In the ten genera described above, the genus *Acetobacter* is characterized chemotaxonomically by Q-9 as a major respiratory quinone, which is quite unique and exceptional while other genera have Q-10 (Yamada et al., 1969).

In the family *Acetobacteraceae*, except for genus *Aceotobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Asaia*, all the other six genera are monotypic, in which only one species is described. The occurrence of strains assigned to some of the six genera is rather rare in common isolation sources such as vinegar, wine fruits, and flowers. Genus-level identification of above mentioned four strains can be easily done by combination of only two conventional tests comprised of acetate and lactate oxidation and acetic acid production from ethanol (Asai et al., 1964; Yamada et al., 1976).

Among ten genera, *Acetobacter*, *Gluconobacter*, and *Gluconacetobacter* are the major ones and well-distinguished in their physiological characteristics. In particular, *Acetobacter* and *Gluconacetobacter* are the most popular and have been used for acetic acid fermentation because of their powerful ability to oxidize ethanol to acetic acid and to tolerate to high acetic acid concentration accumulated in the medium. These AAB involved in acetic acid fermentation exhibit two different acetic acid resistance phases (Mastsushita et al, 2005): one is the ethanol oxidation phase, which is characterized by oxidation of ethanol to acetic acid, where acetic acid resistance occurs without acetate assimilation, and the second phase is the over oxidation phase, which is characterized by oxidation of acetic acid to water and carbon dioxide, where the cells overcome acetic acid by its assimilation. The over oxidation occurs in *Acetobacter* and *Gluconacetobacter*, but not in *Gluconobacter*, which exhibits a relatively weak acetic acid resistance (Sievers and Swings, 2005). *Gluconobacter* is a genus which can oxidize a broad range of sugars, sugar alcohols and sugar acids and accumulate a large amount of corresponding oxidized products in culture medium (Moonmangmee et al, 2000).

2.1.1 Genera and species in AAB

Taxonomic studies of acetic acid bacteria were historically surveyed. The genus *Acetobacter* was first introduced in 1898 with a single species, *Acetobacter aceti*. The genus *Gluconobacter* was subsequelntly proposed 37 years after on 1935 for strains with the ability to intensely oxidize glucose to gluconic acid rather than ethanol to acetic acid and no oxidation of acetate, which were different in these respects from strains of the genus *Acetobacter*. Almost 20 years later from that, the genus "*Acetomonas*" was proposed for polarly flagellated and non acetate-oxidizing strains. In 1984, Yamada and Kondo distinguished Q-10-equipped strains from Q-9-equipped strains in the genus *Acetobacter* at the subgeneric level, and were named as *Gluconacetobacter* later; it was elevated to generic level as *Gluconacetobacter*. The remaining six genera, *Asaia*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia*, *Granulibacter* were recently described. Interesting is that the recently described six genera are all monotypic except for only the genus *Asaia*. As a whole, the family *Acetobacteraceae* accommodates the ten genera for the AAB (Yamada and Yukphan, 2008).

Acetobacter

Acetobacter species occur in flowers, fruits, palm wine, vinegar, kefir, and fermented foods and can cause infections in grape wine, sake, tequila, cocoa wine, cider, beer and fermented meat. It is frequently used for the industrial vinegar production because of their powerful ability to oxidize ethanol and to tolerate high acetic acid concentration accumulated in the medium. Furthermore, *Acetobacter* sp. is a strain which catalyses the biological oxidation of ethanol into acetic acid by the association of two membrane bound enzymes (primary dehydrogenases): a quinoprotein ADH and an ALDH ((Kanchanarach et al. 2010).

The main physiological difference between *Acetobacter* and *Gluconobacter* is that, *Acetobacter* oxidized ethanol into acetic acid and, subsequently, complete the oxidation of acetic acid into water and CO_2.

Gluconobacter

Strains belonging to *Gluconobacter* oxidize a broad range of sugars, sugar alcohols and sugar acids such as D-fructose, D-glucose, D-sorbitol, D-manitol, glycerol, D-gluconate and keto-D-gluconate and excrete high amounts of the corresponding oxidation products into the culture medium (Moonmanhmee et al, 2000). Thus, they could be isolated from sugar-rich environments such as fruits and flowers, honey bees, grapes and wine, palm sap, cocoa wine, cider, beer and soft drinks. The typical feature of *Gluconobacter* species is the ability to oxidize sugars to their corresponding carboxylic acids, 2-keto-L-gluconic acid and 2-keto and 5-keto gluconic acids which can be easily converted to vitamin C. This feature leads to the applications in industry for fermentation of valuable products such as L-sorbose, dihydroxyacetone, D-gluconate and keto-D-gluconates. Such "incomplete" oxidation is carried out by membrane-bound enzymes, whose catalytic sites face the periplasm. All of these enzymes are firmly attached to the cytoplasmic membrane, and the electrons abstracted from the substrates are passed on to ubiquinone and then to terminal ubiqionol oxidases, forming simple respiratory chains which create the membrane potential necessary to produce biological energy for these microorganisms (Saichana et al, 2009).

2.1.2 Sources of AAB

AAB are widespread in nature and a large number of strains have been isolated from variety of sources. AAB belonging to the genus *Acetobacter* have been isolated more frequently than those of *Gluconobacter*, while occurrence of *Acidominas*, *Kozakia*, *Swaminathania*, *Sacchribacter*, *Neoasaia*, and *Granulibacter* strains are rather rare in common isolation sources. For example, the isolation sources of *Acidomonas methanolica* are quite unique. They could be isolated mostly from sludge (Sengun et al. 2011). Isolates belonging to genus Aceobacter and Gluconobacter are found in fruits such as apple, mango, guava, orange, papaya, rose apple, passion fruit, mangoseteen and etc.

Table 2-1 Isolation sources of AAB

Source	Genus	Source	Genus
Coffea Arabica	*Ga. diazotropgicus*	Cherry (*Prunus* sp.)	*G. cerinus*
Coffee plants	*Ga. azotocaptans*	Strawberry	*G. frateurii*
	Ga. johannae	Grape	*G. oxydans*
Fermented cocoa	*A. pasteurianus*		*A. aceti*
beans	*A. syzygii*	Dried fruit, flower	*Ga. liquefaciens*
	A. malorum	(ixora, lantana etc.)	*A. orleanesis*
	G. oxydans		*A. indonesiensis*
	A. aceti		*A. syzygii*
	A. lovaniensis		*A. orientalis*
	A. orleanensis		*G. oxydans*
Tropical fruits	*A. lovaniensis*		*G. frateurii*
(coconut, mango,	*A.syzygii*		*As. bogorensis*
guava, sapodilla etc.)	*A. indonesiensis*		*As. siamensis*
	A. cibinorgensis		*As. indonesiensis*
	A. tropicalis		*F. aurantia*
	A. orientalis	Flowers of red	*Am.*
	G. oxydans	Ginger	*chiangmaiensi*
	G. frateurii	Pollen	*Sa. floricola*
	Ga. hansenii	Beer	*A. cerevisiae*
	F. aurantia		*A. pasteurianus*
	A. malorum		*G. oxydans*
Rooting Apple	*G. azotocaptans*	Sugarcane roots	*Ga.*
Corn roots	*Gs. sacchari*	Palm brown sugar,	*diazotrophicus*
Mealy bug from		Ragi	*K. baliensis*
sugarcane	*Sw. salitolerans*	Wetland rice	
Wild rice			*G. diazotrophicus*
			A. peroxydans

Source: Sengun et al. (2011). Food Control. 22

2.1.3 AAB in food industry

AAB are important microorganisms in food industry because of their ability to oxidize many types of sugars and alcohols to organic acids as the end products during fermentation process. They are widely used in commercial production of vinegar. The oxidation of ethanol to acetic acid, (which is important in vinegar production) is the best known characteristic of AAB. In the early studies, AAB isolated from vinegar fermentations were identified as *A. acidophilum*, *A. polyoxogenes*, *A. hansenii*, and *A. pasteurianus* (Sengun and Karabiyikli. 2011). Also, these bacteria can oxidize glucose to gluconic acid, galactose to galactonic acid, arabinose to arabonic acid and so on. This property of "underoxidation" is been used in manufacturing of ascorbic acid (vitamin C). Ascorbic acid can be formed from L-sorbose. But, chemical synthesis of L-sorbose is difficult. However, it is conveniently obtainable from AAB which oxidized D-sorbitol, a readily available sugar alcohol from a process called bioconversion. Another interesting property of AAB is their ability to synthesize cellulose and the formed cellulose does not significantly differed from plant cellulose.

AAB can play not only a positive role in the production of selected foods and beverages, but also they can also spoil other foods and beverages as wine, beer, soft drinks, and fruits. *Acetobacter* and *Gluconobacter* are known as a spoiling agent of beer and wine. As *Gluconobacter* are resistant to preservatives such as sorbic acid, benzoic acid and dimethyldicarbonate, they are the most frequent cause of bacterial spoilage of soft drinks at low pH. When basic hygienic and technical procedures are not correctly performed, AAB produce acetic acid in beverages under aerobic/ microaerophillic conditions and cause low pH and low ethanol content, swelling of pack, vinegary off flavors, turbidity and ropiness.

2.1.4. Thermotolerant AAB

Thermotolerant bacteria belong to the same genera of so-called mesophilic bacteria but grow at temperatures 5-10°C higher than typical mesophilic strains of the same genus or the same species. These strains are different from so-called thermophilic bacteria, which are defined as strains with as innate ability to grow above 60°C. Therefore, it is conceivable that these thermotolerant strains have acquired their growth phenotype by adapting to a habitat with a higher temperature, such as tropical regions. Such thermotolerant strains that can grow at moderately high temperatures must have some specific mechanism other than the heat-shock response that can be induced at transient high temperatures. In many defense mechanisms, they minimize the damage due to heat stress. In some cases, they contribute to the adaptive response after exposure to minimal or mild stress which results in increased resistance to higher levels of same or other types of stress later. In addition, genetic adaptation with several types of mutations is also related with bacterial survival mechanism.

Isolation, identification, and characterization of thermotolerant AAB were investigated to develop new microbial resources for oxidative fermentation (Saeki et al. 1997a; Moonmangmee et al. 2000; Ndoye et al. 2006; Kanchanarach et al. 2010a). moreover, in recent studies, development of thermotolerant strains useful in acetic acid fermentation (Matsutani et al. 2013) and L-sorbose fermentation (Hattori et al. 2012) were done by thermal adaptation of *A. pasteurianus* SKU1108 and *G. frateurii* CHM43 strains respectively.

In a study by Soemphol et al (2011), 24 different genes were been identified as responsible for thermotolerance in *A. tropicalis* SKU1100. Out of them, ATPR_1619, 0429, 2837, 2097, and 0143 were categorized as heat shock or stress response genes that play major roles in the folding, repair and degradation of denatured proteins. Moreover, ATPR_1965 and 1424 were expected to be involved in cell division, which may be related to DNA replication errors and damage at high temperatures. Genes ATPR_3151, 0874, and 1188 appear to be

related to cell wall or cell membrane biosynthesis, and thus play an important roles as the first protection against stress. Two other genes, ATPR_0071 and 0609 are apparently related to the transport system where ATPR_0071 found to be involved in intracellular pH regulation with K^+ but not with Na^+. According to Soemphol et al (2011), these 24 genes were not found outside of the AAB phylogeny. This resemble that *A. tropicalis* acquired thermotolerant genes by adaptive evolution but not by horizontal gene transfer. Moreover, they conclude that these thermotolerant genes are not essential for cell growth but required in proper growth at higher temperatures. This speculate that *A. tropicalis* has additional homologous genes (paralogous genes) or another metabolic pathway besides the thermotolerant genes or the pathway that involves these genes. Furthermore, they suggest that the thermotolerant genes may produce proteins that are functional at higher temperatures at which proteins produced by mesophilic paralogous genes may not work properly.

On the other hand, a study by Matsutani et al (2011) suggests that the increased number of Arg-based salt bridges may contribute to the thermotolerance of *A. tropicalis* SKU1100. During their study, they have reported that there was a high substitution frequency of large to small residues (Met-Leu, Glu-Asp, Lys- Arg, Glu-Ala, and Ile-Val) from *A. pasteurianus* to *A. tropicalis*. Previous studies suggest that substitution of Lys to Arg affect the thermal stability of proteins in both thermophilic and thermotolerant microorganisms. Furthermore, Arg or Lys residues form a salt bridge with either Glu or Asp residue, and the salt bridge formed with Arg and Glu (or Asp) confers a strong stabilizing mechanism for hyperthermophilic proteins (Vieille and Zeikus, 2001). Other than that, many other substitutions of Asp and Arg were also observed in *A. tropicalis* (Asn-Asp, Gln-Arg, and His-Arg). Increase of the Asp and Arg contributes to the increase of Arg-based salt bridges, which contributes to the thermo-stability of *A. tropicalis*. Moreover, it was shown that the

flexibility of salt-bridge forming regions in *A. tropicalis* is more rigid than that of *A. pasteurianus* (Matsutani et al. 2011).

2.1.5 Potential of Thermotolerant AAB in fermentation industry

The recent hot summer has raised indoor temperatures beyond 30°C, even at night time, in many countries. This is a serious problem for vinegar fermentation and other fermentation industries. Domestic vinegar production by AAB is usually carried out at 30°C and strict temperature control is necessary irrespective of whether the culture is static or submerged. A temperature increase in 2-3°C causes a serious deterioration in both the fermentation rate and fermentation efficiency. In submerged cultures, a large amount of heat is generated during fermentation and thus cooling costs become rather expensive. Thus, if favorable strains of AAB that can work optimally at 37-40°C are available, the cooling expenses can be reduced greatly.

There are several advantages of vinegar fermentation cultures at higher temperatures with thermotolerant AAB. These bacteria can produce acetic acid even at higher temperatures of up to 40°C. When acetate was initially added at 4%, they still oxidized ethanol to accumulate acetic acid, while 2% of the initial acetate was the upper limit for mesophilic strains at higher temperatures. They oxidized higher concentrations of ethanol up to 9% without any appreciable lag time, while alcohol oxidation with mesophilic strains was delayed or became almost impossible under such conditions (Adachi et al, 2003). Marked acetate tolerance as well as high ethanol tolerance was observed with thermotolerant AAB (Saeki et al, 1997a).

Fermentation efficiency of vinegar production with the thermotolerant strains at 38-40°C was almost the same at that of mesophilic strains at 30°C. However, the thermotolerant

strains worked rapidly, with a higher fermentation rate at higher temperatures, at which the mesophilic strains were unable to work. The fermentation rate and efficiency of continuous vinegar fermentation at higher temperatures by the thermotolerant strains in a jar fermentor were more than 2 to 3 times higher than those of mesophilic strains at 30°C (Saeki et al, 1997a). The appearance, taste and fragrance of the vinegar produced by thermotolerant AAB seemed similar to those produced by mesophilic strains (Adachi et al, 2003).

As a whole, thermotolerant AAB are useful in vinegar fermentation at higher temperatures, allowing a possible reduction in cooling expenses and other costs.

Also, the *Gluconobacter* strains, which oxidize a broad range of sugars, sugar alcohols and sugar acids, are useful in industrial production of valuable products such as L-sorbose, dihydroxyacetone, D-gluconate, and keto-D-gluconates. They normally performed at temperature below 30°C and required a large amount of energy for cooling. From a study by Moonmangmee et al (2000) eight thermotolerant AAB belonging to genus *Gluconobacter* were isolated from Thailand and named CHM strains. They can grow and perform oxidative fermentation at 37°C, which is the temperature that mesophilic strains cannot grow. In a study by Hattori et al (2012), they have obtained a strain adapted to higher temperature from a thermotolerant strain, *Gluconobacter frateurii* CHM43, for L-sorbose fermentation. The adapted strain showed higher growth and L-sorbose production at higher temperatures around 38.5-40°C. Moreover, from the experiment on L-sorbose fermentation without temperature control in a jar fermentor, they have found that the adapted strain could keep growing over a high temperature due to mechanical heat and the fermentation heat generation under which conditions another strains cannot grow. Thus, the adapted strain could be used in industrial fermentation of L-sorbose which is important in vitamin C production.

5-keto-D-gluconic acid (5KGA) is a useful raw material for the production of tartaric acid and xylaric acid and is used as a precursor for the synthesis of a number of flavor

compounds, including 4-hydroxy-5-methyl-2,3-dihydroxyfuranone. Moreover, it has been reported that 5KGA can be used to produce vitamin C by Gray's method. From the study by Saichana et al (2009), they have isolated thermotolerant *Gluconobacter* strains which grow and produce 5KGA at temperatures up to 37°C, a temperature at which mesophilic strains neither grow nor produce any product and would be useful for industrial 5KGA production.

2.1.6 Properties of the enzymes from the thermotolerant *Acetobacter* spp.

In acetic acid fermentation, AAB oxidized ethanol by two sequential oxidation reactions of membrane bound ADH and ALDH. ADH isolated from thermophilic microorganism is advantageous over the mesophilic enzyme because of its thermal resistance and tolerance towards the common denaturing agents (Guarggliardi et al, 1996). There are few studies on thermoresistance properties of ADH and ALDH isolated from thermotolerant AAB (Saeki et al, 1997; Chinnawirotpisan et al, 2003, Ndoye et al, 2000; Kanchanarach et al, 2010; Matsutani et al, 2011).

From the past studies, it was suggested that membrane bound ADH and ALDH of thermotolerant strains are much stable to heat treatment than those of mesophilic strains while ALDH shows higher thermostability against heating than ADH (Saeki et al, 1997; Ndoye et al, 2006). At 2010, Kanchanarach et al studied the relationship between high temperature fermentation and thermostability of ADH from thermotolerant strains (*A. pasteurianus* MSU10 and SKU1108), and compared to the mesophilic strain (IFO3191). During the study, the authors have found that all ADHs from above three strains were stable up to 45°C for 30 min but became unstable over 50°C and rapidly inactivated at 55°C. At such higher temperatures, difference in the stability was observed; ADHs of MSU10 and SKU1108 showed a little higher stability than IFO3191 enzyme. In addition, the optimum

temperature of the enzymes from MSU10 and SKU1108 (35°C) was higher than that of IFO3191 (30°C). Furthermore, ADHs from MSU10 and SKU1108 exhibited higher resistance to ethanol and acetic acid than IFO3191 enzyme at elevated temperature. The remaining activity of ADH from MSU10, SKU1108 and IFO3191 has been determined after incubating the enzyme in various concentrations of ethanol at 37°C and 40°C for 30 min. As shown in the fig. 2.2, ADH from IFO3191 lost all the activity at 40°C at 22% ethanol while other two enzymes retained 43% (MSU10) and 29% (SKU1108) of the enzyme activity at the same conditions.

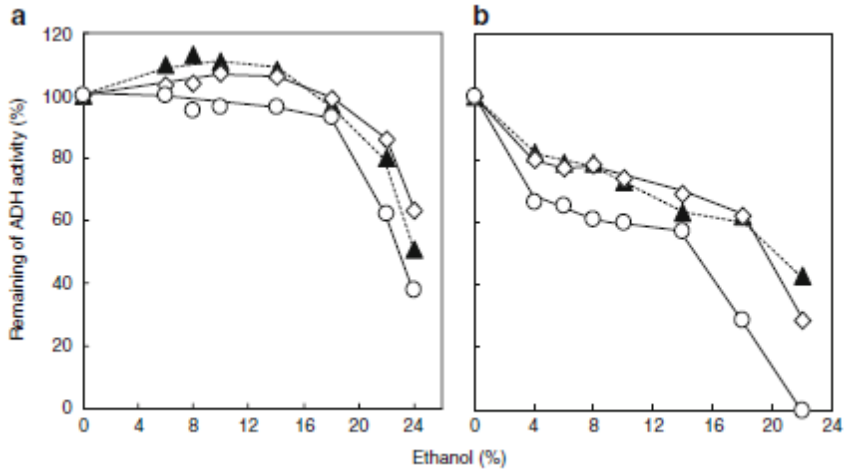

Fig. 2.2 Effect of ethanol on ADHs from *A. pasteurianus* strains. The purified enzymes from MSU10 (black triangle), SKU1108 (white diamond), and IFO319 (white circle) were incubated for 30 min at 37°C (a) and 40°C (b) in 10 mM KPB buffer (pH 6.0) containing different concentrations of ethanol and then the remaining ADH activity was measured.

Source: Kanchanarach et al, (2010a) Bioscience Biotechnology and Biochemistry. 74.

2.1.7 Acetic acid resistance in AAB

Acetic acid inhibits growth of the majority of microorganisms at the minimal concentration of 0.5%. Although most of AAB exhibit natural resistance to acetic acid, the level of tolerance differs among the species. Among the genera in AAB, the genus *Gluconacetobacter* generally exhibit much higher acetic acid resistance than the genus *Acetobacter*, such as *A. pasteurianus* and *A. aceti*. From the study of Treck et al (2006), they have found that *Ga. europaeus* exhibited the highest acetic acid resistance (10%), whereas *Ga. intermedius* and *A. pasteurianus* resisted up to 6% acetic acid. Thus, as well as ethanol oxidation capacity, acetic acid resistance is an important factor for AAB to perform fermentation stably. Many studies of the acetic acid resistance of AAB have been studied.

Many strains in genus *Acetobacter* remove acetic acid from the culture medium through acetate overoxidation. According to Saeki et al (1997b and 1999), enzymes working in the tricarboxylic acid (TCA) cycle are related to acetate overoxidation, together with acetyl-CoA synthase and phospho*enol*pyruvate carboxylase. The acetate resistance genes isolated from *A. aceti* mutant encode citrate synthase and a protein involved in acetate assimilation (Fukuya et al, 1990; 1993), and the amount of aconitase is increased in the presence of acetic acid in the culture medium (Nakano et al, 2004). The genes and proteins involved in the acetate assimilating system which is active during the overoxidation phase are also involved in acetate resistance. However, the mechanism responsible to acetic acid resistance cannot be explained by only acetate assimilation because *Acetobacter* and *Gluconacetobacter* species survive without assimilation of acetic acid under high concentration of acetic acid during fermentation conditions and also due to no acetate utilization is seen in *Acetobacter* spp. during ethanol oxidation and diauxic phase.

The most believable mechanism of acetic acid tolerance is an efflux pump in the cytoplasmic membrane that pumps acetic acid from the cytoplasm to outside.

Biofilm formation of AAB is also another defense mechanism of bacteria to grow under environmental stress. Thus, *Pseudomonas aeruginosa* able to produce biofilm are more resistant to heavy metals than planktonic cells, and biofilmed cells of *Lactobacillus plantarum* subsp. *plantarum* JCM1149 are more resistant to acetic acid and ethanol than planktonic cells. Likewise, *Acetobacter* species also have been found to produce pellicle polysaccharides on their cell surface. As described by Kanchanarach et al (2010b), as well as *A. tropicalis* SKU1100 and *A. aceti* IFO3284, strains in *A. pasteurianus* have two different types of cells; the rough colony (R) and smooth colony (S) strains where the R strain but not the S strain produces the pellicle. In growth curve of *A. pasteurianus* strains, it has found that the cells are having an amorphous layer surrounding the cells during the late ethanol oxidation (EO) and acetate resistance (AR) phases. Also, it was found that have higher sugar content in cells and it might be due to the production of pellicle polysaccharides. Furthermore, from a study by Moonmangmee et al (2002), it has found that a pellicle forming R strain produces non-cellulose-hetero-polysaccharides. Thus, the R strains are expected to become predominant in the late EO and AR phases. On the other hand, in the acetate oxidation (AO) phase, cells have been depressed and perhaps were reduced in sugar content, suggesting increases in S strains. Moreover, it has found that R strains showed high capacity for acetic acid fermentation than the S strain where the R strains produced nearly 3.5% of acetic acid with a typical diauxic growth while, S strains produced only 1.5% of acetic acid (Kanchanarach et al. 2010b). These results suggest that the polysaccharide surrounding the cells is related to acetic acid diffusion into the cells. When the acetic acid diffusion of both S and R strains were compared, it was found that the acetate uptake (influx) was lower in the R strain than in the S strain. When consider all these facts, it can be suggest that pellicle

polysaccharides are involved in the acetic acid resistance of *A. pasteurianus* strains and it functions as a biofilm-like barrier to passive diffusion of acetic acid into the cells.

Hence, acetic acid resistance in AAB is conferred by several different mechanisms as acetate assimilation, proton motive force-dependent and ABC-transporter-like efflux pump system for acetic acid, production of pellicle polysaccharide and additionally by changes in membrane lipid composition.

2.1.8 Acetate oxidation, an unfavorable phenomenon in vinegar fermentation

According to the Bergey's Manual of Systematic Bacteriology, all strains belonging to the genus *Acetobacter* oxidize acetic acid into carbon dioxide and water (Brenner et al, 2005). "Overoxidation of acetate" or "acetate peroxidation" has been used synonymously with acetate oxidation designating the phenomena of aerobic acetate anabolism by acetic acid bacteria. This phenomenon is a serious problem in vinegar industry. An intensive consumption of acetic acid is always accompanied by a corresponding increase in the cell mass of the organisms. Experiences accumulated for centuries have allowed us to terminate vinegar fermentation when a small amount ethanol still remain in the vinegar mash, and thought to be the safest way to avoid acetate oxidation. Vinegar fermentation allowing acetic acid accumulation to be more than 4% has become an alternative way to overcome this. In usual commercial vinegar fermentation, the final concentration of acetic acid is controlled at 4% to 5%. Moreover, selected microorganisms which show the least acetate oxidation have been currently used in fermentation industry, though prolonged incubation after ethanol exhaustion sometimes causes acetate oxidation.

When almost all available carbon and energy sources were exhausted and only acetic acid remained in the late stationary phase, the bacteria started to consume the acetic acid that had been accumulated in the culture medium for vinegar fermentation. They grew rapidly showing the second stationary phase and a typical biphasic growth curve was observed. The cells from the first growth phase were acid tolerant, while the cells from the second growth phase turned over to become acid sensitive. From the results of Saeki et al (1997b), it revealed that acetate oxidation was dramatically observed with thermtolerant *A. methanolicus*, *A. rancens* subsp. *pasteurianus* while no appreciable acetate oxidation was observed with the strains from Institute for fermentation, Osaka (IFO), such as *A. aceti* IFO3283, IFO3284, and *A. rancens* IFO3298 all of which are well known in actual vinegar fermentation. Moreover, they have found that acetate oxidation was observed when the concentration of acetic acid accumulated in the medium was less than 3.7%. but, when only acetic acid was administrated as the sole energy and carbon source, the organisms finally used acetic acid after a long lag time and this could be shorten by adding a small amount of readily usable energy source, such as ethanol. Thus, it can be suggested that ethanol plays an important role as oxidizable substrate generating energy that supported the initial part of microbial growth in a stage where the TCA cycle and NADH dehydrogenase system are not predominant (Saeki et al, 1997b).

For oxidation of acetate to water and carbon dioxide while cell biomass increases, the TCA cycle must be driven smoothly, as enzymes in TCA cycle make citrate with acetyl-CoA. In addition to acetyl-CoA formation *via* pyruvate dehydrogenase complex through catabolism of carbon sources, acetyl-CoA is formed by two different ways from acetic acid. One route is direct acylation of CoA catalyzed by acetyl-CoA synthetase as shown in Fig. 2.3. Acetyl-CoA formation by a coupling reaction of acetate kinase and phosphotransacetylase is an alternative route. Acetyl-CoA thus formed is then combined with oxaloacetate to form citric

acid and incorporated spontaneously into the TCA cycle. Other than that, acetyl-CoA is assimilated in malate formation by combining with glyoxylate from isocitrate by the action of isocitrate lyase (Saeki et al, 1997b).

According to Saeki et al (1997b), acetic acid accumulated outside of the cells are acting directly as a substrate for acetyl-CoA synthetase and the produced acetyl-CoA is incorporated into the TCA cycle and gyoxylate cycle. But, from the results of Saeki et al (1999), the activities of phosphotransacetylase and acetate kinase were higher than the activity of acetyl-CoA synthetase. Thus, the contribution of phosphotransacetylase and acetate kinase to acetate oxidation is much higher than direct acetylation of CoA. Other than that, they have shown increased enzyme activities in the TCA cycle because of the flux of citrate from acetyl-CoA and oxaloacetate. Isocitrate dehydrogenase and fumarase activities are also been increased during acetate oxidation. Moreover, according to Saeki et al (1999), the activity of phospho*enol* pyruvate carboxylase has been stimulated by a trace amount of acetyl-CoA among the enzymes of glycerol catabolism. This suggests that strong acetate oxidation caused by acetyl-CoA synthetase or phosphotransacetylase activity, together with phopho *enol* pyruvate carboxylase, which increased the cell biomass during acetate oxidation.

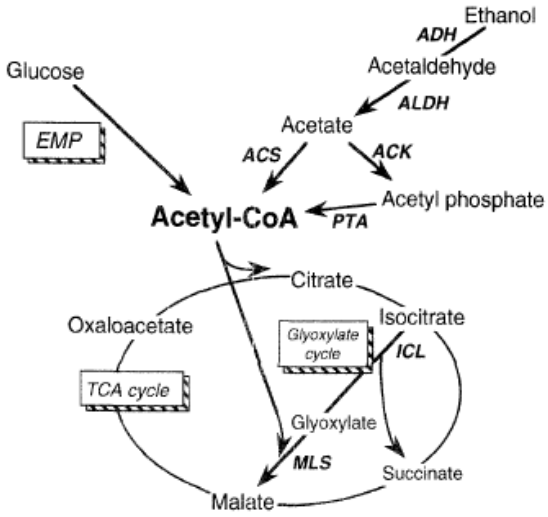

Fig. 2.3 Metabolic pathways of oxidative assimilation of acetate into the TCA cycle and glyoxylate cycle *via* Acetyl-CoA

The enzymes concerning in acetate oxidation are abbreviated in the figure. ACK, acetate kinase; PTA, phosphotransacetylase; ACS, acetyl-CoA synthetase; ICL, isocytrate lyase; MLS, malate synthase.

2.1.9 Pellicle formation of AAB

Polysaccharide formation is a common character among both Gram-positive and Gram-negative bacteria. These polysaccharides are classified according to their cellular association into capsular polysaccharide (CPS), which is permanently attached to outer surface of the cells, and exopolysaccharides (EPS) which is secreted into the growth medium. Attention has been paid to bacterial polysaccharides due to their importance in bacteria-host interaction and biofilm formation, stress adaptation, resistance to desiccation, and their application in food industry.

AAB, a Gram-negative obligate aerobes belonging to α-proteobacteria subdivision is a well known vinegar producer. In order to keep high aerobic state, almost all *Acetobacter*

species have ability to grow floating in static culture by producing a pellicle in the surface of culture medium. This pellicle is an aggregation of cells in the liquid-air interface in which cells are tightly associated with each other by polysaccharides and other extracellular matrix on the cell surface. This is an important phenomenon in vinegar fermentation in static culture.

A. xylinum is well known to produce a pellicle polysaccharide consisting of bacterial cellulose, and it is one of the famous model organisms for studying cellulose biogenesis. Thus, pellicles produced by other *Acetobacter* species pellicle are also been believed to be cellulose or cellulosic material. However, it has been found that the pellicle polysaccharide of *A. aceti* (reclassified as *A. pasteurianus* subsp. Lovaniensis) consists of glucose, and rhamnose (Moonmangmee et al. 2002a) and that of *A. tropicalis* SKU1100 consisting of glucose, galactose, and rhamnose (Moonmangmee et al. 2002b). Moreover, the composition of EPS of other *Acetobacter* species has been studied and found: glucose, mannose, galactose and glucuronic acid in *Acetobacter* sp. (Minakami et al. 1984); glucose, mannose, rhamnose and glucuronic acid in *Acetobacter* sp. NBI1022 (Tayama et al. 1985); glucose, galactose, rhamnose and glyceric acid in *A. methanolicus* (Grimmecke et al, 1994); and glucose, galactose and mannose in *Acetobacter* sp. (MacCormick, 1996). Thus, the pellicle polysaccharides occurs ether as homopolysaccharides or as heteropolysaccharides.

The genetic study of polysaccharide in AAB has shown that the *acs* (Saxena et al. 1994) and *bcs* (Wong et al. 1990) operons are involved in cellulose biosynthesis. Deeraksa et al (2005) has found that the gene cluster, *polABCDE* is required for the formation of capsular polysaccharide of *A. tropicalis* SKU1100. The deduced amino acid sequence of *polABCD* showed a high level of homology to those of *rfbBACD* which involved in dTDP-rhamnose synthesis pathway which is expected to consist of the following four sequential reactions:

Glucose 1-phosphate + dTTP ⟶ dTDP-glucose (RfbA)

dTDP-glucose (RfbA) ⟶ dTDP-4-keto-6-deoxy-L-mannose (RfbB)

dTDP-4-keto-6-deoxy-L-mannose (RfbB) ⟶ dTDP-6-deoxy-L-mannose (RfbC)

dTDP-6-deoxy-L-mannose (RfbC) ⟶ dTDP-L-rhamnose (RfbD)

Moreover, they have found that mutation of *polB* of *A. tropicalis* SKU1100 led to completely defective CPS and EPS synthesis. On the other hand, *polE* exhibited a relatively low level of homology to other gycosyltransferase homologues, compared to *polABCD*. Thus, it was expected that *polE* encodes a rhamnosyltransferase, which transfers a rhamnosyl residue onto the oligosaccharide unit being synthesized. But, the Pel^{-} mutant (Δ*polE*) produces EPS with the same sugar composition as the wild-type CPS (rhamnose, glucose and galactose). By considering all these, the authors concluded that PolE homologues are not a kind of glycosyltransferase or rhamnosyltransferase. During this study, a plasmid harboring the *polE* or *polB* genes could restore pellicle formation in Pel^{-} mutant and S strains, and in the Δ*polB* mutant, respectively. Thus, both *polE* and *polB* are involved in pellicle formation, most likely by anchoring polysaccharide to the cell surface and through the production of dTDP-rhamnose, respectively (Deeraksa et al. 2005).

In addition, the gene *galE* has been identified as the gene encoding UDP-galactose 4-epimerase in *A. tropicalis* SKU1100 (Deeraksa et al. 2006). During this study, the authors have found that Δ*galE* mutant lack CPS production and thus, did not form a pellicle under static condition. But, it produces an EPS, with only glucose and rhamnose, and is different from the sugar composition of CPS which consist glucose, galactose and rhamnose. The production of such mutant polysaccharide is likely to be due to the absence of the precursors, UDP-galactose, and the resulting polysaccharide did not attach to the cell surface.

2.2 Vinegar

2.2.1 General overview

The history of vinegar production dates back to around 2000 BC. Vinegar has been always considered a ‘poor relation’ among fermented food products: it is not considered to be a ‘food’. Vinegar is used as a flavoring agent, as a preservative and in some countries as a healthy drink. It can be made by any fermentable carbohydrate source by a two-step-fermentation process involving yeast as the first agent and the AAB. The most common raw materials are apples, pears, grapes, honey, syrups, cereals, hydrolyzed starch, beer, and wine.

Most vinegar has plant origin except for those produced from whey or honey. Whey, which is the milk serum residual of the cheese-making process, is rich in lactose and/or its corresponding hydrolyzed sugars, galactose and glucose, depending on the cheese making technology. As sour whey is heavily contaminated with lactic acid bacteria (LAB), pasteurization before alcoholic and acetous fermentation is needed. Honey is very rich in sugars (70-80% w/w), mostly sucrose, fructose, and galactose. Thus, it is diluted before alcoholic fermentation occurs.

Vinegar is generally an inexpensive product. Thus, its production requires low cost raw materials, such as substandard fruit, seasonal agricultural surpluses, by-products from food processing, and fruit waste. However there are some very expensive vinegars as traditional balsamic vinegar from Modena in Itlay, sherry vinegar from Spain, and oxos from Greece. Also, there are spirit vinegars obtained directly by acetic oxidation of ethanol derived from distillation of fermented mashes or petrochemical ethanol. There’s another separate group of vinegar consist of flavored vinegars: herbal or fruit vinegar. Herbal vinegars are wine vinegars or white distilled vinegars flavored with garlic, basil, tarragon, cinnamon, cloves,

nutmeg or other herbs. Fruit-flavored vinegars are wine and white vinegars sweetened with fruit or fruit juice to produce a characteristic sweet-sour taste.

2.2.2 Vinegar definitions and legislations

The definition of vinegar itself differs from country to country. FAO/WHO defines vinegar as any liquid, fit for human consumption, produced exclusively from suitable products containing starch and/or sugars by the process of double fermentation, first alcoholic and then acetous. The residual ethanol content must be less than 0.5% in wine vinegar and less than 1% in other vinegars.

In the USA, the Food and Drug Administration (FDA) requires that vinegar products must possess a minimum of 4% acidity. This qualification ensures the minimum strength of vinegars sold in the retail market.

Considering different laws on vinegar, it is clear that acidity and residual ethanol are two main parameters used to establish an all-encompassing vinegar classification. The acetic acid and ethanol contents change on the basis of raw material used, the microorganisms involved in the fermentation process, the technology employed, but mainly on the basis of culture and 'vinegar lore'.

2.2.3 Vinegar processing: the role of fermentation

Fermentation plays a key role in vinegar production. Different microbial species are involved in various stages of the fermentation process, such as LAB, yeasts, molds, and AAB which often colonies vegetables, fruits and other raw materials used in vinegar production.

The great microbial diversity reflects the variety of raw materials, sugar sources and processes, as well as the diversity of the physico-chemical characteristics (e.g. temperature, pH, water activity).

Two steps common to all vinegars: alcoholic and acetic acid fermentation due to yeasts and AAB respectively, whilst other microorganisms, such as molds and LAB are involved only in specific vinegars. Among the yeasts, *Saccharomyces cerevisiae* is the most widespread species in fruit and vegetable vinegars; the lactose-fermenting yeast, *Kluyveromyces marxianus*, is the species responsible for whey fermentation; and a physical association of yeasts, LAB and AAB is involved in the fermentation of kombucha. Even though there are ten genera of AAB, the majority of the species detected in vinegars belong to the genera *Acetobacter* and *Gluconacetobacter*.

In vinegar production, fermentation can be induced either by spontaneous fermentation, by back-slopping or by the addition of starter cultures.

In **spontaneous fermentation**, the raw material is processed and the changed environmental conditions encourage the most appropriate indigenous microflora. The more stringent the growth conditions are, the greater becomes the selective pressure exerted on the indigenous microorganisms. This method is suitable for small-scale production and only for very specific juices. However, the method is difficult to control and there is a great risk of spoilage. In most cases, a microbial succession take place, and quite often LAB and yeast dominate initially. They consume sugars and produce lactic acid and ethanol respectively, which inhibit the growth of many bacteria species, determining prolongation of the shelf life of the goods.

In **back-slopping fermentation**, part of a previously fermented batch is used to inoculate a new batch, and is a primitive precursor of the starter culture method. This

increases the number of desirable microorganisms and ensures a more reliable and faster process than spontaneous fermentation. But, the manufactured goods are still exposed to the risk of fermentation failure, since mold growth or harmful bacteria spoilage can occur. In general, this method is considered a useful practice because it improves the growth of useful yeasts, while inhibiting the growth of pathogenic microorganisms and reducing spoilage, and in addition the laborious and time-consuming starter selection process is avoided. This practice is particularly useful for inoculating AAB, as they are very fastidious microorganisms that need special attention in order to produce true starter cultures. In the semi-continuous submerged acetification process, at least one-third of the vinegar is left in the fermenter to inoculate the new batch, whereas in surface-layer fermentation, a physical transplant of the AAB flim can be easily done in order to preserve the integrity of the cell layer.

2.3 Enzymes responsible in vinegar fermentation

Acetic acid fermentation from ethanol, also known as vinegar fermentation, by AAB is accomplished by two sequential catalytic reactions of membrane bound, pyrroloquinoline quinone dependent alcohol dehydrogenase (PQQ-ADH) and membrane-bound aldehyde dehydrogenase (ALDH) (Fig. 2-4).

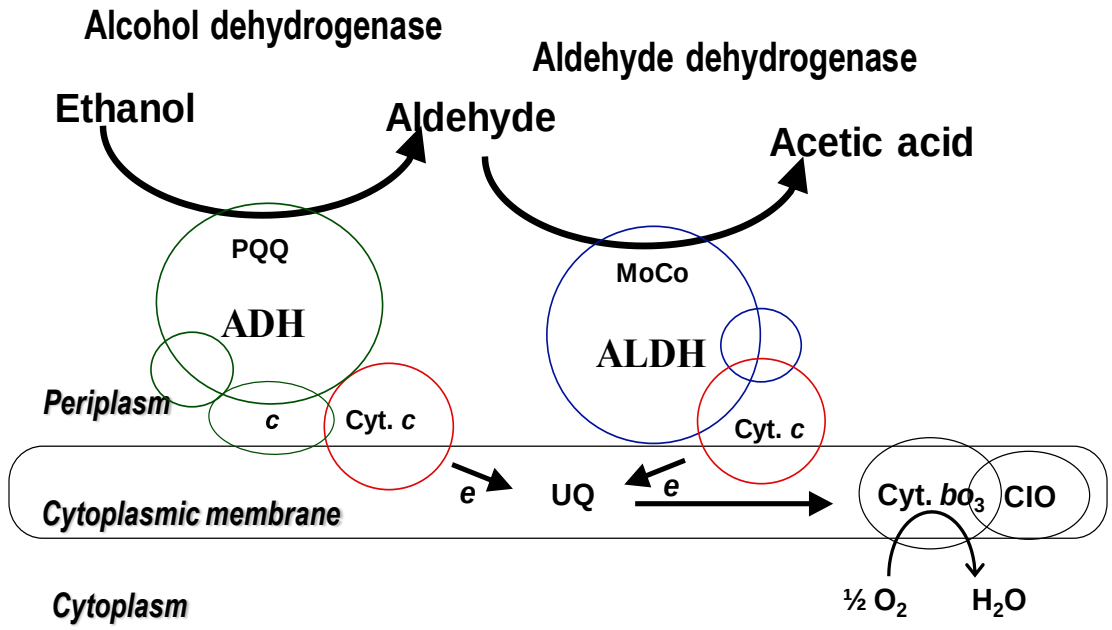

Fig. 2-4 Enzymes responsible in vinegar production.

2.3.1 PQQ dependent alcohol dehydrogenase (PQQ-ADH)

Alcohol dehydrogenase (ADH) is widely distributed in many different types of organisms ranging from bacteria to mammals. It is most often an NAD(P)-dependent enzyme present in the cytoplasm. In contrast, PQQ-dependent ADHs are rather unique and are found in only a narrow range of species of bacteria, α, β and γ-proteobacteria, and are localized only in the periplasmic fraction. PQQ-dependent ADH includes both quinoprotein- and quinohemoprotein type enzymes. Some are soluble in the periplasm and the others are bound to the outer surface of the cytoplasmic membrane (Toyama et al, 2004).

PQQ-ADH is a quinohemoprotein-cytochrome *c* complex bound to the periplasmic side of the cytoplasmic membrane and catalyzes the first step of acetic acid production to oxidize ethanol by transferring electrons to membranous ubiquinone (Q).

PQQ-dependent ADH is the largest enzyme group within the quinoprotein family. Its members consist of 18 different enzymes and are classified into three groups, type I, II, and III ADHs. Type I ADH found in a limited number of Proteobacteria are very similar to quinoprotein methanol dehydrogenase (MDH) in methylotrophs, and are simple quinoproteins having PQQ as the only prosthetic group. They can differentiate with respect to substrate specificity. Unlike type I ADH, type II and III ADHs are quinohemoproteins or contain a quinohemoprotein as one of its subunits, respectively. Type II ADH is an enzyme soluble in the periplasm and has a relatively wide distribution among several proteobacteria. Type III ADH is membrane-bound, working on the periplasmic surface and is unique to acetic acid bacteria.

Type III ADH consist of three subunits, a quinohemoprotein subunit I, a triheme *c*-type cytochrome subunit II, and subunit III that contains no cofactor, except for the enzymes from *Gluconobacter polyoxygens* that does not have the subunit III (Toyama et al, 2004). The

subunit I encoded in the *adhA* gene is approximately 80 kDa in the molecular size and contains PQQ and a heme *c* moiety as the prosthetic groups. Subunit I functions as the catalytic site for ethanol oxidation. The subunit II encoded in the *adhB* gene is approx. 50 kDa in the molecular size and contains three heme *c* moieties. Subunit II works as the electron transfer mediator from subunit I to ubiquinone (Fig 2-5). The subunit III is encoded in the *adhS* gene is approx. 15 kDa in the molecular size and suggested to have no prosthetic groups related to redox reaction from biochemical experiments and its predicted amino acid sequences (Yakushi, 2010). Subunit III exists freely in the periplasmic space besides in ADH complex, and is thought to work as a molecular chaperone (Matsushita, 2008).

The subunit I/III complex has ethanol:ferricyanide oxidoreductase activity, but does not have ethanol:Q-1 (an artificial ubiquinone analogue) oxidoreductase activity. On the other hand, the subunit II has no ADH activity. When the subunit I/III complex is reconstituted with the subunit II, the reconstituted PQQ-ADH complex regains Q-1 reductase activity, as well as ferricyanide reductase activity. When all these were taken together, it can be concluded that the subunit II is responsible for Q reduction (Yakushi, 2010).

According to genetic studies, it is suggested that out of the three subunits, subunit II is a membrane binding subunit. In the absence of subunit II, the subunit I and III, which are in a complex, are detected to be in a soluble fraction rather than a membrane fraction. Besides, the subunit II alone is detected in the membrane fraction of an *adhA* (subunit I) mutant (Yakushi, 2010).

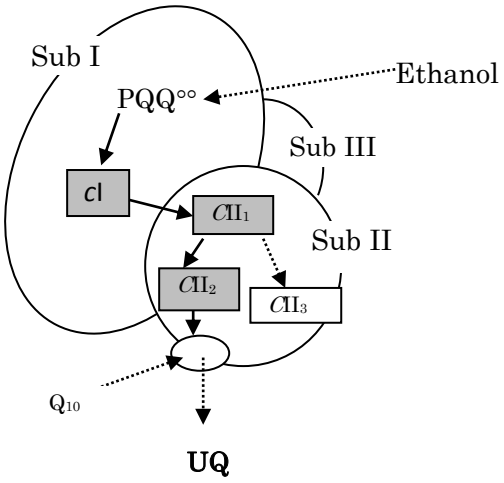

Fig. 2-5 Hypothetical model for intra (solid arrows) and intermolecular (dashed arrows) electron transfer in type III ADHs. *c*I, *c*II$_1$, *c*II$_2$, and cII$_3$ represent the 4 heme c sites in subunit I (I) and subunit II (*c*I, *c*II$_1$, *c*II$_2$, and cII$_{3)}$.

Source: Toyama et al. 2004

2.3.2 Aldehyde dehydrogenase (ALDH)

ALDH is also cytochrome *c* complex with three subunits. Subunit I is the catalytic subunit with molybdenum-molybdopterin as the prosthetic group, the exact structure of which is not determined yet. Subunit II is similar to the one in ADH. Subunit III contains iron-sulfur cluster. The enzymes for which most information is available are the ALDHs isolated with detergent from the membrane of AAB. These usually have pH optimum about 4 and oxidize aldehydes of C chain length C_2-C_4. Their function is to catalyze the oxidation, in the periplasm, of aldehydes produced by the action of the membrane-bound quinohemoprotein ADH (Goodwin and Anthony, 1998).

A mutant of one strain of *Acetobacter*, which were unable to produce PQQ and therefore produce inactive alcohol and glucose dehydrogenases, had the same level of ALDH activity as the parent strain, suggesting that this enzyme cannot be a PQQ-containing quinoprotein. Furthermore, the gene encoding this enzyme has been isolated and its predicted amino acid sequence does not show similarity to other quinoprotein dehydrogenases. The electron acceptor for these membrane ALDHs has not been identified, but as there is usually no cytochrome *c* oxidase in these bacteria, it is probable that electrons are passed to the cytochrome subunit and then to ubiquinone in the membrane, as in the ADHs of these bacteria (Saeki et al, 1997a).

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Chapter 3

Identification and characterization of thermotolerant acetic acid bacteria strains isolated from coconut water vinegar in Sri Lanka

Abstract

From the pellicle formed on top of brewing coconut water vinegar in Sri Lanka, three *Acetobacter* strains (SL13E-2, SL13E-3, and SL13E-4) that grow at 42ºC and four *Gluconobacter* strains (SL13-5, SL13-6, SL13-7, and SL13-8) grow at 37°C were identified as *Acetobacter pasteurianus* and *Gluconobacter frateurii*, respectively. Acetic acid production by the isolated *Acetobacter* strains was examined. All three strains gave 4% acetic acid from 6% initial ethanol at 37°C, and 2.5% acetic acid from 4% initial ethanol at 40°C. Compared with the two other strains, SL13E-4 showed both slower growth and slower acetic acid production. As well as the thermotolerant SKU1108 strain, the activities of the alcohol dehydrogenase (ADH) and the aldehyde dehydrogenase (ALDH) of SL13E-2 and SL13E-4 were more stable than those of the mesophilic strain. The isolated strains were used to produce coconut water vinegar at higher temperatures than typically used for vinegar production.

3.1 Introduction

Vinegar made from fermented coconut water or the sap of coconut palm inflorescence is used extensively as a preservative and flavoring agent in pickles and many dishes in Sri Lanka. Naturally fermented sap collected from tapped tender inflorescence of the coconut palm, known as toddy in Sri Lanka, is a popular alcoholic beverage and is used as the major raw material in traditional vinegar fermentation in rural villages. Coconut water is a waste product from the production of copra and desiccated coconut and is generally discarded, but some is used as a raw material for industrial vinegar production. Like other kinds of vinegar, coconut vinegar is produced by a two-stage fermentation process in which fermentable sugars are first converted into ethanol by yeasts, and then the ethanol is oxidized to acetic acid by AAB.

During industrial coconut vinegar production, up to 10% (w/v) sugar is added to the matured coconut water, since raw coconut water contains only about 3% (w/v) sugar (Jean et al. 2009). The fortified coconut water is then fermented in large vats with baker's yeast (*Saccharomyces cerevisiae*) to an alcohol content of 5-8%. Alcohol fermentation is then stopped by removing the upper yeast layer, and the fermented coconut water is filtered and transferred to a new, aerated vat. After the addition of mother vinegar, the contents are fermented to a total acidity of 4.5% (w/v) and then pasteurized at 60°C for 20 min. The pasteurized product is transferred to aging vats; after aging it is filtered, bottled, and marketed.

AAB are an important group of bacteria that oxidize carbon sources rapidly and incompletely, and hence are widely used in the commercial production of vinegar, D-gluconate, and L-sorbose. Recently, AAB were classified into 10 genera belonging to the family Acetobacteraceae (Yamada and Yukphan. 2008). *Acetobacter* and *Gluconacetobacter* are the primary species used in vinegar fermentation due to their strong ability to oxidize ethanol and to tolerate high acetic acid concentrations. They produce acetic acid from ethanol

by two sequential oxidation reactions involving ADH and (ALDH), which are localized on the outer surface of the cytoplasmic membrane (Adachi et al. 2003). ADH is a quinohemoprotein-cytochrome *c* complex consisting of three subunits with pyrroloquinoline quinone (PQQ) and heme *c* as the prosthetic groups. ALDH is also a cytochrome *c* complex, consisting of three subunits with molybdenum-molybdopterin as the prosthetic group.

A group of microorganisms exists in nature that can grow at temperatures 5-10°C higher than typical mesophilic strains of the same genus, and sometimes even of the same species. These strains, called thermotolerant microbes, are different from thermophiles, which can grow above 60°C and are totally of different genera. Thermotolerant strains may have acquired their growth phenotype by adapting to a habitats characterized by higher temperatures, such as tropical regions. Thermotolerant AAB can produce acetic acid at temperatures of up to 40°C. The fermentation efficiency of thermotolerant strains for vinegar production at 38-40°C is similar to that of mesophilic strains at 30°C, and they exhibit high acetate and ethanol tolerances (Saeki et al. 1997a). Hence, thermotolerant AAB are useful for vinegar fermentation at higher temperatures, allowing a possible reduction in cooling expenses and other costs.

A description of the microbiology and biochemistry of the natural fermentation of coconut palm sap was published by Athputharajah *et al* (1986), but the responsible AAB in Sri Lankan vinegar production is not known. Further, as Sri Lanka is a tropical country, presumably vinegar production would involve AAB that can grow and perform oxidative fermentation at higher temperatures. The aim of this study was to isolate, identify, and characterize thermotolerant AAB from Sri Lankan vinegar, and to use these ABB for vinegar fermentation at higher temperatures.

3.2 Materials and Methods

3.2.1 Sampling, bacterial strains, culture media and culture condition

Samples were collected from the pellicle formed on top of brewing vinegar in fermentation tanks at a coconut water vinegar factory in Panadura, Sri Lanka, in 2010, where vinegar is produced by natural fermentation.

Acetobacter pasteurianus SKU1108 (NBRC101655) isolated in Thailand (optimum growth temperature, approximately 37°C (Saeki et al.1997a)) was used as the thermotolerant reference strain.

Potato medium (1 g of yeast extract, 1 g of polypeptone, 2 g of glycerol, 0.5 g of glucose, and 10 mL potato extract, made up to 100 mL with tap water) was used for stock cultures on agar slants and pre-cultures. YPG medium (0.5 g of yeast extract, 0.5 g of polypeptone, 1.0 g of glycerol, 0.5 g of $CaCO_3$, and 1.5 g of agar) supplemented with 4 mL ethanol and 3 mg of bromocresol purple per 100 mL was used in the isolation of *Acetobacter*-type strains. YPS/YPM medium (0.1 g of yeast extract, 0.1 g of polypeptone, 1 g of D-sorbitol or D-manitol, and 1.5 g of agar) supplemented with 3 mg of bromocresol purple per 100 mL was used in the isolation of the *Gluconobacter*-type strains. YPGD medium (0.5 g of yeast extract, 0.5 g of polypeptone, 0.5 g of glycerol, and 0.5 g of glucose per 100 mL tap water, and 1.5 g of agar for the agar plates), supplemented or not with ethanol or acetic acid, was used in studying acetic acid and ethanol tolerances and acetic acid production. Salt agar (0.02 g of $(NH_4)_2HPO_4$, 0.01 g of $MgSO_4.7\ H_2O$, 0.01 g of $Ca_5(PO_4)_3(OH)$, 0.2 mL of glacial acetic acid and 1.5 g of agar, made up to 100 mL with tap water) was used to study the utilization of various carbon sources (Sokollek et al. 1998).

3.2.2 Isolation of AAB

An inoculating loop-full of sample was streaked onto an YPG (*Acetobacter*-type strains) agar plate supplemented with 4% (v/v) ethanol and onto an YPS/YPM agar plate (*Gluconobacter*-type strains). Colonies with halos on the YPG agar plates containing ethanol, and colonies creating a circular yellow zone on the YPS/YPM agar plates, were selected as *Acetobacter*-type and *Gluconobacter*-type strains, respectively. Pure cultures were obtained by repeated streaking. The cultures were maintained on potato agar slants. Stock cultures of the isolated strains were prepared by mixing equal volumes of the culture and sterile glycerol, and stored at -80°C until use.

3.2.3 Growth and acetic acid production by acetic acid bacteria

The growth of the isolated *Acetobacter* and *Gluconobacter* strains was examined on potato agar plates at 30, 37, and 40°C for 5 d. The acetic acid and ethanol tolerance of the identified *Acetobacter* strains were examined with YPGD agar plates supplemented with 1-4% (v/v) acetic acid or 1-10% (v/v) ethanol at 30, 37, 40, 41, and 42°C for 5 d. Acetic acid production was examined with YPGD medium supplemented with 4 and 6% (v/v) ethanol at 30, 37, 38, and 40°C under both static and shaking (200 rpm) culture conditions.

3.2.4 Production of coconut water vinegar

Simultaneous coconut vinegar production was examined statically at 37, 39, and 40°C by inoculating 0.2 g of commercially obtained dry yeast and the isolated strains together and sequentially in coconut water whose Brix value had been adjusted to 10% (w/v) by the addition of glucose.

3.2.5 Phenotypic and biochemical characterization

Physiological and biochemical characterization of the isolated strains was done following *Bergy's Manual of Systematic Bacteriology* (Brenner et al. 2005) and related publications (Asai et al. 1964; Yamada et al. 1976; Katsura et al. 2002). *Acetobacter* strains were identified by their carbon source utilization (Sokollek et al. 1998).

3.2.6 Preparation of membrane fractions

Cells grown in YPGD medium with and without ethanol at 37°C were harvested by centrifugation at 9,000 × *g* for 10 min at 4°C and washed twice with ice-cold 0.1 M Tris-HCl (pH 8.0) and ice-cold distilled water respectively. The washed cells were suspended on 10 mM KPB (pH 6.0) and passed twice by a French pressure at 16, 000 psi. The intact cells were removed by centrifugation at 9,000 × *g* for 10 min at 4°C. Then the supernatant was ultracentrifuged at 90, 000 × *g* for 90 min at 4°C to separate the membrane fractions from the soluble fraction. The membrane fractions were homogenized in 10 mM KPB (pH 6.0), and were used as sources for the enzyme assay.

3.2.7 Enzyme assay

ADH (EC 1.1.99.8) and ALDH (EC 1.2.99.3) activities were measured with ferricyanide as electron acceptor, as described by Ameyama (1982). One unit of enzyme activity was defined as the amount of enzyme catalyzing oxidation of 1μmol of substrate per min at 25°C with 100 mM substrate and 10 mM ferricyanide. Protein was measured by a modification of the Lowry method with bovine serum albumin as standard (Dulley and Grieve 1975).

3.2.8 Analytical method

The acidity of the culture medium was measured by titration with 0.8N NaOH with phenolphthalein as pH indicator.

3.2.9 16S rDNA gene sequencing and phylogenetic analysis

For sequencing, 16S rDNA was amplified by PCR Go Taq Green Master Mix (Promega, Madison, WI). Cell lysate was used as template DNA without purification. Specific primers 27f (5′-AGAGTTTGATCCTGGCAG-3′) and 1492r (5′-GGCTACCTTGTTACGACTT-3′), selected from highly conserved regions of the nucleotide sequence of 16S rDNA of the proteobacteria, were used (Harish 1992). The PCR product was not obtained from SL13-7 with the 27f and 1492r set of primers, so another set of primers, Alf28f (5′-ARCGAACGCTGGCGGCA-3′) and 1512uR (5′-ACGGHTACCTTGTTACGACTT-3′) (Mühling et al 2008), was used. The amplified PCR products were purified with a LaboPass PCR product purification kit (Cosmo Genetech, Seoul, South Korea), and were sequenced at Sol Gent (Taejon, South Korea). The 16S rDNA nucleotide sequences were aligned and compared to the sequences of representative strains of *Acetobacter* and *Gluconobacter* obtained from GenBank (htts://www.ncbi.nlm.nih.gov/). Multiple alignments of the determined 16S rDNA sequences were performed by CLUSTAL W (Thompson et al. 1994).

3.2.10 Phylogenetic analysis

For phylogenetic analysis of the isolated strains, the 16S rDNA gene sequences of *A. pasteurianus*, *G. frateurii* stains, and related species were obtained from the NCBI website at http://www.ncbi.nlm.nih.gov. To construct the phylogenetic tree of isolated AAB, all the sequences were aligned by CLUSTALW. The Genetyx ver. 10 package was used to generate a

phylogenetic tree to study the phylogenetic relationship based on the 16S rDNA gene by the neighbor-joining (NJ) approach and 1,000 bootstrap replicates.

3.2.11 Amplification and sequencing of the 16S –23S ITS region

PCR amplification of the 16S-23S rRNA gene internal transcribed spacer (ITS) region was done using primers ITS 1 (5′-TGCGGYTGGATCACCTCCT-3′, position 1,522-1,540 on the 16S rDNA, *E. coli* numbering) and ITS 2 (5′-GTGCCWAGGCATCCACCG-3′, position 38-22 on the 23S rDNA, *E. coli* numbering) (Tanasupawwat et al. 2011). The amplified PCR product (0.7 kb) was purified and cloned into pGEM-T^{TM} EASY vector (Promega, Madison, WI) and transformed into competent *E. coli* DH5α cells. Recombinant plasmids with the 16S-23S ITS region were extracted from the cells with a LaboPass plasmid purification kit (Cosmo Genetech, Seoul, South Korea) and sequenced.

3.2.12 Nucleotide sequence accession numbers

The nucleotide sequence data reported here have been deposited in the DNA Data Bank of Japan under accession nos. AB753861 (SL13E-2), AB753862 (SL13E-3), AB753863 (SL13E-4), AB819116 (SL13-5), AB819117 (SL13-6), AB819118 (SL13-7), AB819119 (SL13-8) for 16S rDNA sequences, and AB754591 (SL13E-2), AB754592 (SL13E-3), AB754593 (SL13E-4), AB819120 (SL13-5), AB819121 (SL13-6), AB819122 (SL13-7), and AB819123 (SL13-8) for the 16S-23S ITS sequences.

3.3 Results

3.3.1 Identification and characterization of Acetobacter-type strains from Sri Lankan vinegar

By means of YPG medium containing ethanol, three *Acetobacter*-type AABs (SL13E-2, SL13E-3, and SL13E-4) producing different colony sizes were isolated from the pellicle formed in the fermenter tank. They were Gram-negative, rod-shaped, and catalase-positive. Since they oxidized acetate into CO_2 and H_2O, they were assigned to the genus *Acetobacter* (Table 3-1) (Brenner et al. 2005).

Table 3-1. Differential characteristics of *Acetobacter* strains isolated from Sri Lankan coconut vinegar

Character	1	2	3	4[1]
Production of acetic acid from ethanol/$CaCO_3$ agar	+	+	+	+
Oxidation of acetate to carbonate	+	+	+	+
Growth on potato agar plates at;				
37°C	+	+	+	+
40°C	+	+	+	+
42°C	+	+	+	+
Formation of dihydroxyacetone from glycerol	-	-	-	+
Acid production from				
Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Galactose	+	+	+	+
D-Mannose	+	+	+	+
D-Xylose	+	+	+	+
Sucrose	w	w	w	w
Melibiose	w	+	+	+
L-Rhamnose	-	-	-	-
meso-erythritol	-	-	-	+
Dulcitol	-	-	-	-
L- Arabinose	+	+	+	+
Utilization of C source				
D-Glucose	(+)	(+)	(+)	(+)
D-Mannitol	(+)	(+)	(+)	(++)
Glycerol	(+++)	(+++)	(+++)	(+++)
Ethanol	-	-	-	-
Methanol	-	-	-	-

Strain: 1, SL13E-2; 2, SL13E-3; 3, SL13E-4; 4, *A. pasteurianus*; +, positive; w, weakly positive; -, negative; (+++), Excellent growth; (++), Moderate growth; (+), Poor growth

[1,]Tanasupawat et al. 2009.

For physiological characterization of the three *Acetobacter* strains, their carbon source utilization on salt agar medium was examined. The three isolated strains grew well on glycerol but poorly on D-glucose and D-mannitol. They did not grow on ethanol or on methanol, similarly to strains of *A. pasteurianus*, but differently from *A. pomorum*, which grows well on both methanol and ethanol (Sokollek et al. 1998). As with *A. pasterurianus* (Tanasupawat et al. 2009), all three isolates produced acid from D-glucose, D-fructose, D-galactose, D-mannose, D-xylose, and L-arabinose, and weakly from sucrose, but not from *meso*-erythritol.

The physiological and biochemical characteristics of the *Acetobacter* strains are summarized in Table 3-1. The three isolates were very similar to *A. pasteurianus* reported by Tanasupawat et al (2009).

Next the 16S rDNA from the three isolates was sequenced. According to a BLAST search done on the GenBank database, all three *Acetobacter* strains have greater than 99% identity to several *A. pasteurianus* strains (e.g., SL13E-2, SL13E-3, and SL13E-4 have 100%, 99.85% (2-bp difference), and 99.92% (1-bp difference) identity to *A. pasteurianus* IFO3283 respectively). Thus the three strains were confirmed to belong to *A. pasteurianus*. Figure 3-1 shows the phylogenetic tree and was in agreement that SL13E-2, SL13E-3, and SL13E-4 belonged definitely to the *A. pasteurianus* cluster. A comparison of the 16S rDNA sequence data among the three isolates indicated that SL13E-2 shows a 2-bp and, a 1-bp difference from SL13E-3 and SL13E-4 respectively, while a 3-bp difference was seen between SL13E-3 and SL13E-4 (see Appendix I).

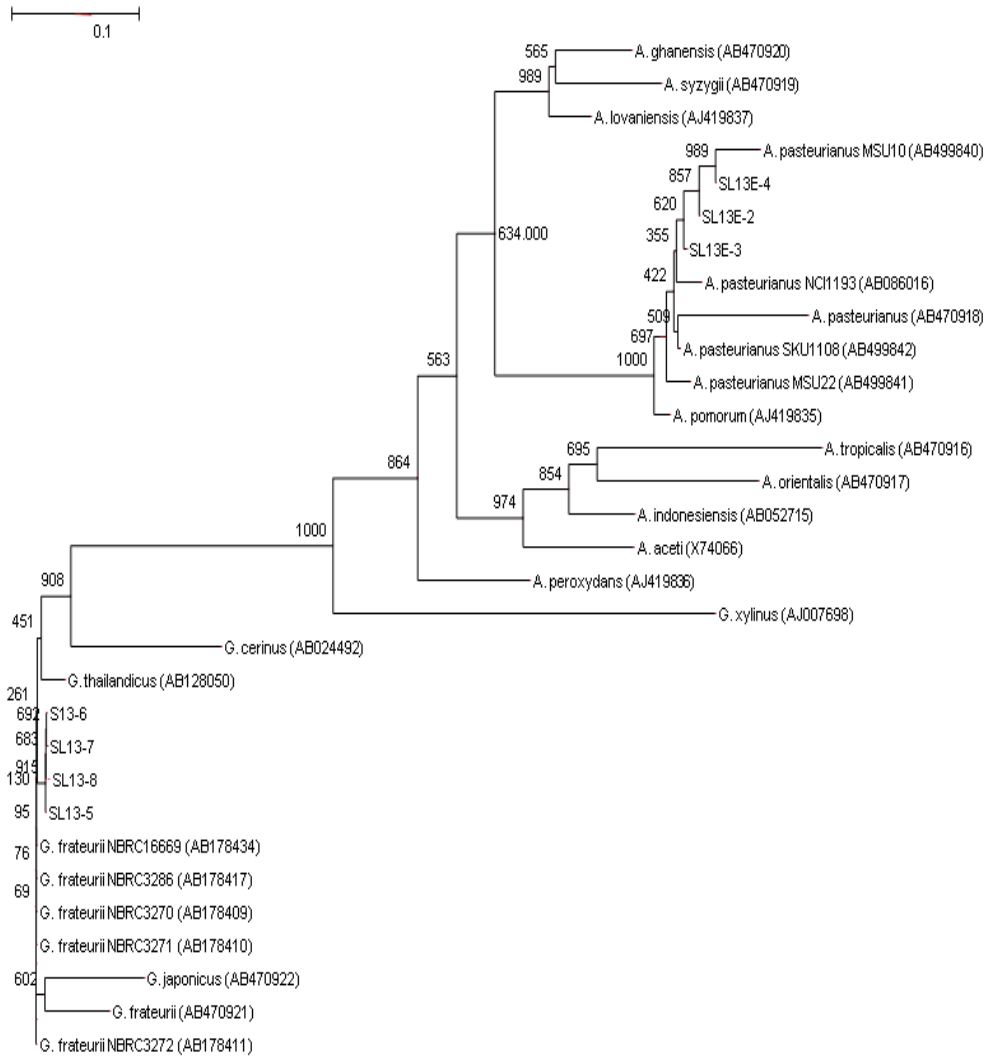

Fig. 3-1 Neighbor joining (NJ) phylogenetic tree calculated with CLUSTALW from 16S rDNA gene sequences of AAB. Phylogenetic tree was constructed using GENETYX Ver.10.

The 16S-23S ITS region of each strain was amplified and their nucleotide sequences (784 bp) were determined. The ITS sequence data of SL13E-2 and SL13E-3 were identical, while that of SL13E-4 was 4-bp different from SL13E-2 and SL13E-3 (see Appendix II).

Since these strains grew well on potato agar plates even at 42°C, the acetic acid and ethanol tolerance of the three *Acetobacter* strains was examined on YPGD agar plates containing either 1-4% (v/v) acetic acid or 1-10% (v/v) ethanol. Their tolerance was compared with that of a thermotolerant *A. pasteurianus* strain, SKU1108, isolated in Thailand (Saeki et al. 1997). Thermotolerant SKU1108 and SL13E-2, SL13E-3, and SL13E-4 tolerated 1% (v/v) acetic acid at 42°C on YPGD agar plates. All three SL13E strains tolerated up to 10% (v/v) ethanol at 37°C and 8% ethanol at 41°C, while SKU1108 grew in up to 10% ethanol at 41°C. At 42°C, SL13E-2, SL13E-3, and SL13E-4 tolerated only up to 3% (v/v) ethanol, while SKU1108 tolerated 4% (v/v) ethanol on YPGD agar plates. Thus, the three isolates obtained in this study were thermotolerant, and highly tolerate to ethanol and acetic acid, compared to SKU1108.

3.3.2 Identification and characterization of Gluconobacter-type strains from Sri Lankan vinegar

From the same samples, four *Gluconobacter*-type AAB (SL13-5, SL13-6, SL13-7, and SL13-8) showing different colony sizes were isolated by means of YPS (SL13-5 and SL13-7) and YPM (SL13-6 and SL13-8) media. These isolates showed no acetate oxidation.

In order to determine the species of the four isolates, their production of acid from pentitols (D-arabitol, L-arabitol, D-ribitol) was examined. This characteristic is useful in discriminating among the three *Gluconobacter* species, *G. cerinus*, *G. frateurii*, and *G. asaii* (Yamada et al. 1999). *G. frateurii* can produce acids from D-arabitol, L-arabitol, and D-ribitol, while *G. cerinus* can produces acids from D-arabitol but not from L-arabitol or ribitol. *G. oxydans* does not produce acids from any of these pentitols (Yamada et al. 1999; Moonmandmee et al. 2000). Since all four isolates showed acid production from D-arabitol, L-arabitol, and D-ribitol, they were identified as *G. frateurii*.

For 16S rDNA sequencing, the PCR products were obtained by using 27f and 1492r as primers from the isolates other than SL13-7. Another set of primers, Alf28f and 1512uR (Mühling et al. 2008), was used to obtain the PCR product for SL13-7. The 16S rDNA sequencing data of all four *Gluconobacter* isolates were identical except for 9 bp in the 5 region of SL13-7. This 16S rDNA sequence was almost identical to those of *G. frateurii* (more than 99% identity, with a 3-bp difference from that of *G. frateurii* NBRC3264, for example). Phylogenetic analysis also indicated that these four strains belong to *G. frateurii* cluster (Fig. 3-1). Moreover, the ITS sequence data for SL13-5 and SL13-6 were identical with to SL13-7 and SL13-8 respectively, while a mere 1-bp difference was seen between SL13-5/SL13-7 and SL13-6/SL13-8 (see Appendix III). In sum, the four isolates were almost identical: the 16S rDNA and the ITS sequences of SL13-6 and SL13-8 were entirely identical, and that of SL13-5 was different only in 1-bp from the other three (see Appendix IV). In the

case of SL13-7, the 5′ region of the 16S rDNA sequence was slightly different from those of the other three isolates.

The growth of the four *Gluconobacter* isolates was examined on potato agar plates at different temperatures. All four strains showed good growth up to 37°C, similarly to the thermotolerant *Gluconobacter* strains isolated in Thailand previously (Monmangmee et al. 2000).

3.3.3 Acetic acid production by isolated Acetobacter strains

Acetic acid production by the three isolated *Acetobacter* strains was compared with that by *A. pasteurianus* SKU1108 strain in YPGD medium with 4% (v/v) ethanol in shaking and static culture at different temperatures (Fig. 3-2). All the strains showed good growth up to 40°C (see Appendix V), although they showed different lag phases at 40°C (SL13E-2, SL13E-3, and SL13E-4 for 2, 3, and 6 days, respectively). SL13E-4 showed slower growth and acetic acid production than the other strains. In shaking culture, as compared to the SL13E-4 strain, the acetic acid production of SL13E-2 and SL13E-3 was lower at 30°C, showing a maximum at about 2% (w/v). On the other hand, SL13E-4 gave more than 3% (w/v) acetic acid production, comparable to the SKU1108 strain. At 37°C, all three strains grew and oxidized ethanol more rapidly than at 30°C and accumulated more than 3% (w/v) acetic acid in the culture medium. At 38°C, all three isolates produced more than 3% (w/v) acetic acid, although they showed a very long lag phase of 5 days in their growth profiles, whereas SKU1108 showed a very short lag phase, less than 1 day. Even at 40°C, SL13E-2 and SL13E-3 gave 3% (w/v) acetic acid production with a longer lag phase, whereas SL13E-4 grew very slowly (a lag phase of 6 days) and gave only 2.5% (w/v) acetic acid production.

Thus, at all temperatures examined, the isolated strains produced significant amounts of acetic acid, but less than SKU1108, in both shaking and static culture.

At all tested temperatures except for 30°C in YPGD medium supplemented with 4% (v/v) ethanol, the three isolated strains and SKU1108 showed acetate oxidation, which is unfavorable for vinegar production. Consistently with these results, Saeki et al. (1997b) reported that acetate oxidation was observed when the final concentration of acetic acid was less than 3.7% (w/v).

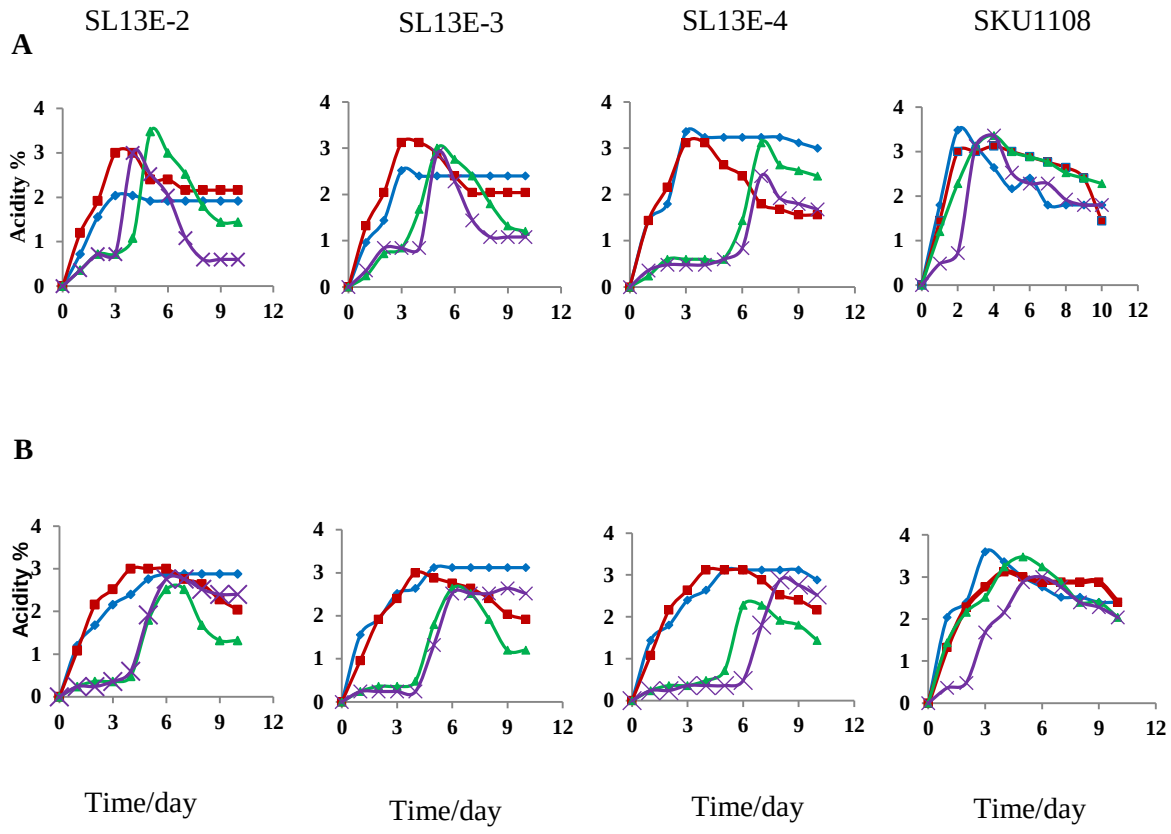

Fig. 3-2 Comparison of acetic acid production in shaking, 200 rpm (A) and static (B) cultures of SL13E-2, 13E-3, 13E-4 and SKU1108 at 30°C (diamonds), 37°C (squares), 38°C (triangles) and 40°C (crosses) in YPGD medium supplemented with 4% ethanol.

All three strains showed their best production of acetic acid at 37°C in shaking and static culture when the initial ethanol concentration was 6% (v/v) (Fig. 3-3). At 38°C and 40°C, even though it showed a long lag phase (6 days), SKU1108 showed significant acid production in a medium containing 6% (v/v) ethanol, whereas all three isolated strains showed very poor production (Fig. 3-3) and growth (data not shown). All three strains yielded the highest acetic acid production (more than 4% w/v) at 37°C in both shaking and static culture (Fig. 3-3). However, at 38°C and 40°C, none of the strains showed significant acetic acid production, while the SKU1108 strain gave more than 2% (w/v) and 3.5 % (w/v) acetic acid production in shaking and static culture respectively. The isolated strains, especially SL13E-2 and SL13E-3, provided a higher concentration of acetic acid at 37°C in shaking culture than SKU1108 did.

When the initial ethanol concentration was 6% (v/v), very little acetate oxidation by all four strains was observed at any temperatures, even though in some cultures the final acetic acid concentration was less than 3.7% (w/v) (Fig. 3-3).

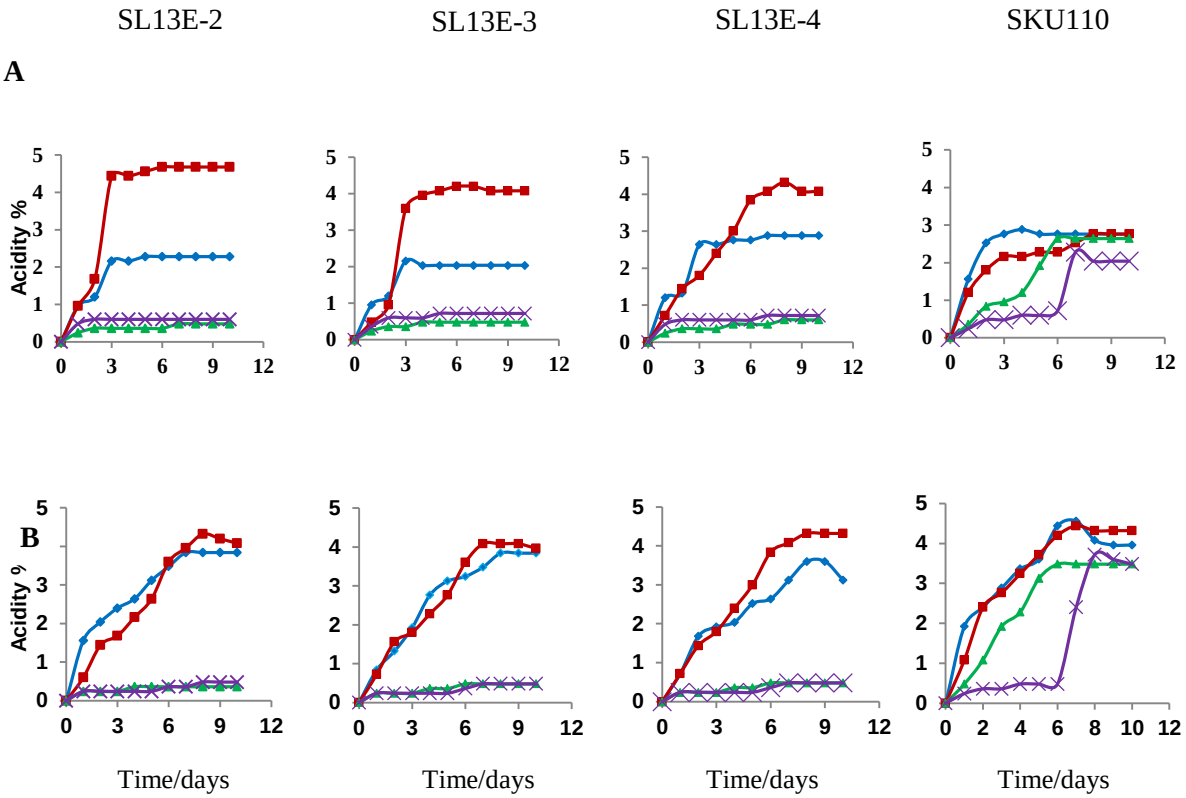

Fig. 3-3 Comparison of acetic acid production in shaking, 200 rpm (A) and static (B) cultures of SL13E-2, 13E-3, 13E-4 and SKU1108 at 30°C (diamonds), 37°C (squares), 38°C (triangles) and 40°C (crosses) in YPGD medium supplemented with 6% ethanol.

3.3.4 ADH and ALDH activities of thermotolerant Acetobacter strains

ADH and ALDH activities in the membrane fractions were measured by means of thermotolerant SL13E-2, SL13E-4, and SKU1108 grown at 37°C for 24 hrs with and without ethanol, and were compared with mesophilic *A. pasteurianus* IFO3284. The results revealed that both the ADH and the ALDH activities of the thermotolerant strains were stronger than those of mesophilic IFO3284 strain when the cells were grown at 37°C (Fig. 3-4), although the activities were quite variable among different batches of membrane fractions, and this probably impaired growth at 37°C. As shown in Fig. 3-4a, by the addition of ethanol to the culture medium, ADH activities of SL13E-2 and SL13E-4 were increased about 3.2-fold and 2.3-fold respectively, but not so greatly (1.2-fold) in the case of SKU1108. In case of mesophilic IFO3284 strain, such as induction in ADH activity was not observed due to the addition of ethanol at 37°C, which is beyond the optimum growth temperature. On the other hand, the ALDH activities of each strain were unchanged by the addition of ethanol (Fig. 3-4b) except for SL13E-2, for which slight induction was observed (up to 1.5-fold). Thus the enzyme activities of ADH were induced in response to the addition of ethanol although induction levels varied among strains, whereas ALDH activities almost unchanged in all strains.

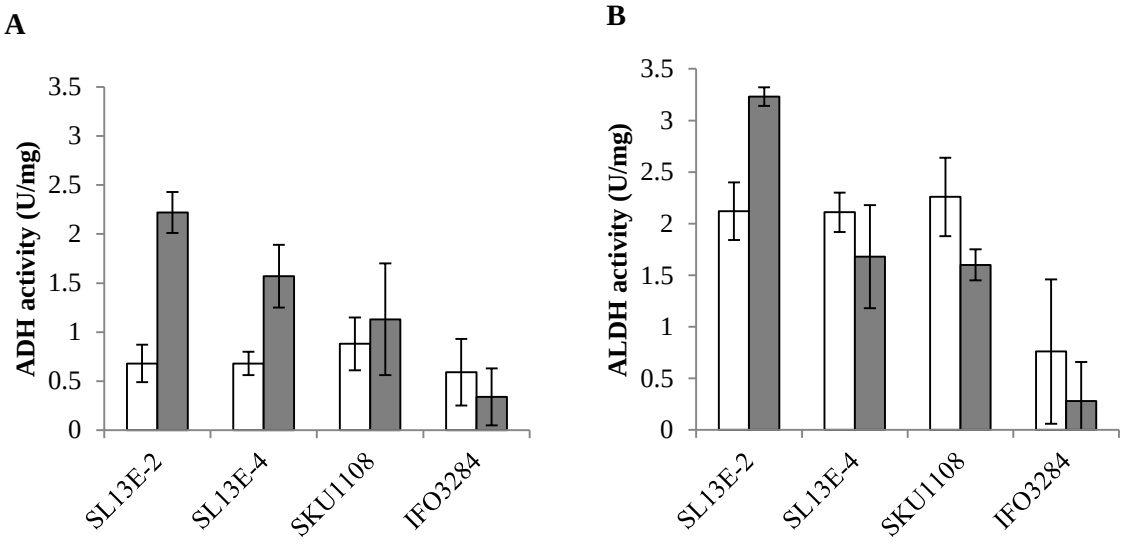

Fig. 3-4 ADH (a) and ALDH (b) activities of *A. pasteurianus* SL13E-2, SL13E-4, SKU1108 and IFO3284 strains grown at 37°C without ethanol (white bar) and with ethanol (ash bar) in the culture medium. Assays were performed in triplicate: the bars indicate averages, and the error indicates standard deviation.

In addition, the thermostability of the ADH and ALDH of thermotolerant SL13E-2, SL13E-4, and SKU1108 were examined by incubation of the membrane fractions at 50°C, 55°C, and 60°C. As shown in Fig. 3-5, as well as the thermotolerant SKU1108 strain, SL13E-2 and SL13E-4 showed almost the same thermal denaturation profiles of both ADH and ALDH. The inactivation of ALDH by thermal treatment was slightly slower than that of ADH. Thus, ALDH in the isolated strains were also more thermostable than ADH as previously reported (Saeki et al. 1997a; Ndoye et al. 2006; Adachi et al. 1988). All three strains accounted for more than 50% remaining ADH and ALDH activities after incubating at 50°C for 30 min. Both the ADH and the ALDH of the three strains became unstable over 55°C, and rapidly inactivated at 60°C, so as the case of SKU1108. Similar results were found for the membrane fraction obtained from the cells grown on the medium in the presence of ethanol (data not shown).

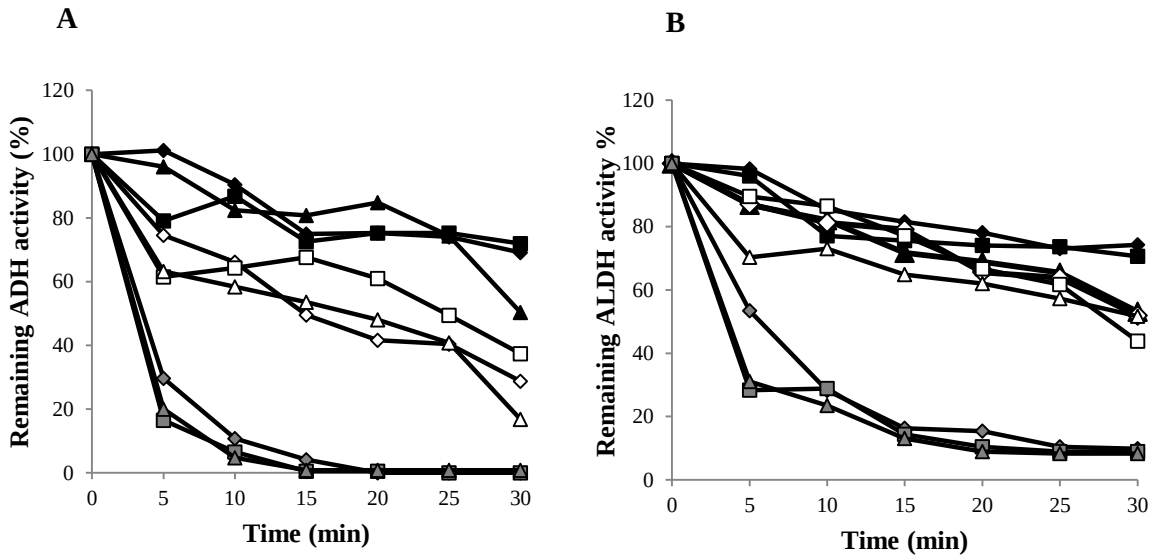

Fig. 3-5 Remaining ADH (A) and ALDH (B) activities of *A. pasteurianus* SL13E-2 (diamonds), SL13E-4 (squares) and SKU1108 (triangles) were assayed after incubating the membrane fractions for 5-30 min at 50°C (black), 55°C (white) and 60°C (ash).

3.3.5 Production of coconut water vinegar

Simultaneous vinegar production by the isolated strains in coconut water with baker`s yeast was examined without shaking at 37, 39, and 40°C (Fig. 3-6).When both yeast and *Acetobacter* strains were added, higher acid production was obtained than when the isolates were added after 1 day of cultivation with baker's yeast alone (Fig. 3-6B). At 37°C, all three isolated strains gave 4% acetic acid production, as SKU1108 did. Although they showed acetate oxidation in YPGD medium with 4% (v/v) ethanol (Fig. 3-2), acetate oxidation was not observed in coconut vinegar production (Fig. 3-6A). Both SL13E-2 and SL13E-3 provided about 3% (w/v) acetic acid production at 39°C and 2.5% (w/v) at 40°C, slightly better than SKU1108. When a mixed culture of the three isolates was used, acid production similar to SL13E-2 or SL13E-3 was observed (data not shown).

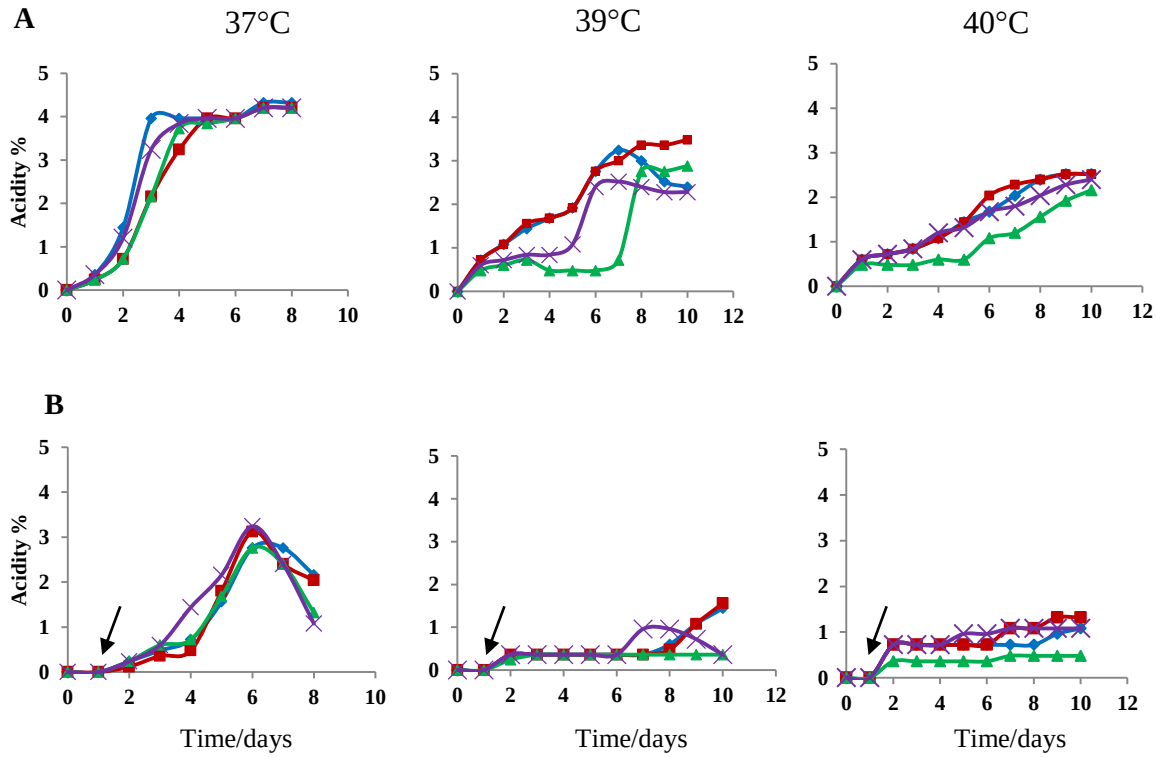

Fig. 3-6 Simultaneous coconut vinegar production by SL13E-2 (diamonds), SL13E-3 (squares), SL13E-4 (triangles) and SKU1108 (crosses) at 37°C, 39°C and 40°C. Dry yeast and AAB were added simultaneously (a) or sequentially (b). Arrows show the point of addition of AAB in sequential addition.

3.4 Discussion

In this study, we isolated, identified, and characterized thermotolerant AAB from Sri Lankan coconut water vinegar. The fermentation capacities of the three *Acetobacter* strains at higher temperatures were compared with that of a thermotolerant *A. pasteurianus* strain, SKU1108, which was isolated in Thailand previously (Saeki et al. 1997a).

The three *Acetobacter*-type isolates (SL13E-2, SL13E-3, and SL13E-4) grew as well at 42°C as the SKU1108 strain on potato agar plates, and tolerated up to 1% (w/v) acetic acid and 8% (v/v) ethanol in YPGD agar medium. The results obtained were similar to those published for other thermotolerant AAB (Ndoye et al. 2006; Adachi et al. 1988; Kanchanarach et al. 2010). On the other hand, mesophilic *A. pasteurianus* IFO3191 did not grow on potato agar plates at 39°C even in the absence of ethanol and of acetic acid, while *A. pasteurianus* IFO3284 grew on a potato agar plate at 39°C but not at 41°C. It tolerated 1% acetic acid at 39°C and only 4% ethanol at 37°C (Kanchanarach et al. 2010). Since SL13E-2, SL13E-3, and SL13E-4 grew well at up to 42°C and tolerated acetic acid and ethanol at elevated temperatures, they are considered "thermotolerant" AAB. Similarly, SL13-5, SL13-6, SL13-7, and SL13-8 are also considered thermotolerant *Gluconobacter* AABs, because although the mesophilic type strain, *G. frateurii* IFO3276, does not grow at 37°C, these strains do grow, like the thermotolerant isolates in Thailand (Moonmangmee et al. 2000). Even though our strains could tolerate up to 8% ethanol at 41°C on agar plates, they were unable to perform acetic acid fermentation at temperatures higher than 38°C with 6% (v/v) ethanol in liquid culture (Fig. 3-3). This might have been due to higher stress caused by both increased temperature and lower oxygen availability in liquid culture.

According to Bergy's Manual of Systematic Bacteriology (Brenner et al. 2005), all *Acetobacter* species can use acetic acid as carbon source. This so-called over-oxidation is unfavorable for vinegar production. According to Saeki et al. (Saeki et al. 1997a), over-

oxidation does not occur when oxidizable ethanol and other carbon sources remain in the culture medium, and no apparent over-oxidation occur when the acetic acid concentration rises above 4.5% (w/v). For many strains, the threshold acetic acid concentration to overcome over-oxidation is around 3.7% (w/v). Thus, in general, over-oxidation can be avoided by terminating vinegar fermentation when a small amount of ethanol remains in the vinegar mash, or by allowing an acetic acid accumulation of in the vinegar mash. In coconut vinegar production, over-oxidation of acetic acid is a frequent problem, since a lower acetic acid concentration is achieved (about 3% (w/v)) than in rice vinegar production (about 5% (w/v)). Our results show significant over-oxidation with all four strains tested on initial ethanol concentration of 4% (v/v), especially at higher temperatures (Fig. 3-2). However, with a 6% (v/v**)** initial ethanol level, no over-oxidation was observed (Fig. 3-3) even though the acetic acid produced was less than 3.7% (w/v). These results indicate that the strains isolated in this study are suitable for the production of coconut water vinegar.

In acetic acid fermentation, ethanol is converted into acetic acid by two membrane-bound dehydrogenases, ADH and ALDH. Hence we also examined the ethanol induction pattern and thermal resistance of ADH and ALDH of the isolated strains and compared them with thermotolerant SKU1108. According to our results, the ADH activity of SL13E-2 and SL13E-4 grown at 37°C has been induced by addition of ethanol in 3.2-fold and 2.3-fold respectively. A study by Takemura et al (1993) reported that the ADH activity of *A. pasteurianus* NCI1380 grown at 30°C was enhanced more than 10-fold by the addition of ethanol to the medium.

The ADHs from all four thermotolerant strains became unstable over 55°C and became inactive at 60°C with irrespective to addition of ethanol in culture medium (Fig. 3-5). Kanchanarach et al (2010) reported that ADH from thermotolerant *A. pasteurianus* MSU10 were stable up to 45°C for 30 min, but become unstable over 50°C and rapidly inactivated at

55°C. Thus, the ADHs from SL13E-2 and SL13E-4 were slightly heat stable than ADH from thermotolerant *A. pasteurianus* MSU10. According to Adachi et al (1988), ALDH from mesophilic *Acetobacter* strains showed already high thermostability, and hence thermostability of ADHs are more important for vinegar production at higher temperature. The ALDH activities of the isolated strains were more thermostable against heating than their ADH activities, and more than 30% of the original ALDH activity was remained after 10 min heating at 60°C (Saeki et al. 1997a; Ndoye et al. 2006; Adachi et al. 1988) (Fig. 3-5).

The potential use of the three *Acetobacter*-type strains for coconut vinegar production at higher temperatures was examined. We tested two procedures: the simultaneous addition of yeast and AAB, and the sequential addition of AAB after yeast was cultivated alone for one day at an ethanol concentration of about 4% (v/v) (data not shown). As shown in Fig. 3-6, simultaneous addition gave better results than sequential addition, up to 4% (w/v) and 2.5% (v/v) acetic acid at 37°C and 40°C respectively, in the case of SL13E-2 and SL13E-3. This is slightly higher than the concentrations produced using SKU1108 (Fig. 3-6). In sequential addition, the yeast grew and produced ethanol rapidly by consuming most of the available glucose (data not shown). This led to poor glucose availability for AAB at the time they are introduced to the medium. All four AAB strains required glucose for better growth. Thus the poor availability of glucose in the medium caused poor growth and acetic acid production in sequential vinegar production. No over-oxidation was observed during coconut water vinegar production within the periods examined, except when sequential addition at 37°C was attempted (Fig. 3-6). These results were inconsistent with the results in Figure 3-2, probably due to the differences in carbon sources in the medium. For over-oxidation, complete exhaustion of ethanol and availability of another carbon source that supplies oxaloacetate to convert acetyl-CoA to citrate are required (Saeki et al. 1997b). In coconut water vinegar production, glucose and sucrose are carbon sources and they are almost completely consumed

by yeast and AAB. As in Figure 3-2, glucose and glycerol were carbon sources, and probably some amount of glycerol remained even after ethanol is completely oxidized to acetic acid.

Our results indicate that as well as thermotolerant *A. pasteurianus* SKU1108 strain, the isolated *Acetobacter*-type strains can be used for coconut water vinegar production at higher temperatures and can be used as starter cultures in the vinegar industry.

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Chapter 4

Pellicle of thermotolerant *Acetobacter pasteurianus* strains: Characterization of polysaccharide and induction patterns

Abstract

Acetobacter species are well known to have an ability to grow floating on the surface of the medium by producing pellicle, which consists of cells and a self-produced matrix of cell-attached polysaccharides. We previously isolated three thermotolerant strains (SL13E-2, SL13E-3, and SL13E-4) from Sri Lankan coconut vinegar, and identified all strains as *A. pasteurianus*. Thus, the pellicle polysaccharides produced by the isolated strains and *A. pasteurianus* SKU1108 were characterized. The monosaccharide composition of pellicle polysaccharides of *A. pasteurianus* strains found to be varied among strains. The pellicle polysaccharide of SL13E-2 composed of rhamnose and glucose in the ratio of 1:8 and that of SL13E-4 and mesophilic *A. pasteurianus* NBRC3191 consisted of rhamnose, glucose, and xylose in the ratio of 1:5:2 and 1:4:2 respectively. On the other hand, the pellicle polysaccharides of SL13E-3 and SKU1108 strains were composed of rhamnose, glucose, and galactose in the ratio of 2:2:1 and 1:5:2.5 respectively. The pellicle formation of thermotolerant SL13E-2, SL13E-3, and SL13E-4 were significantly induced by the addition of ethanol in the medium, while a poor induction was observed in only SKU1108 strain. The molecular or polymerized size and sugar composition of the polysaccharides obtained from the cells induced by ethanol were unchanged from those from the uninduced cells, indicating that number of molecules of the polysaccharides increased and not changed the individual molecule of polysaccharide. On the other hand, addition of carbon sources, such as glucose, sucrose and fructose, showed a slight induction of pellicle formation in SKU1108 strain, especially at 40°C.

4.1 Introduction

Both gram-positive and gram-negative bacterial species produce extracellular hetero- or homopolysaccharides. These polysaccharides are either permanently attached to the outer surface of the cells or secreted into the growth medium, and are known as capsular polysaccharides (CPS) or extracellular polysaccharides (EPS), respectively. These bacterial polysaccharides are a focus of study because of their importance in varied biological processes such as bacteria-host interaction, biofilm formation, and stress adaptation.

Acetobacter and *Gluconacetobacter* species, two genera of acetic acid bacteria (AAB), are well known for their strong ability to oxidize ethanol to acetic acid. This oxidation process is absolutely dependent on highly aerobic respiration using membrane-bound enzymes. In addition, AAB can tolerate high concentrations of acetic acid, and thus they are widely used for vinegar production. In order to maintain a highly aerobic state, *Acetobacter* and *Gluconacetobacter* have the ability to grow floating in static culture by producing a pellicle on the surface of the medium. The pellicle is a collection of cells that are tightly associated through the CPS on the cell surface. The pellicle polysaccharides are a homopolysaccharide of cellulose which is produced by *Gluconacetobacter xylinus*, or as heteropolysaccharides. Examples of the latter include the CPS produced by *A.aceti* (reclassified as *A. pasteurianus* subsp. Lovaniensis) consisting of glucose and rhamnose (Moonmangmee et al. 2002a) or as that of produced by *A. tropicalis* SKU1100 consist of glucose, galactose and rhamnose (Moonmangmee et al. 2002b)

The genetic study on the pellicle polysaccharide produced by *A. tropicalis* SKU1100 showed that a gene cluster, of *polABCDE*, is required for the pellicle formation; *polB* is involved in the production of dTDP-rhamnose, while *polE* involved in anchoring the polysaccharide to the cell surface (Deeraksa et al. 2005). Disruption of *polE* gene caused no pellicle formation in static culture due to the absence of CPS production. Instead, it secret

EPS with the same sugar composition to the culture medium. Further, *galE* gene that encodes UDP-galactose 4-epimerase was identified to be involved in UDP-galactose biosynthesis in *A. tropicalis* SKU1100 (Deeraksa et al. 2006).

Previous studies reported that *A. pasteurianus* IFO3284 produces two different types of colony: a rough-surfaced colony (R strains) that can produce a pellicle and a smooth-surfaced colony (S strain) that cannot produce a pellicle in static culture. The R and S strains are inter-convertible by spontaneous mutation (Matsushita et al. 1992). According to Kanchanarach et al. (2010), the R strain of different *A. pasteurianus* strains gave higher acetic acid production, whereas the S strains could not complete the fermentation. This suggests that the R strain (with pellicle polysaccharide) have a higher acetic acid resistance than the S strains. Moreover, it suggests that the pellicle CPS functions as a barrier-like biofilm against passive diffusion of acetic acid into the cells.

Consequently, formation of a stable pellicle would be beneficial for vinegar production in static culture, and it would be even more useful for thermotolerant strains to produce a pellicle, even at higher temperatures. In this study, we characterized the pellicle produced by thermotolerant *Acetobacter pasteurianus* species (SL13E-2, SL13E-3, SL13E-4, and SKU1108). SL13E-2, SL13E-3, SL13E-4 were isolated from Sri Lanka in a previous study (Perumpuli et al. 2014). We quantitatively analyzed pellicle formation at different temperatures and at different ethanol concentrations to understand relationship between pellicle formation and stress tolerance. We here report that the sugar composition of the pellicle polysaccharide varied among different strains in *A. pasteurianus* species.

4.2 Materials and Methods

4.2.1 Bacterial strains, culture media and culture conditions

Three thermotolerant *A. pasteurianus* strains (SL13E-2, SL13E-3, SL13E-4) isolated from Sri Lankan coconut vinegar (Perumpuli et al. 2014), and thermotolerant *A. pasteurianus* SKU1108 previously isolated in Thailand (Saeki et al 1997), were used in this study. Potato medium (20 g of glycerol, 10 g of yeast extract, 10 g of polypeptone, 5 g of D-glucose, and 100 ml of potato extract in 1 L of tap water) with or without ethanol was used to isolate the pellicle polysaccharide. YPGD medium (20 g of glycerol, 10 g of yeast extract, 10 g of polypeptone, and 5 g of glucose in 1 L tap water) supplemented with a carbon source (ethanol, glucose, sucrose or fructose) was used to quantify the pellicle. Seed culture of test strains were grown in 5 ml potato medium with shaking culture (200 rpm) for 24 h at 37°C. *A. pasteurianus* NBRC3191 was obtained from Biological Resourcce Center, NITE, Japan, and cultured at 30°C.

4.2.2 Isolation of pellicle polysaccharides

Precultures of all strains were performed by 5% (v/v) inoculation of seed culture into 20 ml of same medium in 100 ml Erlenmeyer flask, then shaking on a rotary shaker (200 rpm) at 30°C for 24 hrs. The culture was transferred to 500 ml of same medium with or without 4% (v/v) ethanol in a 2 liter Erlenmeyer flask and incubated with vigorous shaking at 37°C for 48 hrs. Cells were harvested by centrifugation at 9, 000 $\times g$ at 4°C for 10 min and washed twice with 0.1 M Tris-HCl buffer (pH 8.0).

CPS was isolated by two methods: osmotic shock method (Moonmangmee et al. 2002b) and the sonication method of Moonmangmee et al (2002a) with some modifications. First, spheroplasts were prepared as described in Moonmangmee et al. (2002b). The obtained

spheroplasts were suspended in 10 mM potassium phosphate (KPB), pH 6.5 (about 1 g of spheroplasts per 2 ml of buffer), then the spheroplast membranes were collected by ultracentrifugation without sonication. In the sonication method, the suspension was ultrasonically disrupted in 50 ml plastic tube maintained in an ice bath (0-4°C) using an ultrasonic disruptor (Ultra S Homogenizer VP-15S) with a tapered microprobe (0.7 cm diameter) at the maximum setting for 5 min. The disrupted cell suspension was centrifuged at 9,000 ×g for 10 min at 4°C to remove debris and unbroken cells, then the supernatant was further centrifuged at 150,000×g for 90 min at 4°C. To the obtained supernatant, DNase was added to a final concentration of 50 μg/ml and was incubated at 37°C for 24 hrs. Then proteinase K was added to the suspension to a final concentration of 100 μg/ml and incubated for a further 24 hrs under the same conditions. The supernatant was thoroughly dialyzed at room temperature for 24 hrs against 0.1 M NaCl containing 0.1% SDS using a cellulose membrane tube (molecular weight cutoff 12,000-14,000). After removal of the precipitate by centrifuging at 9,000×g for 10 min at 4°C, twice volume of isopropyl alcohol was added to the supernatant and left overnight at 4°C to precipitate polysaccharides. The suspension was centrifuged at 8,000×g for 30 min at 4°C and the resultant precipitate (crude polysaccharide) was suspended in MilliQ water and loaded onto a Sephacryl S-300 column (ø 1.6×60 cm). The column was eluted with MilliQ water at a flow rate of 0.3 ml/min. Polysaccharides were passed through the column, and the fractions containing polysaccharide were determined by measuring sugar contents as described below, then these fractions were combined. The polysaccharides were precipitated with isopropyl alcohol as described above, then the obtained precipitate was dried up and weighed.

4.2.3. Determination of monosaccharide composition by thin layer chromatoghraphy and high performance liquid chromatography

One mg of isolated polysaccharides was hydrolyzed by 2 N trifluoroacetic acid (TFA) for 16 hrs at 100°C in a glass screw-capped vial. The resultant solution was dried in a centrifuged evaporator, dissolved in 1 ml of distilled water, and evaporated to dryness. The hydrolyzed pellet was dissolved in 100 µl distilled water and was applied (~20 µg of starting material) on to a silica gel plate (Silica gel 60, Merk Co., Germany). The plate was developed with 1-propanol:water (85:15, v/v). Spots were detected by spraying 5% H_2SO_4 in ethanol and baking for 10 min at 100°C (Deeraksa et al. 2005).

The monosaccharide composition of CPS samples was confirmed by high performance liquid chromatography (HPLC) analysis. The hydrolyzed monosaccharides were identified by comparing the retention times of the peaks with those of standard sugars using DIONEX HPLC, equipped with a CarboPac PA1 (ø 0.4×25 cm) column. Sugars were eluted at a flow rate of 1 ml/min with 14 mM NaOH.

4.2.4 Measurement of molecular mass of the purified polysaccharide

The average molecular mass of each purified polysaccharide was estimated by high performance liquid chromatography (HPLC) using Shodex $GMPW_{XL}$ column (ø 0.78×30 cm) eluted at a flow rate of 1 ml/min with MilliQ water. A calibration curve was obtained with the following standard pullulans (Showa Denko K.K., Tokyo, Japan): P800 (805 kDa), P400 (366 kDa), P200 (200 kDa), P100 (113 kDa), P50 (48.8 kDa), P20 (21.7 kDa), P10 (9.6 kDa), and P5 (6.1 kDa).

4.2.5 Crystal violet staining method for quantification of the pellicle

Produced pellicle was quantified by crystal violet (CV) assay used in biofilm quantification (Burton et al. 2007; Ren et al. 2005) with some modifications appropriate for *A. pasteurianus* strains. Culture medium (2 ml) under the pellicle was aspirated to remove the planktonic cells, and the pellicle was washed twice with distilled water, then allowed to dry for 15 min. Then, the pellicle was stained with 2 ml of 0.004% (w/v) CV for 15 min. The unbound dye was removed by six washes with distilled water and the pellicle was air-dried for 15 min. The CV absorbed to the pellicle was solubilized by adding 2 ml of 95% ethanol. The amount of pellicle formed was represented by absorbance measured at 540 nm.

To quantify the pellicle, all four strains were precultured in 5 ml potato medium with shaking culture (200 rpm) at 30°C for 24 hrs, and 5% inoculant was added to 2 ml YPGD enrichment medium containing one of several carbon sources (ethanol, glucose, sucrose or fructose). The cultures were statically incubated at 30°C, 37°C and 40°C for 7 days, and the produced pellicle was quantified with the CV staining method as described above.

4.2.6 Other analytical procedures

Sugar content was measured by the phenol sulfuric acid method (Dubois et al. 1956) using glucose as the standard.

4.3 Results

4.3.1 Monosaccharide composition of pellicle polysaccharide

Both the osmotic shock method and sonication method were used to isolate the pellicle polysaccharide from each strain. Data provided by the two methods were in agreement. The pellicle produced by *A. aceti* IFO3284 (Moonmangmee et al. 2002a) and thermotolerant *Acetobacter* sp. SKU1100 (Moonmangmee et al. 2002b), and the pellicle polysaccharide of *A. pasteurianus* strains, are tightly attached to the spheroplast membrane of the cell. The polysaccharide was retained in the spheroplast membrane even when subjected to the sonication method. Since much larger quantities of polysaccharides were obtained using the sonication method, this method was chosen to harvest polysaccharides for analysis.

In our previous study, we isolated three thermotolerant *A. pasteurianus* strains (SL13E-2, SL13E-3, and SL13E-4) from Sri Lankan coconut vinegar, using YPG medium containing ethanol (Perumpuli et al. 2014). In order to compare the polysaccharides of different strains of *A. pasteurianus* species, the pellicle polysaccharides of four thermotolerant strains (SL13E-2, SL13E-3, SL13E-4 and SKU1108) and one mesophilic (NBRC3191) strain were isolated. The molecular sizes of the isolated polysaccharides were in the range 8.4×10^4 -4.8×10^5 Da as determined by gel filtration analysis (Table 4-1). Polysaccharides isolated from cells grown in the presence of ethanol were also analyzed and were found to be of essentially the same molecular size as that of the polysaccharides isolated from cells grown without ethanol (Table 4-1).

Table 4-1: Average molecular mass of pellicle polysaccharides of *A. pasteurianus* strains.

	Retention time (min)	**Provided or estimated molecular mass**
Standards:		
P800	6.61	805,000
P400	7.22	366,000
P200	7.53	200,000
P100	7.81	100,000
P50	8.32	48,000
P20	8.77	21,700
P10	9.14	9,600
P5	9.52	6,100
Sucrose	10.7	360
Polysaccharides from:		
SL13E-2	7.33	250,000
SL13E-2 (with ethanol)	7.697	121,000
SL13E-3	7.872	86,000
SL13E-3 (with ethanol)	7.832	93,000
SL13E-4	7.594	150,000
SL13E-4 (with ethanol)	7.769	105,000
SKU1108	6.996	480,000
SKU1108 (with ethanol)	7.285	274,000
NBRC3191	7.885	84,000

The isolated polysaccharides were hydrolyzed with 2 N TFA and the sugar compositions were determined by both HPLC (Fig.4-1) and thin layer chromatography (TLC) analysis. When many samples were analyzed by HPLC, the time of each sugar increased compared to the standard sugar mix, but the elution order remained unchanged. Both HPLC (Fig. 4-1) and TLC showed that glucose was the main sugar component in all samples. The HPLC retention times of xylose and mannose were very close and provided an overlapped peak in the HPLC chromatogram (Fig. 4-1A). As shown in Fig. 4-1D and F, both SL13E-4 and NBRC3191 provided a peak close to xylose and mannose. Thus, to confirm the composition of polysaccharide from SL13E-4 and NBRC3191, TLC analysis was conducted using xylose and mannose standards (Fig. 4-2). The pellicle polysaccharide of SL13E-2 was found to be composed of rhamnose and glucose in the ratio 1:8, while that of SL13E-4 consisted of rhamnose, glucose, and xylose in the ratio 1:5:2. On the other hand, the pellicle polysaccharides of SL13E-3 and SKU1108 strain was composed of rhamnose, glucose, and galactose in the ratio 2:2:1 and 1:5:2.5 respectively. In addition, the pellicle polysaccharide of mesophilic *A. pasteurianus* NBRC3191 consisted of rhamnose, glucose, and xylose in the ratio 1:4:2. These results indicated that sugar compositions of CPS produced by *A. pasteurianus* strains were quite diverse, even within the same species. Furthermore, the determined compositions were different from those of CPS from other *Acetobacter* species: glucose, galactose, rhamnose, and glyceric acid from *A. methanolicu*s (Grimmecke et al. 1994), rhamnose and glucose from *A. aceti* IFO3284 (Moonmangmee et al. 2002a, and rhamnose, galactose, and glucose from *A. tropicalis* SKU1100 (Moonmangmee et al. 2002b). The determined compositions were also different from the compositions of EPS previously reported for *Acetobacter* species: glucose, mannose, galactose, and glucuronic acid in *Acetobacter* sp. (MInakami et al. 1984), glucose, mannose, rhamnose, and glucuronic acid in

Acetobacter sp. NBI1022 (Tayama et al.1985), and glucose, galactose, and mannose in *Acetobacter* sp. (MacCormick et al. 1996).

The sugar compositions of the polysaccharides obtained from the cells grown with ethanol were also determined and were shown to be unchanged (Appendix V).

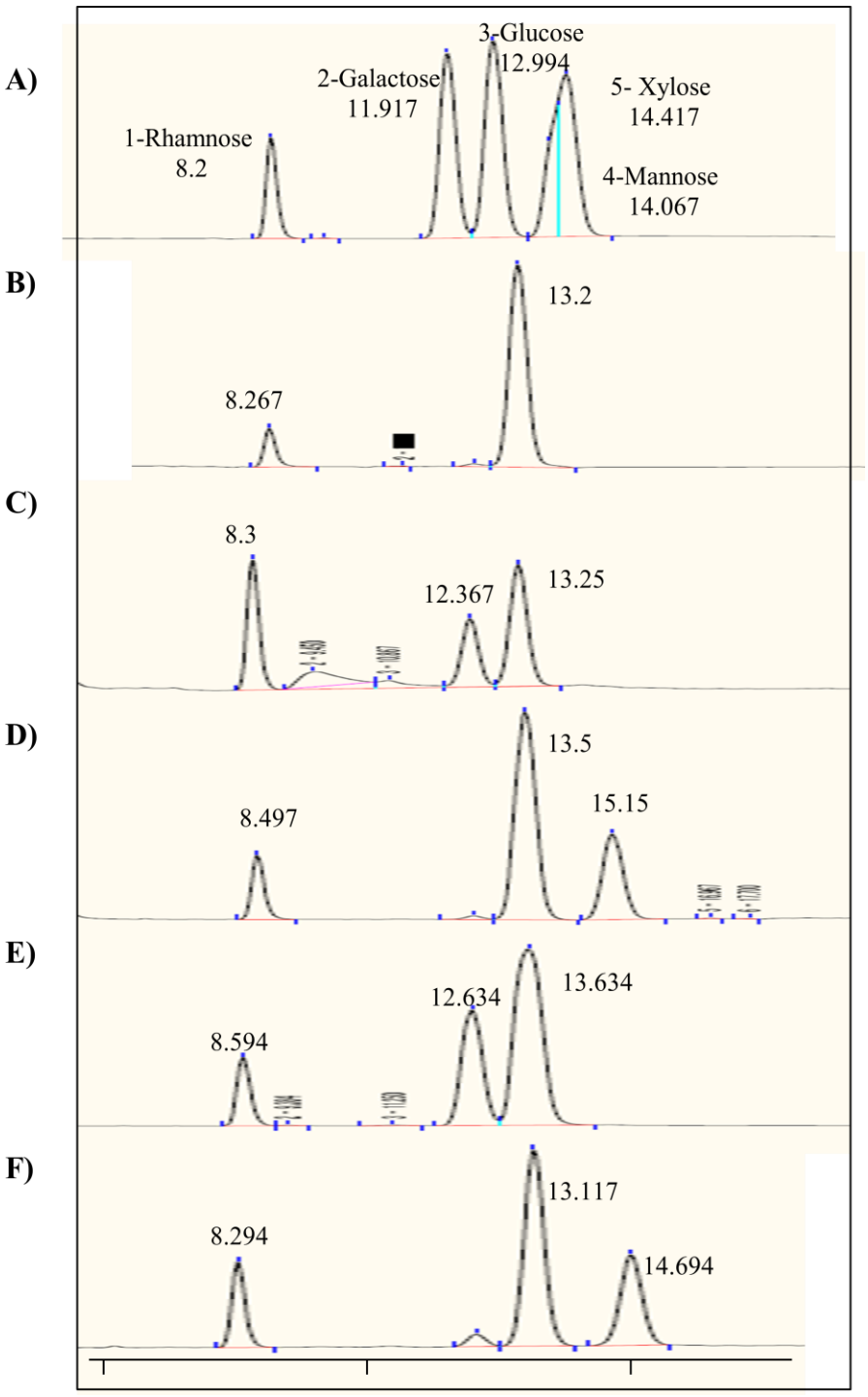

Fig. 4-1 HPLC profiles of acid-hydrolyzed products of purified polysaccharides from *Acetobacter pasteurianus* strains (uninduced by ethanol). A) authentic sugar mix of rhamnose, xylose, mannose, glucose, and galactose; B) *A. pasteurianus* SL13E-2; C) *A. pasteurianus* SL13E-3; D) *A. pasteurianus* SL13E-4; E) *A. pasteurianus* SKU1108; F) *A. pasteurianus* NBRC3191.

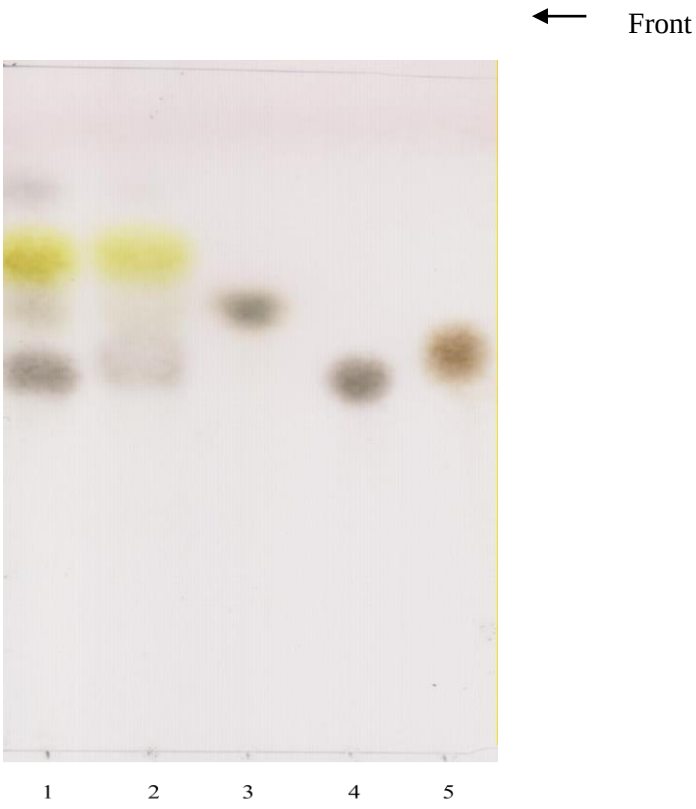

Fig. 4-2 TLC profiles of acid-hydrolyzed products of purified polysaccharides from *A. pasteurianus* SL13E-4 and NBRC3191.

Lane 1, SL13E-4; lane 2, NBRC3191; lane 3, xylose; lane 4, glucose; and lane 5, mannose.

4.3.2 Effect of temperature and ethanol on pellicle production of thermotolerant strains

In order to determine the optimal concentration of CV for quantification of pellicle of *A. pasteurianus* isolates, CV concentration in a range of 0.001%-0.1% (w/v) were tested prior to the actual analysis. If the concentration is too high, the absorbed CV on the pellicle was not dissolved completely in ethanol and would provide inconsistent results (Fig. 4-3). We found that by using 0.004% CV, the adsorbed CV dissolved completely in ethanol and the stained cells were de-stained. The absorbance of CV was proportional to the amount of pellicle and data were reproducible (Fig. 4-4)). Therefore, we used 0.004% CV for staining of the pellicle.

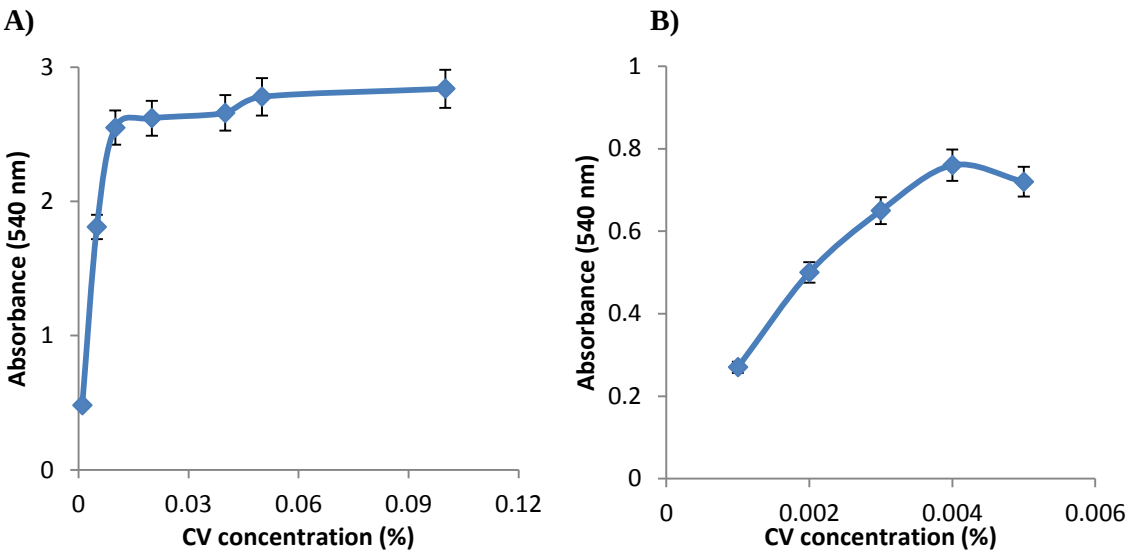

Fig. 4-3 Relationship between the CV concentration and the obtained absorbance for pellicle of *A. pasteurianus* strains. A), CV concentration of 0.001% to 0.1&; B), CV concentrations of 0.001% to 0.005%. Assays were performed in triplicates.

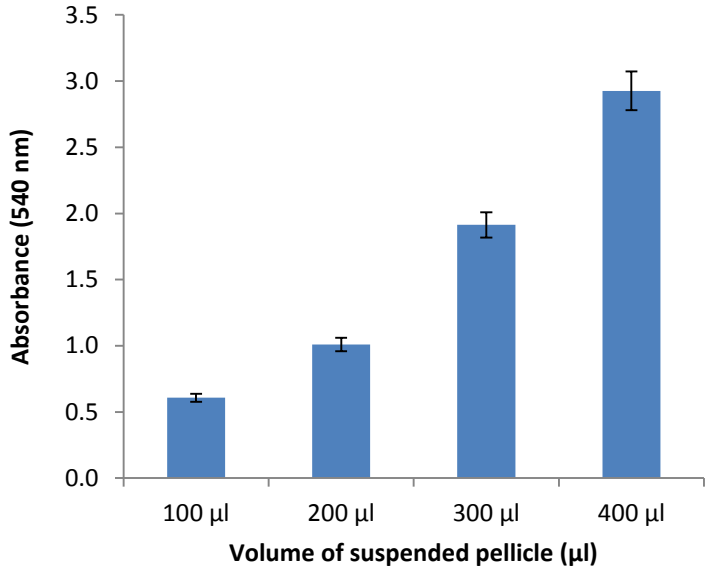

Fig. 4-4 Relationship between the amount of pellicle and absorbance for CV staining method with 0.004% CV. Assays were performed in triplicates and bars indicate averages and standard deviation.

Since production of pellicle is an important phenomenon in thermotolerant *Acetobacter* strains, we examined the relationship between the ethanol stress and pellicle formation of four thermotolerant *A. pasteurianus* strains at 30ºC, 37ºC, and 40ºC (Fig. 4-5). In the absence of ethanol, the isolated strains formed less pellicle as the culture temperature increased, especially at 40ºC. In the case of SKU1108, similar pellicle formation was observed at 30ºC and 37ºC, but much less at 40ºC. We found that better pellicle production by all four strains in the presence of ethanol at all tested temperatures than in the absence of ethanol. Comparison of the growth of the four strains with or without ethanol in shaking culture, showed less growth with ethanol (data not shown), indicating that the pellicle formation is not induced by simple growth stimulation. Of the four tested thermotolerant strains, noticeable induction was observed in SL13E-2, SL13E-3, and SL13E-4 at 1% ethanol; production remained essentially the same at higher ethanol concentrations. Pellicle

production by SKU1108 was poorly induced by the addition of ethanol at 37ºC. All four strains produced comparable amounts of pellicle at 30ºC and 37ºC, and much less pellicle at 40ºC. At 40ºC, both SL13E-2 and SKU1108 produced pellicle up to 2% ethanol during 7 days of incubation, while SL13E-3 showed pellicle production only at 1% ethanol. SL13E-4 did not give any pellicle production at 40ºC after 7 days of incubation. The results clearly show that all strains produced more pellicle in the presence of ethanol, and that the induction ratio remained similar at ethanol concentrations between 1% and 4%.

Both the average molecular mass of polysaccharides (Table 4-1) and the sugar composition (Figure 4-1) of all strains were unchanged in the presence of ethanol, indicating that concentration of the pellicle polysaccharide might increase in the presence of ethanol but that no new or elongated of polysaccharides were produced.

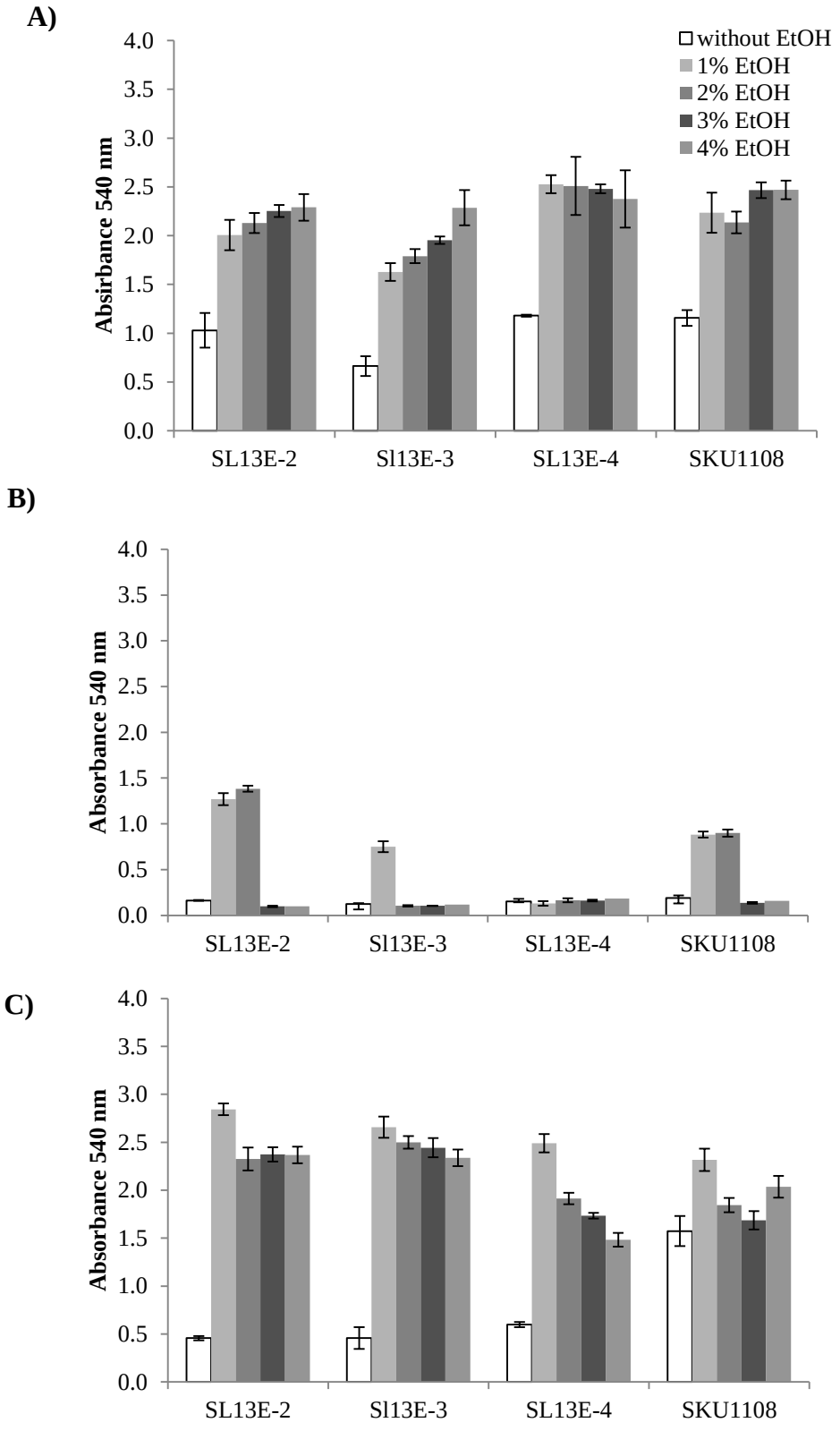

Fig. 4-5 Pellicle production of thermotolerant *A. pasteurianus* strains in YPGD enrichment media containing 1-4% ethanol at 30°C (A), 37°C (B), and 40°C (C). Produced pellicle was measured by crystal violet staining method as described in material and methods in 7 days incubation. Assays were performed in three replicates, and bars indicate averages and standard deviations.

4.3.3 Effect of different sugar sources on production of pellicle of thermotolerant strains

The effect of the addition of different sugar sources (glucose, sucrose, or fructose) to the same culture medium on the production of pellicle was examined at 30ºC, 37ºC, and 40ºC (Fig. 4-6). The results revealed that the sugar source affects pellicle formation less than the addition of ethanol for the four strains at the tested temperatures. At 30ºC and 37ºC, the sugars had no effect on the production of pellicle and were even inhibitive in some cases. However, at 40ºC, the addition of sugar increased the production of pellicle especially by SKU1108.

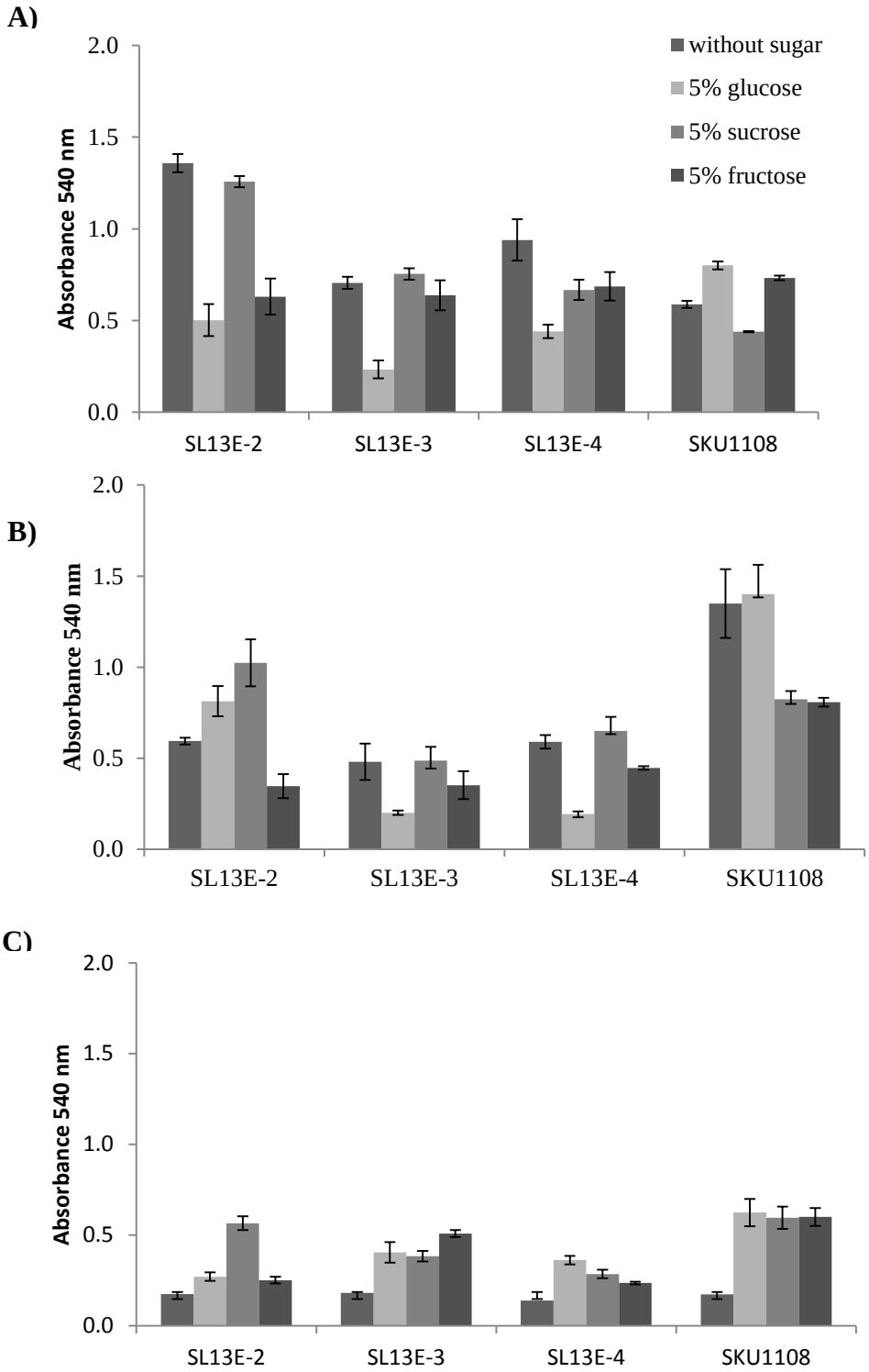

Fig. 4-6 Pellicle production by thermotolerant *A. pasteurianus* strains in YPGD enrichment medium supplemented with 5% glucose, sucrose and fructose at 30°C (A), 37°C (B), and 40°C (C). The produced pellicle was measured by crystal violet staining method as described in material and methods in 7 days incubation. Assays were performed in three replicates, and bars indicate averages and standard deviations.

4.4 Discussion

Acetobacter species is a food-grade microorganism which oxidizes ethanol to acetic acid and thus is used to produce vinegar. *A. pasteurianus* is an AAB species traditionally used for vinegar production. Previous studies reported the composition of CPS in *Acetobacter*: rhamnose and glucose (in a 1:1 ratio) in *A. aceti* (Moonmangmee et al. 2002a) , and rhamnose, galactose, and glucose (in a1:1:1 ratio) in *A. tropicalis* SKU1100 (Moonmangmee et al. 2002b). The composition of EPS of other *Acetobacter* species has been studied: glucose, mannose, galactose and glucuronic acid in *Acetobacter* sp. (Minakami et al. 1984); glucose, mannose, rhamnose and glucuronic acid in *Acetobacter* sp. NBI1022 (Tayama et al. 1985); glucose, galactose, rhamnose and glyceric acid in *A. methanolicus* (Grimmecke et al. 1994); and glucose, galactose and mannose in *Acetobacter* sp. (MacCormick et al. 1996). Thus, the composition of the polysaccharides produced by *Acetobacter* species is quite variable. The stability of the pellicle depends on its structure and monosaccharide composition. As the production of a stable pellicle is an important characteristic for thermotolerant AAB, we examined the sugar composition of CPS of different thermotolerant strains of *A. pasteurianus*. Diversity in the appearance of pellicle was observed during static growth of the thermotolerant strains SL13E-2, SL13E-3, SL13E-4, and SKU1108, so we identified the composition of their CPS and found that they varied even within the same species. The sugar composition data showed that glucose and rhamnose were common to all the tested strains, while xylose was found in SL13E-4 and NBRC3191, and galactose was found in SL13E-3 and SKU1108 (Fig. 4-1). It can thus be assumed that the main chain of polysaccharides in all five strains are the same as the one proposed for *A. tropicalis* by Ali et al. (2011) and consists of glucose and rhamnose, with galactose or xylose branches.

Deeraksa et al. (2005) studied the gene involved in pellicle formation by the thermotolerant *A. tropicalis* strain, SKU1100, and found that the *polABCDE* genes have a high level of homology with enzymes involved in polysaccharide synthesis by various microorganisms. *polABCD* showed significant homology to the *rfbBACD* genes involved in the dTDP-rhamnose synthesis pathway in other Gram-negative bacteria. Also, they found that the *polE* gene is involved in anchoring the pellicle polysaccharide to the cell surface of bacteria in order to synthesize CPS. Furthermore, it has been reported that the synthesis of dTDP-L-rhamnose, which serves as the backbone for the rhamnose-glucose polysaccharide (RGP) of *Streptococcus mutans*, is regulated by four genes: *rmlA*, *rmlB*, *rmlC*, and *rmlD* (Yamashita et al. 1998). The *A. pasteurianus* strains used in this study have genes similar to *polABCD* or *rmlABCD* for the production of main chain polysaccharides, and the polysaccharides are anchored by the product of a gene similar to *polE*. In *A. tropicalis* SKU1100, *galE* is involved in the attachment of galactose residues to the polysaccharide (Deeraksa et al. 2006), and in *S. mutans*, the *rgpE* gene, whose sequence has features typical of glycosyltransferases, is involved in glucose side chain formation (Yamashita et al. 1998). Therefore, the variety of polysaccharides in *A. pasteurianus* might be derived from differences in genes like *galE* or *rgpE*, which are involved in the attachment of the sugar moiety to the main chain polysaccharides.

Not only the composition of the CPS, but also the induction patterns of CPS in response to ethanol and additional sugars, differed among the strains of *A. pasteurianus* tested (Fig 3 and 4). In SL13E-2, SL13E-3 and SL13E-4, ethanol is a fairly strong inducer of pellicle formation at 37˚C, but less so in SKU1108. On the other hand, the addition of glucose, fructose or sucrose enhanced pellicle formation by SKU1108 at 40˚C, but less so in the other strains. The results of growth studies confirmed that this induction of pellicle production by thermotolerant strains was not due to an enhancement of cell growth by the

addition of ethanol (data not shown). It is interesting to analyze how and why these induction differences occur.

Kanchanarach et al. (2010) suggested that the pellicle of *A. pasteurianus* strains is involved in acetic acid resistance, and that the pellicle functions as a biofilm-like barrier to the passive diffusion of acetic acid into the cells. It was recently shown that thermotolerant AAB are better at oxidative fermentation at higher temperatures than mesophilic strains. It would be more beneficial if thermotolerant strains could produce a stable pellicle even at higher temperatures in the presence of higher ethanol levels and thus increased environmental stress. We found that ethanol acts as a strong inducer for pellicle formation, especially at 37°C, by SL13E-2, SL13E-3 and SL13E-4. Thus, the induction of pellicle production by thermotolerant strains by the addition of ethanol would be another stress adaptive mechanism in *A. pasteurianus* which could be similar to the σ^E stress-response system in Gram-negative bacteria (Lima et al. 2013).

Clearly, strains which could produce a higher amount of pellicle at higher temperatures in the presence of ethanol would show higher stress tolerance and would be beneficial in the production of acetic acid, especially in static culture.

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Appendix I

Comparison of 16S rDNA sequences of three isolated *Acetobacter* strains (SL13E-2, SL13E-3, and SL13E-4). Mismatched bases are marked in red.

```
SL13-2 16S     1: GTGGGGTCAGCGACTGGGCTGGCACCAGCCGACGCAGGAACGCCAAGCGAGTGGCCGAAT  60
SL13-3 16S     1: GTGGGGTCAGCGACTGGGCTGGCACCAGCCGACGCAGGAACGCCAAGCGAGTGGCCGAAT  60
SL13-4 16S     1: GTGGGGTCAGCGACTGGGCTGGCACCAGCCGACGCAGGAACGCCAAGCGAGTGGCCGAAT  60

SL13-2 16S    61: TCCAGTTTGGTTGAGGGTACCACACTGCCCGCCACACATGTTCCGGGCCCTTGCATAAGT  120
SL13-3 16S    61: TCCAGTTTGGTTGAGGGTACCACACTGCCCGCCACACATGTTCCGGGCCCTTGCATAAGT  120
SL13-4 16S    61: TCCAGTTTGGTTGAGGGTACCACACTGCCCGCCACACATGTTCCGGGCCCTTGCATAAGT  120

SL13-2 16S   121: GGCGCCGTACGACTAGGCGCTAATGATCGCTAAGGTGGAAGTACGTGAGCTCAACGTCTC  180
SL13-3 16S   121: GGCGCCGTACGACTAGGCGCTAATGATCGCTAAGGTGGAAGTACGTGAGCTCAACGTCTC  180
SL13-4 16S   121: GGCGCCGTACGACTAGGCGCTAATGATCGCTAAGGTGGAAGTACGTGAGCTCAACGTCTC  180

SL13-2 16S   181: ACGTTAGGCTTGACTCTGCCGAAAATCTCTAGTCGTACCACAGTGGTGGATCGAAGGGTG  240
SL13-3 16S   181: ACGTTAGGCTTGACTCTGCCGAAAATCTCTAGTCGTACCACAGTGGTGGATCGAAGGGTG  240
SL13-4 16S   181: ACGTTAGGCTTGACTCTGCCGAAAATCTCTAGTCGTACCACAGTGGTGGATCGAAGGGTG  240

SL13-2 16S   241: ACAGTGGCGGTAACATCGTGCACACATCGGGTCCTGTATTCCCGGTACTCCTGAACTGCA  300
SL13-3 16S   241: ACAGTGGCGGTAACATCGTGCACACATCGGGTCCTGTATTCCCGGTACTCCTGAACTGCA  300
SL13-4 16S   241: ACAGTGGCGGTAACATCGTGCACACATCGGGTCCTGTATTCCCGGTACTCCTGAACTGCA  300

SL13-2 16S   301: GTAGGGGTGGAAGGAGGCCGAACAGTGGCCGTCAGAGAGATCTCACGGGTCGGGTTGGAC 360
SL13-3 16S   301: GTAGGGGTGGAAGGAGGCCGAACAGTGGCCGTCAGAGAGATCTCACGGGTCGGGTTGGAC 360
SL13-4 16S   301: GTAGGGGTGGAAGGAGGCCGAACAGTGGCCGTCAGAGAGATCTCACGGGTCGGGTTGGAC 360

SL13-2 16S   361: TACCGTTGATTTCTATCCCCAACGCGAGCAACGCCCTGAATTGGGTTGTAGAGTGCTGTG  420
SL13-3 16S   361: TACCGTTGATTTCTATCCCCAACGCGAGCAACGCCCTGAATTGGGTTGTAGAGTGCTGTG  420
SL13-4 16S   361: TACCGTTGATTTCTATCCCCAACGCGAGCAACGCCCTGAATTGGGTTGTAGAGTGCTGTG  420

SL13-2 16S   421: CTCGACTGCTGTCGGTACGTCGTGGACACAATCTCCAGGGAACGCCCTTTGTTTGTAGAG  480
SL13-3 16S   421: CTCGACTGCTGTCGGTACGTCGTGGACACAATCTCCAGGGAACGCCCTTTGTCTGTAGAG  480
SL13-4 16S   421: CTCGACTGCTGTCGGTACGTCGTGGACACAATCTCCAGGGAACGCCCTTTGTTTGTAGAG  480

SL13-2 16S   481: ACGAACGTCGGAGATGTAAGTTCGGGACCATTCCAAGACGCGCAACGAAGCTTAATTTGG  540
SL13-3 16S   481: ACGGACGTCGGAGATGTAAGTTCGGGACCATTCCAAGACGCGCAACGAAGCTTAATTTGG  540
SL13-4 16S   481: ACGAACGTCGGAGATGTAAGTTCGGGACCATTCCAAGACGCGCAACGAAGCTTAATTTGG  540

SL13-2 16S   541: TGTACGAGGTGGCGAACACGCCCGGGGGCAGTTAAGGAAACTCAAAGTTGGAACGCCGGC  600
SL13-3 16S   541: TGTACGAGGTGGCGAACACGCCCGGGGGCAGTTAAGGAAACTCAAAGTTGGAACGCCGGC  600
SL13-4 16S   541: TGTACGAGGTGGCGAACACGCCCGGGGGCAGTTAAGGAAACTCAAAGTTGGAACGCCGGC  600

SL13-2 16S   601: ATGAGGGGTCCGCCACACGAATTGCGCAATTGACGCTGTGACTTACTGATTCAGTGGGTT  660
SL13-3 16S   601: ATGAGGGGTCCGCCACACGAATTGCGCAATTGACGCTGTGACTTACTGATTCAGTGGGTT  660
SL13-4 16S   601: ATGAGGGGTCCGCCACACGAATTGCGCAATTGACGCTGTGACTTACTGATTCAGTGGGTT  660

SL13-2 16S   661: GTAGATCGTGTGTAGCAAATGTCGCACCTGATGGTCCCATAGATTAGGACAAACGAGGGG  720
SL13-3 16S   661: GTAGATCGTGTGTAGCAAATGTCGCACCTGATGGTCCCATAGATTAGGACAAACGAGGGG  720
SL13-4 16S   661:GTAGATCGTGTGTAGCAAATGTCGCACCTGATGGTCCCATAGATTAGGACAAACGAGGGG  720

SL13-2 16S   721: TGCGAAAGCGCGGAGTCGCAGTCATTACTCGGTCCAACGGCGGAAGCGGTGGCCACAAGA 780
SL13-3 16S   721: TGCGAAAGCGCGGAGTCGCAGTCATTACTCGGTCCAACGGCGGAAGCGGTGGCCACAAGA 780
SL13-4 16S   721: TGCGAAAGCGCGGAGTCGCAGTCATTACTCGGTCCAACGGCGGAAGCGGTGGCCACAAGA 780

SL13-2 16S   781: AGGGTTATAGATGCTTAAAGTGGAGATGTGACCCTTAAGGTGTTGGGAGAGAGTGTGAGA  840
SL13-3 16S   781: AGGGTTATAGATGCTTAAAGTGGAGATGTGACCCTTAAGGTGTTGGGAGAGAGTGTGAGA  840
SL13-4 16S   781: AGGGTTATAGATGCTTAAAGTGGAGATGTGACCCTTAAGGTGTTGGGAGAGAGTGTGAGA  840

SL13-2 16S   841: TCAGACGTGCATAGTTTACGTCGAGGGTCCAATTCGGGCCCCTAAAGTGTAGACTGACAT  900
SL13-3 16S   841: TCAGACGTGCATAGTTTACGTCGAGGGTCCAATTCGGGCCCCTAAAGTGTAGACTGACAT  900
SL13-4 16S   841: TCAGACGTGCATAGTTTACGTCGAGGGTCCAATTCGGGCCCCTAAAGTGTAGACTGACAT  900
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SL13-2 16S    901: GTTTGGCGGATGTGCGGGAAATGCGGGTCAGTAAGGCTCGTTGCGATCGGGGGAAGCATA  960
SL13-3 16S    901: GTTTGGCGGATGTGCGGGAAATGCGGGTCAGTAAGGCTCGTTGCGATCGGGGGAAGCATA  960
SL13-4 16S    901: GTTTGGCGGATGTGCGGGAAATGCGGGTCAGTAGGGCTCGTTGCGATCGGGGGAAGCATA  960

SL13-2 16S    961: ATGGCGCCGACGACCGTGCTTCAATCGGCCCCGAAGAAGATGCCCATGGCAGTAGTAGCA  1020
SL13-3 16S    961: ATGGCGCCGACGACCGTGCTTCAATCGGCCCCGAAGAAGATGCCCATGGCAGTAGTAGCA  1020
SL13-4 16S    961: ATGGCGCCGACGACCGTGCTTCAATCGGCCCCGAAGAAGATGCCCATGGCAGTAGTAGCA  1020

SL13-2 16S   1021: GGGGCAGCTTTCACGAAATGTTAGGCTTCTGGAAGAAGTGTGTGCGCCGTAACGACCTAG   1080
SL13-3 16S   1021: GGGGCAGCTTTCACGAAATGTTAGGCTTCTGGAAGAAGTGTGTGCGCCGTAACGACCTAG   1080
SL13-4 16S   1021: GGGGCAGCTTTCACGAAATGTTAGGCTTCTGGAAGAAGTGTGTGCGCCGTAACGACCTAG   1080
SL13-2 16S   1081: TCCCAACGGGGGTAACAGGTTATAAGGGGTGACGACGGAGGGCATCCTCAGACCCGGCAC 1140
SL13-3 16S   1081: TCCCAACGGGGGTAACAGGTTATAAGGGGTGACGACGGAGGGCATCCTCAGACCCGGCAC 1140
SL13-4 16S   1081: TCCCAACGGGGGTAACAGGTTATAAGGGGTGACGACGGAGGGCATCCTCAGACCCGGCAC 1140

SL13-2 16S   1141: AGAGTCAGGGTCACACCGACTAGTAGGAGAGTTTGGTCGATAACTAGTAGCGGAACCATC  1200
SL13-3 16S   1141: AGAGTCAGGGTCACACCGACTAGTAGGAGAGTTTGGTCGATAACTAGTAGCGGAACCATC  1200
SL13-4 16S   1141: AGAGTCAGGGTCACACCGACTAGTAGGAGAGTTTGGTCGATAACTAGTAGCGGAACCATC  1200

SL13-2 16S   1201: CGGAAATGGGGTGGTTGATCGATTAGTTTGCGTCCGAGGAGGTGTCCGCTGAACGCGGAA  1260
SL13-3 16S   1201: CGGAAATGGGGTGGTTGATCGATTAGTTTGCGTCCGAGGAGGTGTCCGCTGAACGCGGAA  1260
SL13-4 16S   1201: CGGAAATGGGGTGGTTGATCGATTAGTTTGCGTCCGAGGAGGTGTCCGCTGAACGCGGAA  1260

SL13-2 16S   1261: ACTGGGAGTCCACAGTACGCCATAATCGTGGTCAAAGGGTCACAATAGGGGGTGGGTACC  1320
SL13-3 16S   1261: ACTGGGAGTCCACAGTACGCCATAATCGTGGTCAAAGGGTCACAATAGGGGGTGGGTACC  1320
SL13-4 16S   1261: ACTGGGAGTCCACAGTACGCCATAATCGTGGTCAAAGGGTCACAATAGGGGGTGGGTACC  1320

SL13-2 16S   1321: TATCTATGGATGCGCAATGAGTGGGCAGGCGGTGATTCCGGCTTTGGAAGCACGCTGAAC  1380
SL13-3 16S   1321: TATCTATGGATGCGCAATGAGTGGGCAGGCGGTGATTCCGGCTTTGGAAGCACGCTGAAC  1380
SL13-4 16S   1321: TATCTATGGATGCGCAATGAGTGGGCAGGCGGTGATTCCGGCTTTGGAAGCACGCTGAAC  1380

SL13-2 16S   1381: GTACACAATTCGTACGGCGGTCGCAAGCGA                              1418
SL13-3 16S   1381: GTACACAATTCGTACGGCGGTCGCAAGCGA                              1418
SL13-416S    1381:GTACACAATTCGTACGGCGGTCGCAAGCGA                              1418
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Appendix II

Comparison of ITS sequence data of SL13E-2, SL13E-3 and SL13E-4.

```
SL13E-2 ITS   1 GTGCCTAGGCATCCACCGAATGCCCTTCTCATACTCACTCACCCCATGCACAGAAACCAT 60
SL13E-3 ITS   1 ............................................................ 60
SL13E-4 ITS   1 ............................................................ 60

SL13E-2 ITS  61 CCATACCTACTTACGCAGATACAAACAGCTCCAAAGCATGACGCATCCGAGTACGTCTAC 120
SL13E-3 ITS  61 ............................................................ 120
SL13E-4 ITS  61 ...........C................................................ 120

SL13E-2 ITS 121 TTCTTCAAAACGCTTCTGAACGCTTACACCAGAAATGCTTAACGCATGAGCAAATCTCTT 180
SL13E-3 ITS 121 ............................................................ 180
SL13E-4 ITS 121 ..........................T................................. 180

SL13E-2 ITS 181 TGCTCAATACGGGTCAGACCAACCCACATTTCTGATACGCCCAGACGCGCACCAACCGAT 240
SL13E-3 ITS 181 ............................................................ 240
SL13E-4 ITS 181 ............................................................ 240

SL13E-2 ITS 241 TCACACTGACAAAGATCAACACCAGACACACACCCATACGTGCCGCTATCGCAGCGCCGC 300
SL13E-3 ITS 241 ............................................................ 300
SL13E-4 ITS 241 ............................................................ 300

SL13E-2 ITS 301 ATAAATGAAGTCCGATCCACAAACTCCCTTGCAACAGATACTCTCAACACCACAAAACCA 360
SL13E-3 ITS 301 ............................................................ 360
SL13E-4 ITS 301 ............................................................ 360

SL13E-2 ITS 361 CTCTGGATCCTTGGTGGAGACGGACGGGTTCGAACCGACGACCCCCTGCTTGCAAAGCAG 420
SL13E-3 ITS 361 ............................................................ 420
SL13E-4 ITS 361 ............................................................ 420

SL13E-2 ITS 421 GTGCTCTCCCAGCTGAGCTACGCCCCCATAGGAACCGGCAGTCTCAAATGGTGGTGGGCC 480
SL13E-3 ITS 421 ............................................................ 480
SL13E-4 ITS 421 ............................................................ 480

SL13E-2 ITS 481 AGGGAGGACTTGAACCTCCGACCCCACGCTTATCAAGCGTGTGCTCTAACCAACTGAGCT 540
SL13E-3 ITS 481 ............................................................ 540
SL13E-4 ITS 481 ............................................................ 540

SL13E-2 ITS 541 ACTAGCCCAAAACCCGACTGAATAACCTAGACAATACATAAAAACGTATCAGCCCAAATC 600
SL13E-3 ITS 541 ............................................................ 600
SL13E-4 ITS 541 ..........................G..................C.............. 600

SL13E-2 ITS 601 ACTCAGACATCTGTTGCAGAAAGGGATATGTTGACGGCGCCCCCGATGCACAAACACATC 660
SL13E-3 ITS 601 ............................................................ 660
SL13E-4 ITS 601 ............................................................ 660

SL13E-2 ITS 661 AGAAACTGACAGACAGCGCCTTTGCCGATCCATAAGCAAAGGACTTTTTATTCAGAACAT 720
SL13E-3 ITS 661 ............................................................ 720
SL13E-4 ITS 661 ............................................................ 720

SL13E-2 ITS 721 TCCAAATCAATCAGTAAACCAATTAACTCAGAACAGTTCCTTGAAAGGAGGTGATCCAGC 780
SL13E-3 ITS 721 ............................................................ 780
SL13E-4 ITS 721 ............................................................ 780

SL13E-2 ITS 781 CGCA                                                     784
SL13E-3 ITS 781 ....                                                     784
SL13E-4 ITS 781 ....                                                     784
```

Appendix III

Comparison of 16S rDNA sequences of four *Gluconobacter* strains (SL13-5, SL13-6, SL13-7, and SL13-8).

```
SL 13-5 16S    1: GTGGGGTCAGCGACTGGGCAGGCACCAGCCGACGCAGGAACGCCAAGCGAGTGGCCGAAT   60
SL 13-6 16S    1: GTGGGGTCAGCGACTGGGCAGGCACCAGCCGACGCAGGAACGCCAAGCGAGTGGCCGAAT   60
SL 13-7 16S    1: GTGGGGTCAGCGACTGGGCAGGCACCAGCCGACGCAGGAACGCCAAGCGAGTGGCCGAAT   60
SL 13-8 16S    1: GTGGGGTCAGCGACTGGGCAGGCACCAGCCGACGCAGGAACGCCAAGCGAGTGGCCGAAT   60

SL 13-5 16S   61: TCCAGCTTGGTTGAGGGTACCACACTGCCCGCCACACATGTTCCGGGCCCTTGCATAAGT   120
SL 13-6 16S   61: TCCAGCTTGGTTGAGGGTACCACACTGCCCGCCACACATGTTCCGGGCCCTTGCATAAGT   120
SL 13-7 16S   61: TCCAGCTTGGTTGAGGGTACCACACTGCCCGCCACACATGTTCCGGGCCCTTGCATAAGT   120
SL 13-8 16S   61: TCCAGCTTGGTTGAGGGTACCACACTGCCCGCCACACATGTTCCGGGCCCTTGCATAAGT   120

SL 13-5 16S  121: GGCGCCGTACGACTAGGCGCTAATGATCGCTAAGGTGGAAGTACATGAGCTCAACGTCTC   180
SL 13-6 16S  121: GGCGCCGTACGACTAGGCGCTAATGATCGCTAAGGTGGAAGTACATGAGCTCAACGTCTC   180
SL 13-7 16S  121: GGCGCCGTACGACTAGGCGCTAATGATCGCTAAGGTGGAAGTACATGAGCTCAACGTCTC   180
SL 13-8 16S  121: GGCGCCGTACGACTAGGCGCTAATGATCGCTAAGGTGGAAGTACATGAGCTCAACGTCTC   180

SL 13-5 16S  181: ATGTTAGGCTTGACTCTGCCGAAAATCTCTAGTCGTGACACAGTGGTGTATCGAAGGGTG   240
SL 13-6 16S  181: ATGTTAGGCTTGACTCTGCCGAAAATCTCTAGTCGTGACACAGTGGTGTATCGAAGGGTG   240
SL 13-7 16S  181: ATGTTAGGCTTGACTCTGCCGAAAATCTCTAGTCGTGACACAGTGGTGTATCGAAGGGTG   240
SL 13-8 16S  181: ATGTTAGGCTTGACTCTGCCGAAAATCTCTAGTCGTGACACAGTGGTGTATCGAAGGGTG   240

SL 13-5 16S  241: ACAGTGGCGGTAACATCGTGCACACATCGGGTCCTGTATTCCCGGTACTCCTGAACTGCA   300
SL 13-6 16S  241: ACAGTGGCGGTAACATCGTGCACACATCGGGTCCTGTATTCCCGGTACTCCTGAACTGCA   300
SL 13-7 16S  241: ACAGTGGCGGTAACATCGTGCACACATCGGGTCCTGTATTCCCGGTACTCCTGAACTGCA   300
SL 13-8 16S  241: ACAGTGGCGGTAACATCGTGCACACATCGGGTCCTGTATTCCCGGTACTCCTGAACTGCA   300

SL 13-5 16S  301: GTAGGGGTGGAAGGAGGCCGAACAGTGGCCGTCAGAGAGATCTCACGGGTGGACTTTCAC  360
SL 13-6 16S  301: GTAGGGGTGGAAGGAGGCCGAACAGTGGCCGTCAGAGAGATCTCACGGGTGGACTTTCAC  360
SL 13-7 16S  301: GTAGGGGTGGAAGGAGGCCGAACAGTGGCCGTCAGAGAGATCTCACGGGTGGACTTTCAC  360
SL 13-8 16S  301: GTAGGGGTGGAAGGAGGCCGAACAGTGGCCGTCAGAGAGATCTCACGGGTGGACTTTCAC  360


SL 13-5 16S  361: GACCGTTGATTTCTGTTCCCAACGCGAGCAACGCCCTGAATTGGGTTGTAGAGTGCTGTG   420
SL 13-6 16S  361: GACCGTTGATTTCTGTTCCCAACGCGAGCAACGCCCTGAATTGGGTTGTAGAGTGCTGTG   420
SL 13-7 16S  361: GACCGTTGATTTCTGTTCCCAACGCGAGCAACGCCCTGAATTGGGTTGTAGAGTGCTGTG   420
SL 13-8 16S  361: GACCGTTGATTTCTGTTCCCAACGCGAGCAACGCCCTGAATTGGGTTGTAGAGTGCTGTG   420

SL 13-5 16S  421: CTCGACTGCTGTCGGTACGTCGTGGACACGCCCTCCAGGCTTCTTTATGGGTAGAGACTC   480
SL 13-6 16S  421: CTCGACTGCTGTCGGTACGTCGTGGACACGCCCTCCAGGCTTCTTTATGGGTAGAGACTC   480
SL 13-7 16S  421: CTCGACTGCTGTCGGTACGTCGTGGACACGCCCTCCAGGCTTCTTTATGGGTAGAGACTC   480
SL 13-8 16S  421: CTCGACTGCTGTCGGTACGTCGTGGACACGCCCTCCAGGCTTCTTTATGGGTAGAGACTC   480

SL 13-5 16S  481: ATGCAGGAGGGGTACGTTCGGGACCATTCCAAGACGCGCAACGAAGCTTAATTTGGTGTA   540
SL 13-6 16S  481: ATGCAGGAGGGGTACGTTCGGGACCATTCCAAGACGCGCAACGAAGCTTAATTTGGTGTA   540
SL 13-7 16S  481: ATGCAGGAGGGGTACGTTCGGGACCATTCCAAGACGCGCAACGAAGCTTAATTTGGTGTA   540
SL 13-8 16S  481: ATGCAGGAGGGGTACGTTCGGGACCATTCCAAGACGCGCAACGAAGCTTAATTTGGTGTA   540

SL 13-5 16S  541: CGAGGTGGCGAACACGCCCGGGGGCAGTTAAGGAAACTCAAAGTTGGAACGCCGGCATGA  600
SL 13-6 16S  541: CGAGGTGGCGAACACGCCCGGGGGCAGTTAAGGAAACTCAAAGTTGGAACGCCGGCATGA  600
SL 13-7 16S  541: CGAGGTGGCGAACACGCCCGGGGGCAGTTAAGGAAACTCAAAGTTGGAACGCCGGCATGA  600
SL 13-8 16S  541: CGAGGTGGCGAACACGCCCGGGGGCAGTTAAGGAAACTCAAAGTTGGAACGCCGGCATGA  600

SL 13-5 16S  601: GGGGTCCGCCACACGAATCGCGCAATCGAAGCTGTGACTCATTGATTCAATGGGTTGTAG   660
SL 13-6 16S  601: GGGGTCCGCCACACGAATCGCGCAATCGAAGCTGTGACTCATTGATTCAATGGGTTGTAG   660
SL 13-7 16S  601: GGGGTCCGCCACACGAATCGCGCAATCGAAGCTGTGACTCATTGATTCAATGGGTTGTAG   660
SL 13-8 16S  601: GGGGTCCGCCACACGAATCGCGCAATCGAAGCTGTGACTCATTGATTCAATGGGTTGTAG   660
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SL 13-5 16S   661: GTCGTGTGTAGCAAATGTCGCACCTGATGGTCCCATAGATTAGGACAAACGAGGGGTGCG   720
SL 13-6 16S   661: GTCGTGTGTAGCAAATGTCGCACCTGATGGTCCCATAGATTAGGACAAACGAGGGGTGCG   720
SL 13-7 16S   661: GTCGTGTGTAGCAAATGTCGCACCTGATGGTCCCATAGATTAGGACAAACGAGGGGTGCG   720
SL 13-8 16S   661: GTCGTGTGTAGCAAATGTCGCACCTGATGGTCCCATAGATTAGGACAAACGAGGGGTGCG   720

SL 13-5 16S   721: AAAGCGCGGAGTCGCAGTCATAGCTCGGTCCAACGGCGGAAGCGGTGGCCACAAGAAGGG   780
SL 13-6 16S   721: AAAGCGCGGAGTCGCAGTCATAGCTCGGTCCAACGGCGGAAGCGGTGGCCACAAGAAGGG   780
SL 13-7 16S   721: AAAGCGCGGAGTCGCAGTCATAGCTCGGTCCAACGGCGGAAGCGGTGGCCACAAGAAGGG   780
SL 13-8 16S   721: AAAGCGCGGAGTCGCAGTCATAGCTCGGTCCAACGGCGGAAGCGGTGGCCACAAGAAGGG   780

SL 13-5 16S   781: TTATAGATGCTTAAAGTGGAGATGTGACCCTTAAGGTGTTGGGAGAGAGCTTGAGATCAG    840
SL 13-6 16S   781: TTATAGATGCTTAAAGTGGAGATGTGACCCTTAAGGTGTTGGGAGAGAGCTTGAGATCAG    840
SL 13-7 16S   781: TTATAGATGCTTAAAGTGGAGATGTGACCCTTAAGGTGTTGGGAGAGAGCTTGAGATCAG    840
SL 13-8 16S   781: TTATAGATGCTTAAAGTGGAGATGTGACCCTTAAGGTGTTGGGAGAGAGCTTGAGATCAG    840

SL 13-5 16S   841: TTACGCAGAGTTTACGTCAAGGGTCCAATTCGGGCCCCTAAAGTGTAGACTGACGTAGTT    900
SL 13-6 16S   841: TTACGCAGAGTTTACGTCAAGGGTCCAATTCGGGCCCCTAAAGTGTAGACTGACGTAGTT    900
SL 13-7 16S   841: TTACGCAGAGTTTACGTCAAGGGTCCAATTCGGGCCCCTAAAGTGTAGACTGACGTAGTT    900
SL 13-8 16S   841: TTACGCAGAGTTTACGTCAAGGGTCCAATTCGGGCCCCTAAAGTGTAGACTGACGTAGTT    900

SL 13-5 16S   901: GGCGGATGCGCGGGAAATGCGGGTCAGTAAGGCTCGTTGCGATCGGGGGAAGCATAATGG    960
SL 13-6 16S   901: GGCGGATGCGCGGGAAATGCGGGTCAGTAAGGCTCGTTGCGATCGGGGGAAGCATAATGG    960
SL 13-7 16S   901: GGCGGATGCGCGGGAAATGCGGGTCAGTAAGGCTCGTTGCGATCGGGGGAAGCATAATGG    960
SL 13-8 16S   901: GGCGGATGCGCGGGAAATGCGGGTCAGTAAGGCTCGTTGCGATCGGGGGAAGCATAATGG    960

SL 13-5 16S   961: CGCCGACGACCGTGCTTCAATCGGCCCCGAAGAAGATGCCCATGGCAGTAGTAGCAGGGG   1020
SL 13-6 16S   961: CGCCGACGACCGTGCTTCAATCGGCCCCGAAGAAGATGCCCATGGCAGTAGTAGCAGGGG   1020
SL 13-7 16S   961: CGCCGACGACCGTGCTTCAATCGGCCCCGAAGAAGATGCCCATGGCAGTAGTAGCAGGGG   1020
SL 13-8 16S   961: CGCCGACGACCGTGCTTCAATCGGCCCCGAAGAAGATGCCCATGGCAGTAGTAGCAGGGG   1020

SL 13-5 16S  1021: CAGCTTTCACGAAATGTTAGGCTTCTGGAAGAAGTGTGTGCGCCGTAACGACCTAGTCCG   1080
SL 13-6 16S  1021: CAGCTTTCACGAAATGTTAGGCTTCTGGAAGAAGTGTGTGCGCCGTAACGACCTAGTCCG   1080
SL 13-7 16S  1021: CAGCTTTCACGAAATGTTAGGCTTCTGGAAGAAGTGTGTGCGCCGTAACGACCTAGTCCG   1080
SL 13-8 16S  1021: CAGCTTTCACGAAATGTTAGGCTTCTGGAAGAAGTGTGTGCGCCGTAACGACCTAGTCCG   1080

SL 13-5 16S  1081: AACGCGGGTAACAGGTTATAAGGGGTGACGACGGAGGGCATCCTCAGACCCGGCACAGAG   1140
SL 13-6 16S  1081: AACGCGGGTAACAGGTTATAAGGGGTGACGACGGAGGGCATCCTCAGACCCGGCACAGAG   1140
SL 13-7 16S  1081: AACGCGGGTAACAGGTTATAAGGGGTGACGACGGAGGGCATCCTCAGACCCGGCACAGAG   1140
SL 13-8 16S  1081: AACGCGGGTAACAGGTTATAAGGGGTGACGACGGAGGGCATCCTCAGACCCGGCACAGAG   1140

SL 13-5 16S  1141: TCAGGGTCACACCGACTAGTAGGAGAGTTTGGTCGATAGCTAGTAGCGGAACCATCCGGA   1200
SL 13-6 16S  1141: TCAGGGTCACACCGACTAGTAGGAGAGTTTGGTCGATAGCTAGTAGCGGAACCATCCGGA   1200
SL 13-7 16S  1141: TCAGGGTCACACCGACTAGTAGGAGAGTTTGGTCGATAGCTAGTAGCGGAACCATCCGGA   1200
SL 13-8 16S  1141: TCAGGGTCACACCGACTAGTAGGAGAGTTTGGTCGATAGCTAGTAGCGGAACCATCCGGA   1200


SL 13-5 16S  1201: AATGGGGTGGTTGATCGATTAGCTTGCGTCCAAGGAGGTGTCCGCTGAACGCGGAAACTG   1260
SL 13-6 16S  1201: AATGGGGTGGTTGATCGATTAGCTTGCGTCCAAGGAGGTGTCCGCTGAACGCGGAAACTG   1260
SL 13-7 16S  1201: AATGGGGTGGTTGATCGATTAGCTTGCGTCCAAGGAGGTGTCCGCTGAACGCGGAAACTG   1260
SL 13-8 16S  1201: AATGGGGTGGTTGATCGATTAGCTTGCGTCCAAGGAGGTGTCCGCTGAACGCGGAAACTG   1260

SL 13-5 16S  1261: GGAGTCCATAGTACGCCATAATCGAGGTCAAAGGGCCTCAACAGGGGGTGGGTACCTATC   1320
SL 13-6 16S  1261: GGAGTCCATAGTACGCCATAATCGAGGTCAAAGGGCCTCAACAGGGGGTGGGTACCTATC   1320
SL 13-7 16S  1261: GGAGTCCATAGTACGCCATAATCGAGGTCAAAGGGCCTCAACAGGGGGTGGGTACCTATC   1320
SL 13-8 16S  1261: GGAGTCCATAGTACGCCATAATCGAGGTCAAAGGGCCTCAACAGGGGGTGGGTACCTATC   1320

SL 13-5 16S  1321: TAGGGATGCGCAATGAGTGGGCAGGCGGTGATTAGGGCTTTCTAGGCACGCTGAACGTAC   1380
SL 13-6 16S  1321: TAGGGATGCGCAATGAGTGGGCAGGCGGTGATTAGGGCTTTCTAGGCACGCTGAACGTAC   1380
SL 13-7 16S  1321: TAGGGATGCGCAATGAGTGGGCAGGCGGTGATTAGGGCTTTCTAGGCACGCTGAACGTAC   1380
SL 13-8 16S  1321: TAGGGATGCGCAATGAGTGGGCAGGCGGTGATTAGGGCTTTCTAGGCACGCTGAACGTAC   1380

SL 13-5 16S  1381: ACAATTCGTACGGCGGT                                            1397
SL 13-6 16S  1381: ACAATTCGTACGGCGGT                                            1397
SL 13-7 16S  1381: ACAATTCGT                                                    1389
SL 13-8 16S  1381: ACAATTCGTACGGCGGT                                            1397
```

Appendix IV

Comparison of ITS sequence data of four *Gluconobacter* strains (SL13-5, SL13-6, SL13-7, and SL13-8).

```
SL13-5   1 GTGCCTAGGCATCCACCGAACGCCCTTCTCATTCTCACACCATCAAATCATCATTGCTGA 60
SL13-6   1 ............................................................ 60
SL13-7   1 ............................................................ 60
SL13-8   1 ............................................................ 60

SL13-5  61 TGAAACACATGCACAGAAACCAGCCACACCCCGAGAGGTATGGACCGACTTCTGTCCACG 120
SL13-6  61 ............................................................ 120
SL13-7  61 ............................................................ 120
SL13-8  61 ............................................................ 120

SL13-5 121 ACAGTGTCAGTCACACTCTTTTTGTCACCGTCTGAATGCTTCATCCGTTCTCTTAACGCC 180
SL13-6 121 ............................................................ 180
SL13-7 121 ............................................................ 180
SL13-8 121 ............................................................ 180

SL13-5 181 GCCACCTGACAAAGCCAGACAGCAGACGGGTCAGACCAACCCGAGAACGGTACACGCAGA 240
SL13-6 181 ............................................................ 240
SL13-7 181 ............................................................ 240
SL13-8 181 ............................................................ 240

SL13-5 241 CAGTGCACCAACCTATTCACACTCTAAAAGACCCAACATCCCTGACGCTTCATCCGACTA 300
SL13-6 241 ............................................................ 300
SL13-7 241 ............................................................ 300
SL13-8 241 ............................................................ 300

SL13-5 301 TGCGGTGACAGGATCAGGAAACAGACTTCCCATTTCCTATGATTTTCCCAGTGACCAGAT 360
SL13-6 301 ............................................................ 360
SL13-7 301 ............................................................ 360
SL13-8 301 ............................................................ 360

SL13-5 361 TTTGAGCTCTTCACCACTTCAGGAGACCGAAATCTCAACCAAAGGTGGTGGAGGCAGACG 420
SL13-6 361 ............................................................ 420
SL13-7 361 ............................................................ 420
SL13-8 361 ............................................................ 420

SL13-5 421 GGTTCGAACCGACGACCCCCTGCTTGCAAAGCAGGTGCTCTCCCAGCTGAGCTATGCCCC 480
SL13-6 421 ............................................................ 480
SL13-7 421 ............................................................ 480
SL13-8 421 ............................................................ 480

SL13-5 481 CAAACTCTGGTGGGCCAGGGAGGACTTGAACCTCCGACCCCACGCTTATCAAGCGTGTGC 540
SL13-6 481 ............................................................ 540
SL13-7 481 ............................................................ 540
SL13-8 481 ............................................................ 540

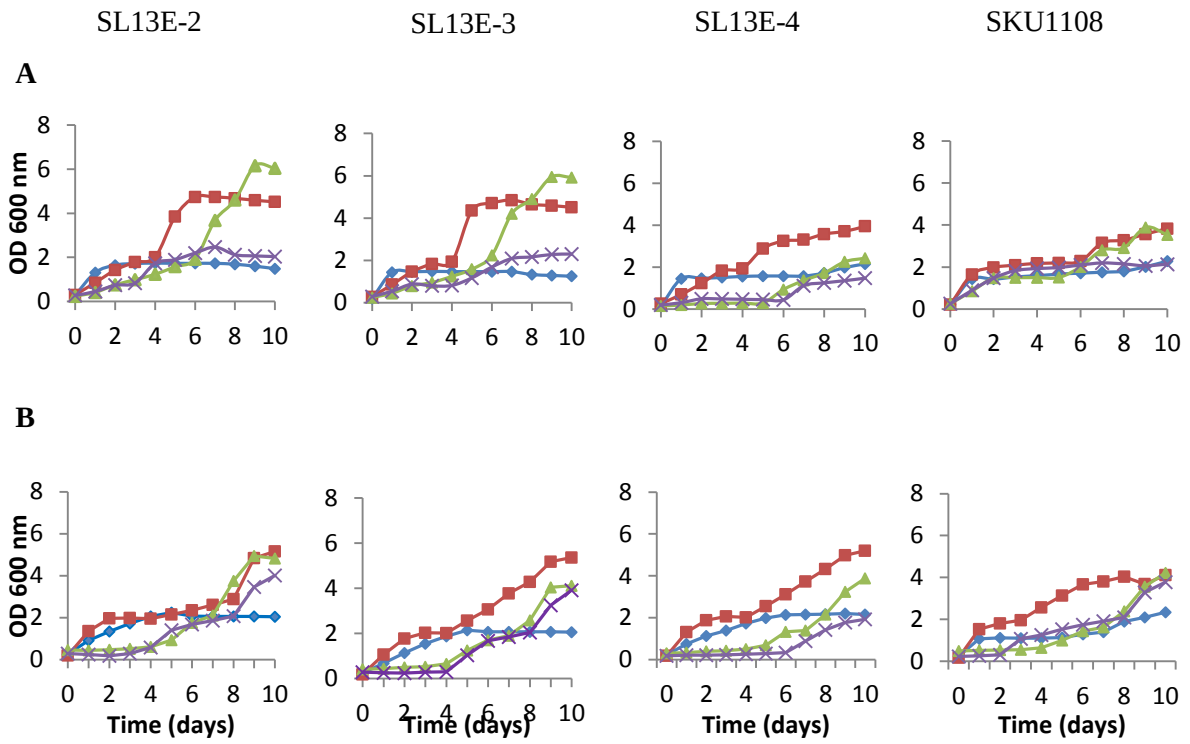
SL13-5 541 TCTAACCAACTGAGCTACTAGCCCGGGAAAATATAGGAAGGGATATGTTGACGGCGCCTT 600
SL13-6 541 ............................................................ 600
SL13-7 541 ............................................................ 600
SL13-8 541 ............................................................ 600

SL13-5 601 GAACTCCAGGTTCCAGGATCGTCTGGCGCCTTGGCTGATACAACCAAGGACTTTTATTTC 660
SL13-6 601 ............................................................ 660
SL13-7 601 ............................................................ 660
SL13-8 601 ............................................................ 660

SL13-5 661 GGAGATCTCCAACGTATCGGAGAACATCCTTGAAAGGAGGTGATCCAGCCGCA        713
SL13-6 661 ..............................................A.....        713
SL13-7 661 ..............................................A.....        713
SL13-8 661 ..............................................A.....        713
```

Appendix V

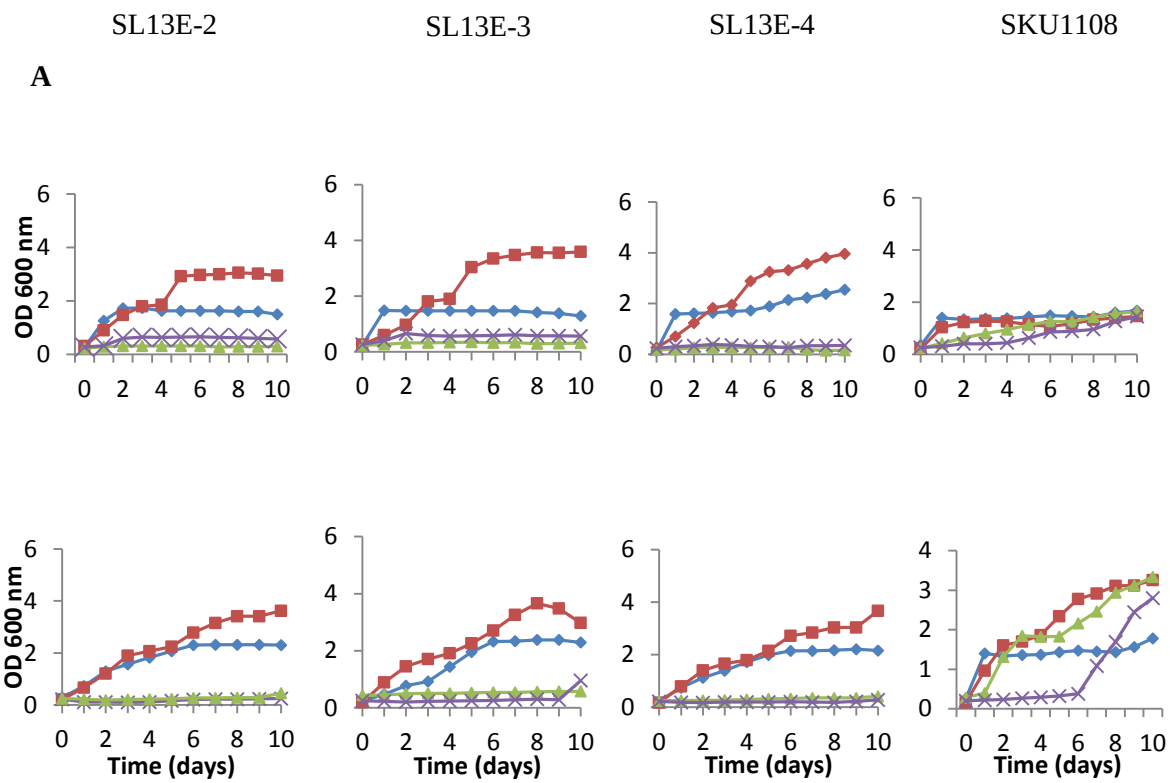
Comparison of growth of thermotolerant strains at different temperatures with 4% initial ethanol.



Comparison of growth in shaking, 200 rpm (A) and static (B) cultures of SL13E-2, 13E-3, 13E-4 and SKU1108 at 30°C (diamonds), 37°C (squares), 38°C (triangles) and 40°C (crosses) in YPGD medium supplemented with 4% ethanol.

Appendix VI

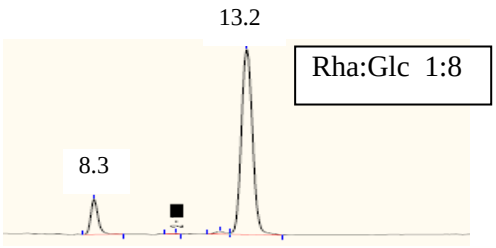
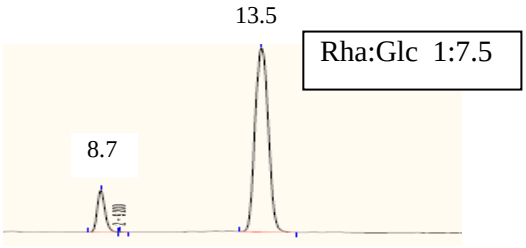
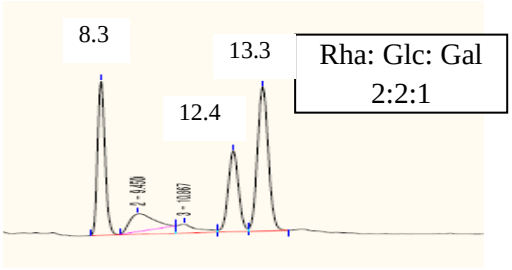
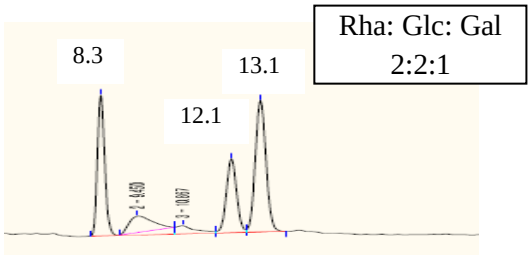
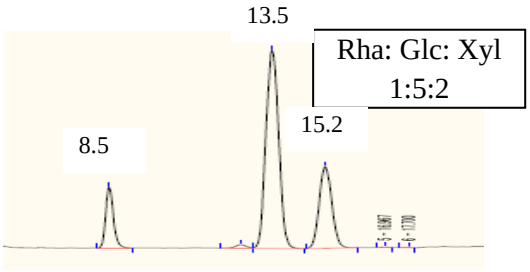
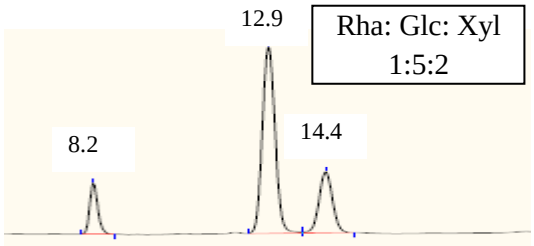
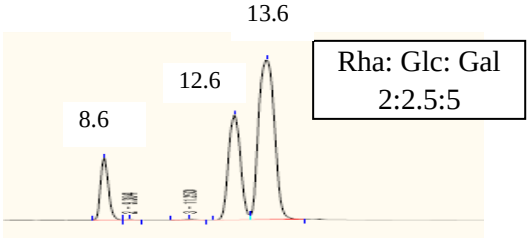
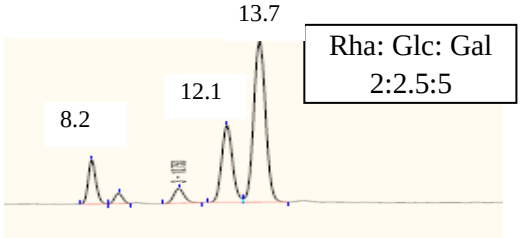
Comparison of growth of thermotolerant strains at different temperatures with 6% initial ethanol.



Comparison of growth in shaking, 200 rpm (A) and static (B) cultures of SL13E-2, 13E-3, 13E-4 and SKU1108 at 30°C (diamonds), 37°C (squares), 38°C (triangles) and 40°C (crosses) in YPGD medium supplemented with 6% ethanol.

Appendix VII

HPLC profiles of acid-hydrolyzed products of purified polysaccharides of ethanol uninduced and induced cells from *Acetobacter pasteurianus* strains

Without addition of ethanol	With addition of ethanol
	
	
	
	

Appendix VIII

Ferricyanide assay for ADH and ALDH

McIlavain Buffer (pH 4.0)	(800-x) µl
Ethanol/Sample	100 µl
Enzyme (Membrane fraction)	10 µl

↓ Pre-incubation 5 min

Add 100 µl of 0.1 M potassium ferricyanide

↓ Leave for 5 min

Add 500 µl of Dupanol reagent

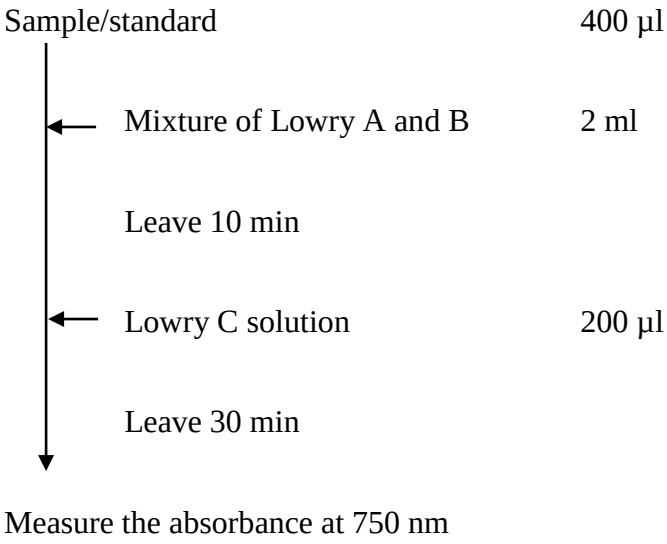
↓ Leave 20 min for colour development (Prucian blue)

Add 3.5 ml of distilled water

Measure the absorbance at 660 nm

Appendix IX

Lowery method for Protein assay



Lowry A solution: 2% Na_2CO_3, 0.1 N NaOH, 0.5% SDS

Lowry B solution: 0.5% $CuSO_4.5H_2O$, 1% sodium tartarate

Lowry C: Phenol reagent (×2)

Appendix X

Phenol-sulfuric method for total sugar content