ADAPTATION OF HIGH TOLERANT ACID STRAIN AND PROCESS DEVELOPMENT FOR HIGH TEMPERATURE ACETIFICATION

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Thesis	Adaptation of High Tolerant Acid Strain and Process Development for High Temperature Acetification
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ABSTRACT

In this study, *Acetobacter pasteurianus* strain UMCC 2951 was tested as a microbial starter to conduct acetification processes by repeated cultivation cycles under high initial acetic acid concentration (HAAi). And high temperature acetification at 40 ± 1°C in internal Venturi injector (iVi) bioreactor and in reciprocal shaking bioreactor with out temperature control were investigated. Acid production (AP) and acetification rate (ETA) increased with repeated cultures under high temperature acetification (HTA) as adaptation period increased, but were still lower than acetification at 30 ± 1°C. However, the addition of 0.15% calcium chloride ($CaCl_2$) reduced the negative effects of 40 ± 1°C on both acid production and acetification rate compared to 30 ± 1°C.

The combination of luffa sponge matrices (LSM) (50% w/v) and 0.15% $CaCl_2$, called as LSM- $CaCl_2$ could provide the highest AP and ETA under HTA, but still slightly lower than 30°C. The AP of LSM- $CaCl_2$ at total concentration TC8, TC9 and TC10 were 29 ± 1, 21 ± 1, 15.33 ± 0.6 g/L while ETA were 3.96 ± 0.19, 2.18 ± 0.23, 1.44 ± 0.07 g/L/d, respectively, during the last three cycles. In case of reciprocal shaking bioreactor for quick acetification process, LSM (50% w/v) also affected on AP and ETA (consisting of at TC8 with AP 20.33 ± 1.5 g/L, ETA 1.83 ± 0.1 g/L/d, TC9 with AP 11.00 ± 1.0 g/L, ETA 0.72 ± 0.04 g/L/d, and at TC10 with AP 7.33 ± 0.6 g/L, ETA, 0.38 ± 0.04 g/L/d).

A strong decrease in membrane-bound enzyme, fatty acids and phosphatidylethanolamine and increases in phosphatidylcholine and phosphatidylglycerol in cell membranes were found under high acid and high temperature acetification. In addition, transmission electron microscope images revealed a more compact cell wall when $CaCl_2$ was added to the cultivation medium. While, three strains (HT-adapted, RT-adapted, RTN-wild type strain) belonging *A. pasteurianus* species were analyzed. The comparative analysis

16S rRNA gene sequence, show that no substantial differences occurs in the genes set for all the three gene sequence. The high similarity of all three gene sequence with *A. pasteurianus* species was 99 % identical analysis. However, all three strains were significant differences in terms of physical and morphological properties in microbial cells.

While, volatile organic components (VOCs) produced during acetification under high temperature (40°C)-high acid conditions of upland rice vinegar (URV-HT) was conducted using GC-MS. Six major VOCs were found; acetic acid (AA), ethyl acetate, isoamyl acetate, isoamyl alcohol, phenethyl acetate and phenethyl alcohol with AA (50.48 ± 2.12 mg/L) and ethyl acetate (31.43 ± 1.01 mg/L) at the higher levels, but these levels were lower than those found during acetification at 30°C (URV-RT). The VOCs in vinegar was applied on antifungal and antibacterial. Our results showed that UVR-HT and some of its VOCs were effective as antimicrobial agents, which are suitable for application safe in foods and feeds to control some microorganisms.

Thus, it is suggested that the thermo adapted cells with $CaCl_2$ addition could become strong and resistant to high temperature acetification (HTA), and thus could be useful for large-industrial acetification. Moreover, the strategy used in this study confirmed that the use of acetic acid bacteria as microbial starter could be effective also at temperature above the optimal values, when acetification processes are managed through repeated semi-continuous cycles.

Keywords: *Acetobacter pasteurianus* UMCC 2951 strain, $CaCl_2$, Luffa sponge matrices, high temperature acetification, high initial acetic acid concentration (HAAi) process, quick acetification process, vapor-phase VOCs of vinegar

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RUTTIPRON POTHIMON

2020

CONTENTS

Page

CONTENTS (cont'd)

CONTENTS (cont'd)

LIST OF TABLES

LIST OF TABLES (cont'd)

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (cont'd)

LIST OF FIGURES (cont'd)

LIST OF FIGURES (cont'd)

LIST OF ABBREVIATIONS

AA	Acetic acid
AAB	Acetic Acid Bacteria
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
AP	Acid production
BC	Bacterial cellulose
CA	Citric acid
CDW	Cell dry weight
$CaCl_2$	Calcium chloride
ET	Ethanol
ETA	Acetification rate
FAMEs	Fatty acid methyl esters
FDA	The U.S. Food and Drug Administration
FID	Fame ionization detector
FM	Fermentation medium
GC-MS	Gas chromatography–mass spectrometry
GWAS	Genome-wide association analysis
HAAi	High initial acetic acid concentration
HT	High temperate (40 °C)
HTA	High temperature acetification
HTA-C	High temperature acetification with $CaCl_2$ addition
iVi	Internal Venturi Injector
LA	Lactic acid
LSM	Luffa sponge matrices.
ML	Maximum-likelihood
PAA	Pure acetic acid
PDA	Potato Dextrose Agar
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
QAP	Quick acetification process

RT	Room temperate (30 °C)
SEM	Scanning Electron Microscopy
SPME	Solid phase micro-extraction
TC	Total concentration
TEM	Transmission Electron Microscopy
TSA	Trypticase soy agar
URV	Upland rice vinegar
URV-HT	High acid conditions of upland rice vinegar
VOCs	Volatile organic components
VRBGA	Violet red bile glucose agar

Strains adaptation

adapted HT-strain; HT	*Acetobacter pasteurianus* UMCC 2951
wild type strain; RTN	*Acetobacter pasteurianus* UMCC 2952
adapted RT-strain; RT	*Acetobacter pasteurianus* UMCC 2953

CHAPTER 1

INTRODUCTION

Acetic acid bacteria (AAB) are Gram negative and strictly aerobic bacteria which are able to produce acetic acid from ethanol (Sievers and Swings, 2005; Kersters et al., 2006; Komagata et al., 2014; Yamada, 2016). Mostly, *Acetobactor* and *Gluconacetobater* are recommended to use for produce acetic acid with high acid than other strains . Normally, *Acetobater aceti* and *A . pasteurianus* species are used for vinegar production, the optimum temperature is usually regulated to about 30 °C (Lu et al., 1999). Their ability to oxidize carbon sources, such as ethanol to acetic acid, varies according to genera and species, due to the different stability of the membrane-bound enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) at high acetic acid content (Treck et al., 2006; Wu et al., 2017). The performance of strains is then the results of their ability to tolerate the initial ethanol content and to resist to increasing acetic acid (Kanchanarach et al., 2010; Gullo et al., 2014; Krusong et al., 2015a; La China et al., 2018; Yin et al., 2018). However, the large-scale industrial fermentation is carried out in a closed fermentor (semi-continuous acetification system), which generates a lot of heat to oxidize alcohol into acetic acid (Matsutani et al., 2013). High temperature in system effect to decrease the acetification rate and disturbs cells including effect to long fermentation period which is a problem in large - scale industrial fermentation (Denich et al., 2003). Therefore, use cooling system with acetification process to reduce negative effect over temperature is focussed, but it is able to high power consumption, high cost. The chiller used to control cooling system is usually found as the problem of electric power failures and chiller not working. Microorganisms can make an adaptation for optimum function physiological properties environment (Herbert, 1989) and can adapt to tolerate small changes over time in an environment such as minutes, hours, or days (Hill et al., 1995). Adaptability of microorganism under high temperature is the most important on efficacy of acetification, including adapted microbial cells to long time effect to gene mutation (Matsutani et al., 2013). Recent studies in vinegar production showed that *A. pasteurianus*, one of the most used species in producing vinegar, has the capability to conduct stable fermentations if adapted to environmental stressors of the fermentation conditions. In particular, *A. pasteurianus* was shown to be able to activate tolerant mechanisms against high acidity conditions and high temperatures and is considered a highly versatile microorganism (Qi et al., 2013; Phathanathavorn et al., 2019). The activation of tolerance mechanisms is the result of genetic mutations that occur with target genes. For application of vinegar in terms of volatile organic components (VOCs) have been used to control infections of crops for many years. VOCs containing acetic acid (AA), alcohol, aldehydes, acetates and esters were shown to have antibacterial have antifungal properties (Krusong et al., 2015b; Pornpukdeewattana et al., 2017). Many studies have shown that loofa (Luffa cylinddrica) sponge is an excellent immobilization carrier for microorganisms due to offset negative of aeration, and provide cell to high bacterial biomass (Chen et al., 2014., Krusong et al., 2007, 2010, 2014, 2016). And the addition of CaCl2 to improve thermotolerant properties of cell microorganism, decrease negative effect of high temperature and as a source of Ca^{2+} binding the enzyme substrate on the membrane and cell-wall call thickness (Nedjimi and Daoud, 2009; Zeng et al., 2010). Moreover, to increase the content of phospholipids, fatty acids and the activities of membrane-bound enzymes involved in acetification, and cell stability at acetification of high temperature at 35 °C (Krusong et al., 2015b). In this study, we used *A. pasteurianus* UMCC 2951 which was isolated from fully ripen pineapple fruit in Thailand and that shows strong ability to resist to acetic acid during fermentation. The aim of this study was to improve a thermotolerant strain for high temperature, at 40 °C, acetification and to clarify the mechanisms of bacterial properties and gene mutation for its thermal adaptation in internal Venturi injector bioreactor and to develop acetification process with no use cooling system conjugate with LSM in Quick acetification process. In addition, vinegar inhibitory impact of its main components on antimicrobial and antifungal were investigated.

1.1 Objectives

The objectives of this research were:

1. To adapt *Acetobacter pasteurianus* UMCC 2591 cells as thermotolerant strain, 40 °C.
2. To monitor the effect of $CaCl_2$ on the change of physical properties of cell-wall thickness, fatty acid and the membrane-bound enzymes activities involve of *A. pasteurianus* UMCC 2591 during acetification under high temperature, 40 °C.
3. To apply the cells adsorbed on luffa sponge matrices (LSM) in conjunction with $CaCl_2$ to improve thermotolerant property of *A . pasteurianus* UMCC 2591 for

high initial acetic acid concentration (HAAi) acetification in internal Venturi injector bioreactor 100 L at 40 °C.

4. To apply the adsorbed cells on LSM for HAAi in quick acetification process with out control temperature condition.
5. To investigate antifungal properties of volatile organic components produced during the high temperature/high acetification processing of upland rice vinegar and their inhibitory impacts of main components on mold.
6. To determine basic data of gene mutation of adapted thermotolerant *A .pasteurianus* UMCC 2591 compared with wild-type strain.

1.2 Scope of research

1. To study the application a thermotolerant properties of AAB strain for acetification at high temperature 40 °C . Application of immobilization cell on luffa sponge matrix and $CaCl_2$ addition on application both Internal Venturi injector bioreactor, and on quick acetification process in Packed-bed bioreactor under non control temperature condition.
2. To investigate mechanism change of bacterial properties under HTA after adaptation.
3. To investigate the main component of volatile compound present in vinegar which ferment at 40 °C and determine basic data of gene adaptation of adapted *A .pasteurianus* UMCC 2591.

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CHAPTER 2

LITERATURE REVIEWS

2.1 Acetic Acid Bacteria (AAB)

2.1.1 Characteristics of acetic acid bacteria

AAB are Gram-negative, rod-shaped and obligate aerobic bacteria use for vinegar production, to oxidize ethanol into acetic acid and tolerant high concentration of acetic acid, in addition to some strain convert glucose to gluconic acid (Asai 1968; Sievers and Swings, 2005; Kersters et al., 2006; Komagata et al., 2014). AAB are classified in the family *Acetobacteraceae* of the class of *Alphaproteobacteria* based on phylogeny, physiology, and ecology (Gillis and de Ley, 1980). Nowaday, AAB are classified into ten genera comprising 52 recognized species. Three major genera namely *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* are most important of industrial fermentation. *Acetobacter* and *Gluconacetobacter* and the best produce acetic acid and shown high concentration acidity of acetic acid, and *Gluconobacter* is a producing of cellulose (Yamada et al., 1997; Nakano and Fukaya, 2008; Matsutani et al., 2011). Three major strain shown Phylogenetic relationships on Fig. 2.1

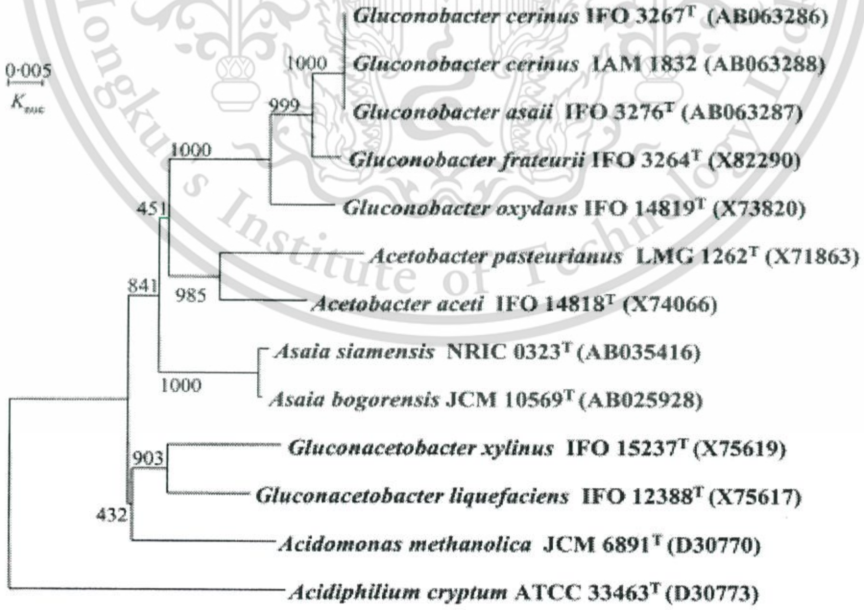

Figure 2.1 Phylogenetic relationships of some species of acetic acid bacteria, based on 16S rRNA gene sequences.

Source: Katsura (2002)

The optimal temperature for growth is around 30°C, some species grow at 37°C, but not at 45 °C. There are several in food fermentation products especially vinegar, cocoa, kumbucha and nata de coco. Other then, AAB germinate in food spoilage from beer, wine and cyder (Greenberg et al., 2006; Kersters et al., 2006). AAB oxidize ethanol into acetic acid by two step reactions by the alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) enzymes in membrane (Yakushi and Matsushita, 2010).

2.1.2 Genera and Species in Acetic Acid Bacteria

2.1.2.1 Acetobacter spp.

Acetobacter is the oldest genus in the classification of AAB. The major species of *Acetobacte*r there are *Acetobacter aceti*, *A. pasteurianus*, and *A. peroxydans*, with nine subspecies (Skerman *et al*., 1980). Cells are ellipsoidal to rod shaped, measuring about 0.4-1.0 by 1.2-3.0 µm, rarely longer. The optimal growth temperature is around 30 °C, but not at 45 °C and grows at pH 3.5 (Komagata et al., 2014). Generally, colonies are circular, smooth, entire, convex, cream color to beige and germinate on medium composition of D-glucose, yeasts extract, ethanol and peptone agar. *Acetobacter* strains are several in fermentation foods such as vinegar, beer, wine, de coco, pickles, sake, nata, palm wine, tofu curd and tempeh. And found in some fruits there are mangosteen, banana, papaya, pine apple and found in some flowers. Mostly, *Acetobacter* species were isolated from fermented foods, fruits, and flowers in Indonesia and Thailand (Lisdiyanti et al., 2001; Charoenyingcharoen et al., 2015).

(1) *Acetobacter aceti*

A. aceti isolated from beechwood shavings of a vinegar plant, is a Gram-negative, obligate aerobe bacteria with peritrichous flagella. The history of safe use for *A. aceti* is importantly for food grade acetic acid of vinegar productions. Strain of *A. aceti* is the most economically important for industrial fermentation, to oxidize ethanol into acetic acid and tolerant high acetic acid (Komagata et al., 2014). The optimal growth temperature is around 25 to 30°C, pH that ranges 5.4 to 6.3 (Ming et al., 2016). *A. aceti* survival in the environment, fermentation food, some fruits, some flower and on honey bees, and it is a common contaminate in wine and beer affecting to turbidity and off-flavor in product (Jiménez-Alvarez and Téllez-Jurado, 2010).

(2) *Acetobacter pasteurianus*

A. pasteurianus first isolated from beer, Netherlands. It is a Gram-negative bacteria can produce acetic acid by oxidative reaction to convert ethanol to acetic acid

(Beijerinck, 1898). One of the most frequently isolated AAB from fermented cacao beans is *A. pasteurianus* (Camu et al., 2007; Garcia-Armisen et al., 2010). Strain of *A. pasteurianus* were isolate from Chinese vinegar and Ivorian palm wine, it is a thermotolerant several to high temperature, tolerant of high ethanol concentration about 10%, produced acetic acid at above 30g/L (Matsutani, 2013; Konate et al., 2014; Chen et al., 2016) and *A. pasteurianus* is a common traditional Japanese black vinegar (Hashimoto, 2018).

2.1.2.2 Gluconobacter spp.

The genus of *Gluconobacter* appeared for the first time in 1935 by Asai, the classified of genus *Gluconobacter* use phenotypic and the chemotaxonomic characteristics species (Yamada et al., 1999; Tanaka et al., 1999; Huong et al., 2007). The genus of *Gluconobacter* genera seven species, there are *G. oxydans, G. frateurii, G. thailandicus, G. cerinus, G. asaii, G. albidus,* and *G. kondonii* (Yamada and Akita, 1984; Tanasupawat et al., 2004, 2005; Malimas et al., 2007, 2008). *Gluconobacter* oxidized D-glucose more than ethanol different from genus *Acetobacter* which focus on acetic acid (Asai, 1935). Normally, optimal growth temperature is around 35°C, and a few species grow at 37°C, pH around 5.5 and most of species grow at pH 3.5 (Komagata et al., 2014). Colonies are smooth, raise to convex and germinate on medium composition: ethanol, glucose, yeast extract, calcium carbonate and agar. Some strains produce pink colonies. *Gluconobacter* strains are isolated from a variety of fruits (strawberry, grape and peach etc.) and flowers and other sugar-rich environments.

(1) *Gluconobacter oxydans*

G. oxydans isolated from beer, is a Gram-negative, incompletely oxidize sugar of monosaccharides, sugar acids polyols and alcohols (De Ley et al., 1984). *G. oxydans* used in several biotechnological processes, to convert L-sorbose and or D-sorbitol into 2-keto-L-gulonic acid in a medium such as produce vitamin C, 6-amino-L-sorbose and produce anti-diabetic drug miglitol (Schedel, 2000; Adachi et al., 2003; Deppenmeier et al., 2002). Substance xilitol from D- arabitol is are sweetener instead of sucrose which use for food and drug industrial especially oral products (Suzuki et al., 2002; Sugiyama et al., 2003)

(2) *Gluconobacter frateurii*

G. *frateurii* isolated from strawberry, can oxidize allitol to L-psicose at a very high rate, and produce L-psicose from natural sugar and o-fructose. The L-psicose can used for produced and as a raw material for chemical or fermentation reactions such as use to produce vitamin C (Mason and Claus, 1989).

2.1.2.3 Gluconacetobacter spp.

Gluconacetobacter are Gram negative, ellipsoidal to rod-shaped, measuring 0.5-0.8 by 1.0-3.0 mm, obligate aerobic bacteria, using peritrichous flagella. Some species colonial color an light brown to brownish such as *G. azotocaptans* and *G. johannae*. They can germinate on glutamate agar and mannitol agar. The optimal growth temperature is 30°C but not at 37°C. They are growth in pH 5.5 and some species no growth at pH 7.0. The genus *Gluconacetobacter* is the most species and various physiological capabilities such as nitrogen fixation, organic acid and carbohydrate polymer synthesis. However, the classified of species focus mainly on metabolism mainly of cell. (Quintana-Quirino et al., 2019) The genus *Gluconacetobacter* consisted of two major groups there are *Gluconacetobacter liquefaciens* group and the *Gluconacetobacter xylinus* group (Yamada and Kondo, 1984). The strains are able to oxidize ethanol into acetic acid and other acids produce from D-fructose, D-glucose, D-mannitol, sucrose and ethanol, oxidizes acetate and lactate. The *Gluconacetobacter* strains are isolated from vinegar, fruits and dried fruits etc. (Yamada et al., 2011).

(1) *Gluconacetobacter entanii*

G. entanii is a Gram-negative, ellipsoidal to rod-shaped, straight or slightly curved, non-motile, occurring singly. Colonies are round, regular, soft, glossy, umbonate, and with a diameter of 1–2 mm on AE agar. Growth on mix acetic acid and ethanol concentration 6% start up for vinegar production. The strain isolated form vinegar production in submerged fermentor and not detected produce cellulose on solid media, oxidize of ethanol, diols and sugars is prevalent (Dórame-Miranda et al., 2019).

(2) *Gluconacetobacter xylinnus*

G. xylinus (formerly *Acetobacter xylinum*) is a Gram-negative, aerobic bacteria, can produce cellulose which is highly concentration in the group of cellulose (Du et al., 2018). Most stains of *G. xylinus* can convert of glucose to gluconic acid and ketogluconic acids extracellularly by enzyme responsible dehydrogenase (GDH) in membrane-bound. The production of cellulose is the most important the species providing a means of floatation and thus access to air/liquid interfaces where the supply of oxygen is abundant. The production of gluconic acid removes glucose from the medium at the expense of cellulose production (Shigematsu, 2005). Normally, found this species several in some fruits and sugar (Verschuren et al., 2000; Ishihara et al., 2002; Bae and Shoda, 2004), coconut water, pineapple and sugar cane juices and syrup (Kongruang, 2008; Castro et al., 2011).

2.1.3 High tolerant acid strain and thermotolerant acetic acid bacteria

2.1.3.1 *Acetobacter aceti* WK

A. aceti WK is a Gram-negative, aerobic bacteria, isolate from pineapple, a tropical fruit in Thailand. The strain was screened over a 10-year period in internal Venturi injector bioreactor 100 litters. *A. aceti* WK produce acetic acid shown of the high acid concentration about 8-12% and Krusong et al. (2015a) improved the semi-continuous process developed for acetification of high temperature which make this the strain can produce acetic acid at 36°C (Krusong et al., 2014a, 2014b, 2014c, 2015a, 2015b). The thermotolerant properties of *A. aceti* WK is the most important of the large-industrial fermentation because the large scale has a problem about of a lot of heat when AAB oxidize ethanol into acetic acid, and reduce overall production cost from cooling system.

2.1.3.2 *Acetobacter pasteurianus* SKU1108

A. pasteurianus SKU1108 was isolated in Thailand (Saeki et al., 1997). The strain can produce acetic acid at high temperature about at 37 °C, and growth at the wide range temperature is about 39 °C to 41 °C (Kanchanarach et al., 2010a and Saeki et al., 1997). The thermotolerance of *A. pasteurianus* SKU1108 considered several at the highest temperature (Matsutani et al., 2013).

2.2 Adaptation of Microorganism

2.2.1 Definition of adaptation

The application of physical stress to the most important method for adaptation of microorganisms. The application method uses to reduce cell microorganism to inactivation and promote food stability. Microorganism are adapted to functioning normal physiological and changes in environmental conditions just a little from the optimal value resulting the induction of stress responses of cell (Russell et al., 1995; Matsushita et al., 2016). Generally, the environment function efficiency directed at survival rather than growth so the control of microorganisms. Additionally, temperature can improve thermotolerant properties of AAB cells for acetification at high temperature (Matsutani et al., 2012).

2.2.2 Effect of physical stress to microorganism

2.2.2.1 High temperature and Low temperature

Temperature is the one of the most important factor of survival microorganism. An increase temperature of 2-3°C from optimal growth can disturb cell, inhibited growth and the high temperature causing phenotypic changes to the membrane lipids which affect to decrease acetification rate, especially AAB (Denich et al., 2003). Some bacteria can improve small changes in an environmental parameter and can adapt over the time scale of minutes, hours, or days (Hill, 1995). The first report on isolation of thermotolerant AAB was published in 1980 by Ohmori et al. AAB were isolated from surface vinegar fermentation, soil from vinegar factory and spoiled fruits and tested for their ability to produce acetic acid at higher temperatures. Isolation from rice vinegar identified as *A. aceti* can survive at high temperature. The isolate No. 1023 from rice vinegar mash from surface acetification is identified as *A. aceti* that show a normal growth and fermentation ability at 35 to 38°C. In 1997, Saeki et al. report the thermotolerant AAB strains isolated from fruit samples in Thailand. Thermotolerant AAB isolate strain are species of *A. pasteurianus*, oxidize ethanol into acetic acid at 38 to 40°C and show high acid produce about 9% (v/v). Furthermore, Kanchanarach et al. (2010) found the species AAB classified as *A. pasteurianus* SKU1108 which is a strain produced of high acetic acid at high ethanol contain. The application of physical stress to microorganism in terms of adapted cell to thermotolerant strain of AAB is a develop process for acetification at high temperature for acetification.

Calcium chloride ($CaCl_2$) can improve thermotolerant properties of cell microorganism, decreases negative effect of high temperature and as a source of Ca^{2+} binding the enzyme substrate on the membrane (Nedjimi and Daoud, 2009; Zeng et al., 2010). Krusong et al. (2015a) reported the improve thermotolerant properties of *A. aceti* WK by addition 0.15% $CaCl_2$ which affects the increment the content of phospholipids, fatty acids and the activities of membrane-bound enzymes involved in acetification both of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). Moreover, the improvement of cell wall with strong thickness was also investigated. The strain can oxidize ethanol into acetic acid at 36°C with no negative effect.

Low temperature can change metabolism in cell microorganism. As the temperature decrease, show the negative effect on growth rate is decreased, and the final cell numbers may be decreased. Low temperature effect the physiological change on cell during the lag phase including a decrease in the saturation of fatty acids and inhibition of DNA, RNA, and protein synthesis. (Demailly et al., 2019). Low temperature can inhibit of activity of enzymes such as lipase and proteinase produced by *Pseudomonas* and certain other genera (Machado et al., 2018). And low temperature causes in the change of fatty acid on the membrane (Russell et al., 1995). However, some species can tolerate to reduced temperatures such as *Streptococcus thermophilus* (Wouters et al., 1999).

2.2.2.2 Weak acid preservative

Weak lipophilic acids found in many fruits, vegetables, fruit juices, beverages, and wines which is a antimicrobial form natural (Dua and Paul, 2020; Chahardoli et al., 2020). Weak acid affect preservatives to the microorganism cells and can inhibit and disturb cells. Generally, Gram-negative bacteria are more resistant to weak acid preservatives than Gram-positive bacteria (Russell, 1991). However, some species can tolerance to weak acid preservative.

2.2.2.3 Low pH

pH is an important factor directly sensitive to survival of cell. Microorganisms growth and survival at optimum pH. And application on food, use pH to control growth and inhibit metabolism in cell of microorganisms. The change of supstate by pH inside organisms shows the stronger effect than external pH, although significant changes in either will lead to loss of viability. However low pH effect in external when detrimental to food sensory which shown to food quality (Lund et al., 2020). In general, for growth and survival, bacteria require pH range of 4 to 8, while yeasts and molds are able to grow and survive at a wider range of pH between 2 and 11 (Wheeler et al., 1991). However, microorganisms may survive in conditions of low pH, and although growth may have been stopped but the cells may still be metabolically active. However, microorganisms can adapt to tolerant stress of low pH.

2.2.2.4 Osmotic stress

Increasing the osmotic pressure (lowering Aw) is among one of the most widely used methods to preserve food products. The Aw of food product is the most effect directly on growth and survival of microorganisms. Microorganisms have minimum Aw limits, below which growth cannot occur (Csonka, 1989). Addition of salts (NaCl and KCl) and sugars (glucose and sucrose) to product can inhibit to growth of microorganisms (Gould, 1989). The osmotic a hyper osmotic shock in Aw surrounding the cell, may affect to the restoration and stabilization of the membrane lipid bilayer phase (Gutierrez et al., 1995). However, many microorganisms tolerance on osmotic stress.

2.2.3 Evolution of genetics microorganism

Many bacterial strains have fluid genetic traits that can be presented to suitable growth environments. In genetic evolution of high temperature, some species of AAB possess high mutability during growth in certain conditions (Deeraksa et al., 2005; Azuma et al., 2009). Phenotypic modifications by transposing on insertion are reported in ethanol oxidation, acetic acid resistance and cellulose formation (Matsushita et al., 2016). Some groups of researchers have performed are adaptive mutagenesis in AAB. Matsutani et al. (2013) report to the mutations of *A. pasteurianus* SKU1108 to thermotolerant of high temperature at 41°C in vitro evolution that forced the bacterial cells to survive under stressful conditions.

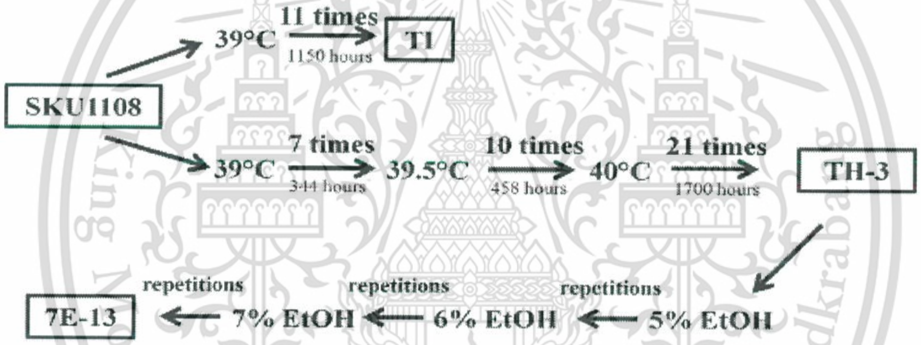

Figure 2.2 Diagram of adaptive mutagenesis of *A. pasteurianus* SKU1108 to obtain TI, TH-3 and 7E-13 strains.
Source: Matsutani et al. (2013)

The adapted strain showed a large number of mutations in highly diversified genes which could be categorized into groups related to cell surface functions, ion or amino acid transporters and some transcriptional factors. Adaptive mutation of this strain resulted in the generation of TI and TH-3 strain which showed higher thermotolerance than the SKU 1108 wild-type strain. Mutagenesis of *A. pasteurianus* SKU 1108 was performed by repeated cultivation at 39°C or step-wise increase in the adaptive cultivation temperature from 38.5 to 40°C shown on Fig. 2.2.

2.3 Immobilization of microorganisms

2.3.1 Definition of immobilization

The technique of immobilization, usually using a various substance as the support of cell as physical confinement of intact cells to defined space for preserving the metabolic or catalytic activity (Kourkoutas et al., 2001; Guo et al., 2010). Traditional vinegar is produced by taking a long time about 4-5 weeks. Therefore, cell immobilization is applied to reduced cost and time for vinegar production. It improves the acetification process by increasing biomass, option of reusability, protection of cells from toxic effects of low pH, temperature, inhibitors, etc besides keeping the aerobic conditions necessary for acetic acid bacteria (Miller et al., 1994; Krusong et al., 2020) which helps in early clarification of the product (Kocher et al., 2006). However, cell immobilization can minimize the problem associating with oxygen diffusion (Park et al., 1989; Miller et al., 1994). The matrix material for immobilization composite of monolithic support (European Patent EP0121981, 1981) are porous cellulose carrier (Sakurai et al., 2000), chitosan-treated polypropylene (Krishnan et al., 2001), fibrous inert support (WO, 2002) and polyurethane foam (De Ory et al., 2003), etc. At the same time, the interesting of natural materials for immobilization material in the form of inexpensive and easily available inert biological materials can help to reduce the cost, there are wood shavings (De Ory et al., 2003), corn cobs, bagasse (Kocher et al., 2006) and loofa sponge (Ogbonna et al., 1997; Liu et al., 1999; Akhtar et al., 2003; Chen et al., 2003; Pekdemir et al., 2003; Vignoli et al., 2006; Ganguly et al., 2007; Kang et al., 2007; Krusong et al., 2007, 2010, 2014b, 2016, 2020).

2.3.2 Natural materials for immobilization

2.3.2.1 Luffa sponge

Luffa cylindrica is classified in the Order: Cucurbitales, Family: Cucurbitaceae, Luffa sponge is a netting-like fibrous vascular system. When it is dried, the fibrous network structure serves like an open cell foam material (Chen et al., 2014) it is a natural material (Chen et al., 2018). The components chemical of fibers are cellulose, hemicellulose and lignin which support strongly stable structure. As shown in Fig. 2.3, the physical properties in luffa sponge are stiffness, strength and energy absorption. Normally, it is used as the matrix material to immobilize the cell, which show the most important for industry fermentation (Kocher and Dhillon, 2013). The composition of luffa sponge divided into four parts there are (1) outer surface (O), a thin layer live in external and combine with sponge; (2) middle part (M), composition of complex fiber internal structure, (3) inner surface (I), a parallel and long way of structure; and (4) core part (C), the main liner middle to combine other part in the structure (Chen et al., 2014).

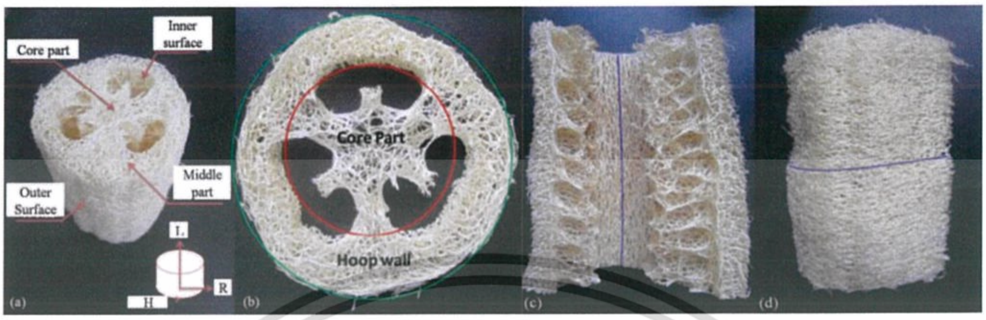

Figure 2.3 Structure of luffa sponge: (a) definition of four parts of the luffa sponge; (b) core part and hoop wall; (c) longitudinal section; (d) outer surface.
Source: Chen et al. (2014)

2.3.2.2 Wood chips

Wood chips are a natural material and high stable structure use for cell immobilization cell and substrate such as immobilized Nitrogen (Homyak et al, 2008). Normally, pore size of the matrix is small for absorption cell. Mostly, wood chips use for isemi-continuous process in considered (De Ory et al., 2003). Characteristics of wood chips (oak chips; 1.5 mm length; 1 mm width; average pore size 15 μm) show on Fig.2.4.

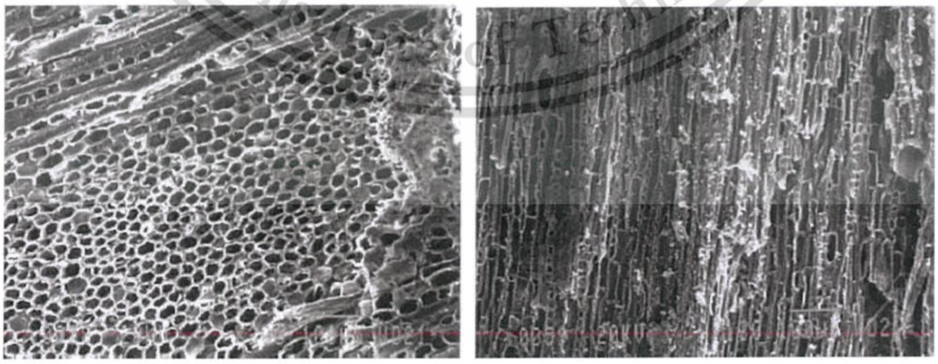

Figure 2.4 Scanning electron micrographs cuts in WOOD CHIPS, longitudinal (×130) and transversal (×200).
Source: De Ory et al. (2003)

2.3.2.3 Sugarcane bagasse

Sugarcane bagasse is a natural material for absorbing cell and developing the semi-continuous process and other process. The recycling bagasse adsorbed cells, can improve fermentation process, option of reusability, increasing biomass for fermentation, protection of cells from negative effects of low pH, temperature, inhibitors etc. (Kocher et al., 2006). Fig. 2.5 shows that the Sugarcane bagasse image by FE-SEM micrographs of the as received ground (30 mess screen).

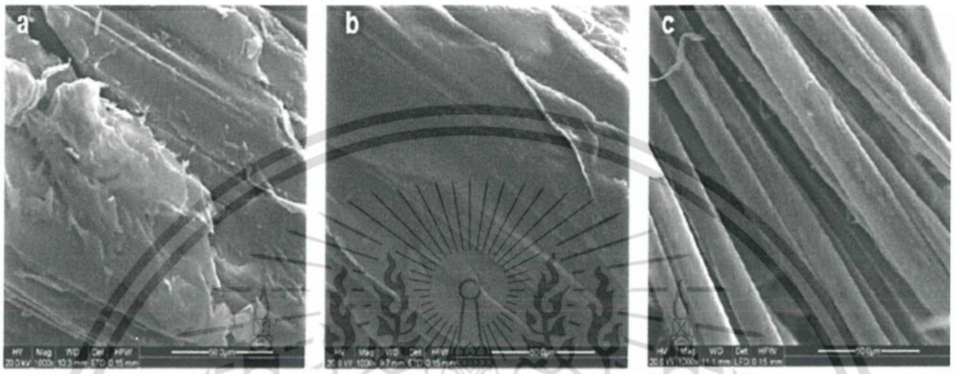

Figure 2.5 FE-SEM images of (a) sugarcane bagasse as raw-material; (b) de-lignified sugarcane bagasse; (c) chemically purified cellulose fibers.
Source: Kumar et al. (2014)

2.3.3 Characteristics of immobilize cells

Kocher et al. (2006) reported the use of bagasse, corn cobs, wood shavings and entrapped (calcium alginate) cells of *Acetobacter aceti* NRRL 746 on semi-continuous acetification. The results show that all matrix can absorb cell causing to the better fermentation. Krusong et al. (2016) report the use of luffa sponge to immobilize AAB cells for bacterial cellulose (BC). Results show that luffa sponge matrix (LAM) can offset negative of aeration, and provide cell to high bacterial biomass. Kumar et al. (2014) report the use of wood shavings for adsorption *A. aceti* under semi-continuous acetification. Result its show the immobilize cell can be used for production of sugarcane vinegar, as potential applications of the developed economical technology in food industry.

In addition, Krusong et al. (2014b) report the use of luffa sponge for adsorption of AAB for acetification in reciprocating shaker and reveal the 50% LSM-AAB give the highest acetification rate (ETA) due to optimal acetification conditions. Fig. 2.6 shown the consistency of acetification using LSM-AAB, were taken at random during the 3rd cycle for SEM analysis.

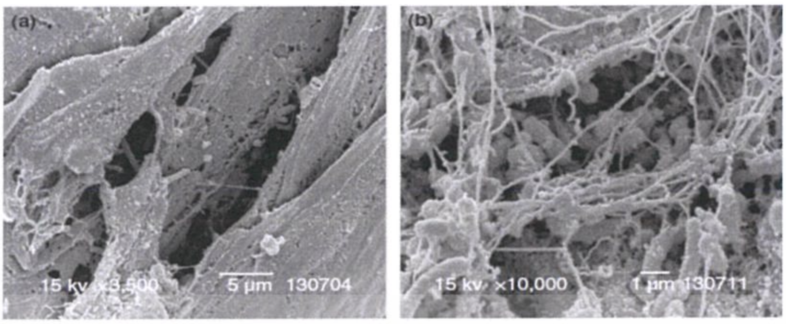

Figure 2.6 SEM images of adsorbed *Acetobacter aceti* WK cells on loofa sponge matrices (LSM): (a) on surface of LSM at 93500 magnification and (b) inside LSM at 910000 magnification.
Source: Krusong and Tantratian (2014b)

2.4 Vinegar

2.4.1 Definition of vinegar

The history of vinegar products vinegar started more than 10,000 years ago (Johnston and Gaas, 2006; Tan, 2005). Vinegar is applied as a seasoning or preservative agent in salad dressings, Previously, vinegar is considered as a food by-product from the spoilage of wine (Tesfaye et al., 2002). Far way back material for product is a low cost such as substandard fruits, seasonal agricultural surpluses and by products from food process. The definition of vinegar is different from country to country.

FAO/WHO defines vinegar as any liquid, fermentation material from starch or sugars. Vinegar production have two steps for fermentation, first step oxidize sugar into ethanol, second step convert ethanol to vinegar. The ethanol content must be less than 0.5% in wine vinegar and less than 1% in other vinegars (Joint FAO/WHO Food Standards Program, 1998).

The U.S. Food and Drug Administration (FDA) defines the vinegar are no standards of identity for vinegar established under the Federal Food, Drug and Cosmetic Act. However, the FDA considers that a satisfactory guideline for vinegar that are natural vinegars is that it must contain in excess of 4 g of acetic acid per 100 mL. Vinegar is made by the alcoholic and subsequent acetous fermentation of fruit juice (USFDA, 1977).

Nowadays, mixing of vinegar and wine to improve flavor and food safety is pound in China. In Europe, vinegar is used for improving flavor and use for preservative. In Asia and Africa, vinegar is also a drink with a less sour taste.

2.4.2 Vinegar production

2.4.2.1 Starter culture fermentation

The starter culture is defined as a microorganism for adding to the raw material to produce vinegar fermentation (Leroy and de Vuyst, 2004). Normally, true starter cultures of *S. cerevisiae* strains are used for producing the alcoholic bases for vinegars, such as beer, wine and cider (Maris et al., 2006). It is mainly used in rice vinegar fermentation. In addition, there is also *Amylomyces cerevisiae* use for ferment starch into sugar is a most important for rice wine production. For fermented acetic acid use starter cultures AAB especially *A. aceti* can produce high acid and tolerant at high temperature.

2.4.2.2 Raw material preparation

Substrate for the preparation of sugar, which the used for ferment ethanol. Normally, preparation source of sugar with some fruits such as: mangoes, pineapple, apple, peach, grape, etc. The extraction of juice; are mixed with molasses, corn syrup or honey to increase sugar content. However, the mold *Aspergillus oryzae* can produce *koji* growth on rice in Japan, the source of amylolytic enzyme in the rice vinegar productin (Hajar-Azhari et al., 2018). Commercially produced concentration of the microbial enzyme amylase and amylopectin are now widely used industrial starch converting process, including the production of vinegar (Adams, 1998).

Research on producing new types of vinegars has been ongoing. Using a new material for fermentation such as onions and bamboo for the production of vinegar. The onion produced organic acids, amino acids and minerals (Horiuchi et al., 2000b). However, onions and bamboo are low sugar content which the addition of sugar for fermentation to ethanol (Mu et al., 2006).

2.4.2.3 Alcoholic-Acetic fermentation

The vinegar production has two step, first step is the use yeast under anaerobic condition to convert sugar into ethanol, normally strains of *Saccharomyces cerevisiae* (Budak et al., 2014). Secondly in acetous fermentation steps, the AAB oxidize ethanol to acetic acid. Vinegar production shown on Fig. 2.7 (Mounir et al., 2016; Ho et al., 2017).

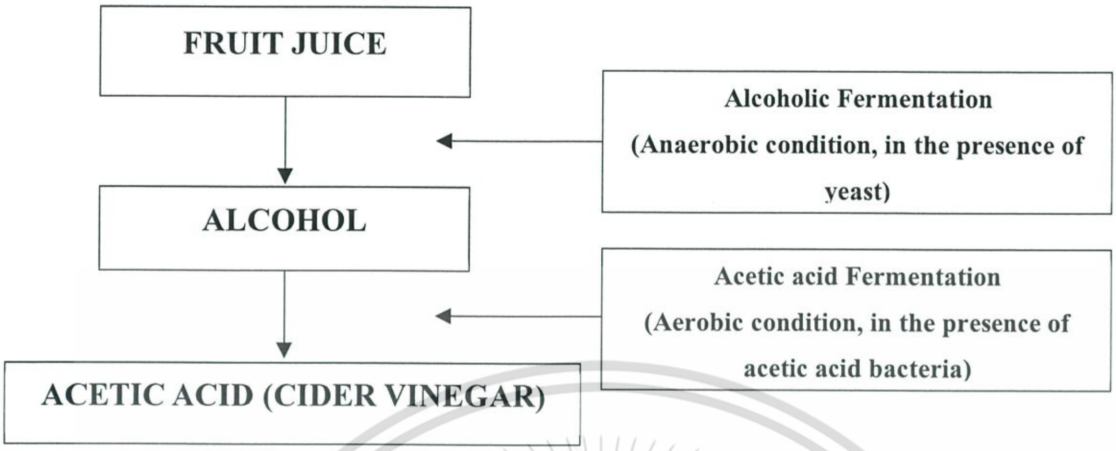

Figure 2.7 The process of cider vinegar-making from fruit juice
Source: Ho et al. (2017)

2.4.3 Acetification Process

2.4.3.1 Submerged acetification

Process of submerged acetification is the most popular important of industrial process. The design of bioreactor is an aeration system which optimize to oxygen transfer. Aeration accounts for most of the Acetator is power for support cell convert ethanol into acetic acid, AAB cells are distribute in bioreactor.

The material for produce bioreactor is stainless steel. However, a food grade with no corrosive. Normally, Frings acetator is wildly used in various producer Krusong et al. (2014a) improve the process by addition aeration system with fermented water and called as internal Venturi injector bioreactor, shown on Fig. 2.8 (Krusong et al., 2014c).

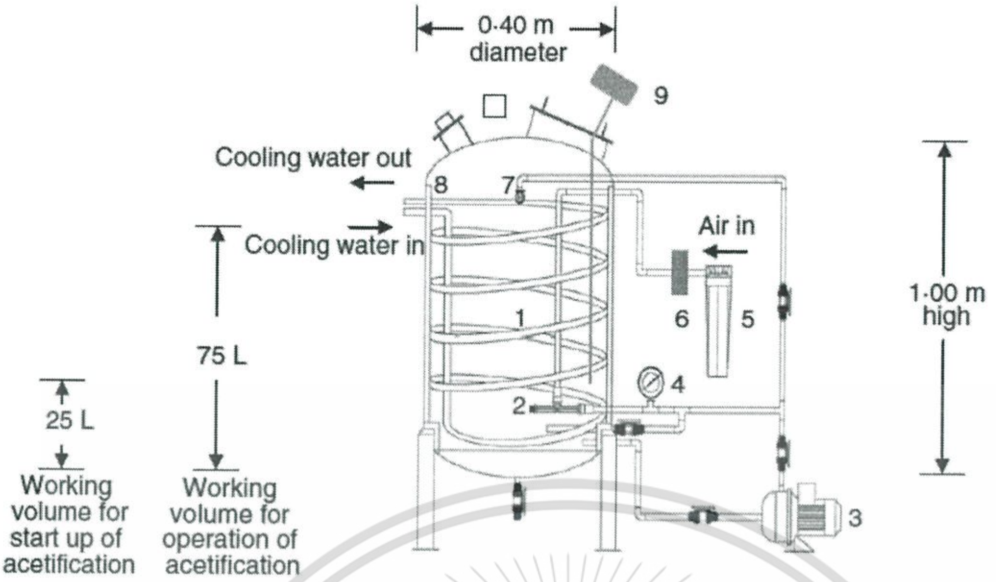

Figure 2.8 Cross-section of internal Venturi injector bioreactor for acetification by Krusong et al. (2014) is shown that: (1) 100 l stainless steel bioreactor; (2) internal Venturi injector; (3) centrifugal pump; (4) pressure gauge; (5) air filter; (6) rotameter; (7) antifoam sprinkler; (8) internal cooling coil; (9) dissolved oxygen meter

Source: Krusong et al. (2014c)

2.4.3.2 Quick acetification process

The quick acetification process (QAP) is commonly used in small-scale vinegar production. The process achieves faster rate of acetification than surface culture techniques by increasing the area of bacteria film and improving the oxygen of the stock. The process design of the fixed bed microorganisms film reactor which a film bacteria support to medium pack is focused. The matrix substrate for the adsorption of AAB to produce vinegar such as luffa sponge, wood chips, cotton towel cloth, bagasse and polyurethane is considered, the basic process of quick acetification shown on Fig. 2.9 (Okuhara, 1985; Lotong et al. 1989; Horiuchi et al., 2000a; De Ory et al., 2004; Kocher et al., 2006; Kocher and Dhillon 2013; Krusong et al., 2014b).

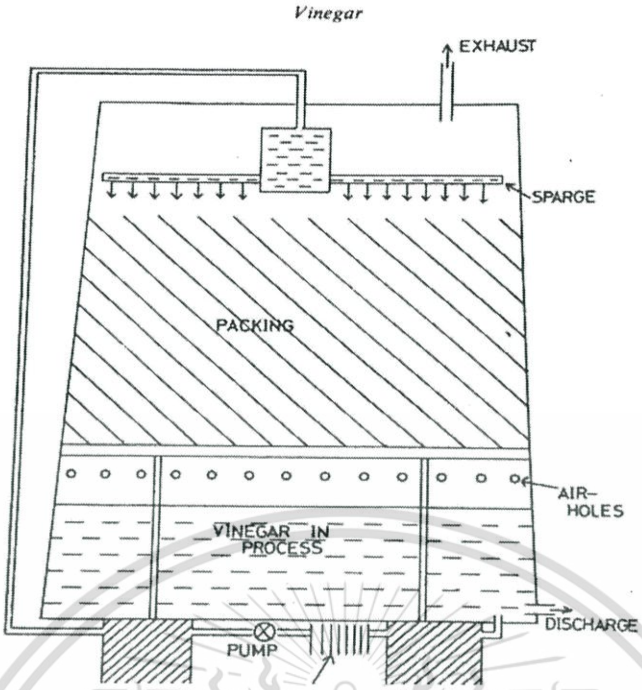

Figure 2.9 The basic process of quick acetification.
Source: Adams (1998).

2.4.4 Volatile compounds in vinegars

The flavors of vinegar are mainly attributed. The main volatile compound in vinegar is acetic acid. The material, for fermented is a source of flavor and aroma in the product. However, most content presented as acetic acid, while other volatile such as 2-butanol, 2-propen-1-ol, 4-ethylguaiacol and benzeneethanol (Ozturk et al., 2015; Ubeda et al., 2011).

Madrera et al. (2010) also reported that the volatile compound and organic acid in strawberry vinegar, are lactic, acetic, succinic and volatile compounds included 2-butanol, 2- propen-1-ol, 4-ethylguaiacol and eugenol.

Yu et al. (2012) also reported the volatile compound, of nutty, roasty and toasty tones in vinegars. There are heterocycle compounds, among which 2,3-dimethyl-5-ethylpryazine, tetramethyl-pyrazine, alkylpyrazines including 2,3,5-trimethyl pyrazine, and 2,3,5-trimethyl-6-ethylpyrazine are the major compounds.

Al-Dalali et al. (2019) was investigated the characterization of volatile compounds in three commercial Chinese vinegars by using SPME-GC-MS and GC-O. Main volatile chemical groups was found in Table 2.1, there are alcohols, esters, acids, ketones, aldehydes, furans, pyrazines, phenols, sulfides and others compound.

Pornpukdeewattana et al. (2017) reported the case of upland rice as substrate for vinegar production, Result shown in the chromatogram of chromatography-mass spectrometry (GC-MS) reveal that the main contain of composition volatile compound on upland rice vinegar, are acetic acid, ethyl ester of acetic acid; 1-butanol, 3-methyl-, acetate; isoamylalcohol, b-phenylethyl acetate; benzeneethanol and hexadecanoic, methyl este.

Table 2.1 Statistical analysis of total concentrations (means ± SD) of volatile chemical groups in three commercial Chinese vinegars.

Groups	Concentration (μg/L)			P>0.05
	ZRV	ZAV	LSV	
Alcohols	217.51 ± 0.39^{c}	5275.85 ± 1.25^{a}	539.00 ± 0.86^{b}	0.0001
Esters	1111.62 ± 0.97^{b}	3408.69 ± 1.09^{a}	801.00 ± 0.54^{c}	0.0001
Acids	14744.76 ± 1.71^{c}	32963.41 ± 1.98^{b}	194178.85 ± 1.83^{a}	0.0001
Ketones	399.77 ± 0.43^{c}	1713.52 ± 0.55^{a}	531.09 ± 0.29^{b}	0.0001
Aldehydes	5627.17 ± 1.12^{c}	44435.20 ± 2.66^{a}	33260.16 ± 1.89^{b}	0.0001
Furans	0^{c}	32.48 ± 0.22^{a}	1.26 ± 0.01^{b}	0.0001
Pyrazines	10397.09 ± 0.85^{c}	13513.00 ± 0.97^{b}	20017.40 ± 1.54^{a}	0.0001
Phenols	175.33 ± 0.76^{a}	5.37 ± 0.18^{c}	41.34 ± 0.11^{b}	0.0001
Sulfides	14.29 ± 0.08^{a}	0^{c}	3.95 ± 0.04^{b}	0.0001
Others	1815.60 ± 0.16^{a}	1614.44 ± 0.33^{c}	1792.26 ± 0.65^{b}	0.0001

Means with same letters within the same row are not significantly different at the 95% confidence level.

ZRV, Zhengrong rice vinegar. ZAV, Zhenjiang aromatic vinegar. LSV, Longmen smoked vinegar.

Source: Al-Dalali et al., 2019

2.4.5 Application of vinegar on antimicrobial

Application of volatile organic components (VOCs) has been used to control infections of crops for many years. VOCs containing acetic acid (AA), alcohol, aldehydes, acetates and esters was shown to have antibacterial and antifungal properties (Krusong et al., 2015b; Pornpukdeewattana et al., 2017).

These weak acids are relative safe for human use (Ricke, 2003) and have been extensively used pre-harvest and post-harvest in agriculture as well as in the food and drinks industry for their antimicrobial properties. Dickson and Anderson (1992) reported that reduction in levels of bacterial contamination was directly proportional to acid concentration and temperature and can be more effective if it was applied in combinations.

Bjornsdottir et al. (2006) and Krusong et al. (2015b) described the properties of VOCs as natural low molecular weight substances with high vapor pressure and low polarity that provide high activity to inhibit fungi and bacteria. Several investigators have reported that many species of fungi and bacteria on fruits and animal feeds, including maize seeds, tomatoes, grapes, sweet cherries and strawberries, can be protected by treatment with vinegar in either the liquid or vapor phases (Sholberg et al., 2000; Pornpukdeewattana et al., 2017; Tzortzakis, 2010) or with specific VOCs (Ando et al., 2012; Siri-udom et al., 2017).

The mode of action of VOCs as antifungal agents, has been described as severe damage on dehydrated surfaces, shrinking cell bodies on cell-walls, interference of the membrane function and nutrient transport, inhibition of enzyme activity and overall metabolic (Blackburn and McClure, 2002). Additionally, changing genes expressions in aflatoxin biosynthesis pathways has also been shown to reduce transcript levels after exposure to volatile components (Al-Amiery et al., 2012; Gong et al., 2019).

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CHAPTER 3

Primary adaptation of *Acetobacter pasteurianus* cells as thermo-tolerant strain to high temperature acetification process, 40°C

3.1 Abstract

In this study *Acetobacter pasteurianus* UMCC 2951 was tested as a microbial starter to conduct acetification processes by repeated cultivation under high temperature and high acetic acid acetification at 40 ± 1°C. Acid production (AP) and acetification rate (ETA) increased as adaptation period (AAP) increased, but were still lower than acetification at 30 ± 1°C. The acetification rate was lower at 40 °C respect to 30 °C. At 40 °C, the production of acetic acid was slightly lower (47-49 g/L) than that produced at 30 °C (50-52 g/L). However, the addition of 0.15% calcium chloride ($CaCl_2$) reduced the negative effects of 40 ± 1°C. On both acid production and acetification rate when compared to 30 ± 1°C. It is confirmed that acetic acid bacteria cells under the long cultivation with stress environment could physically adapt to activate at high temperature. For high initial acetic acid acetification, the acetification process was handled at 40 °C at TC8 (45 ± 1 g/L AA and 35 ± 1 g/L ET), TC9 (55 ± 1 g/L AA and 35 ± 1 g/L ET) and TC10 (65 ± 1 g/L AA and 35 ± 1 g/L ET). Meanwhile, $CaCl_2$ addition could improve thermo-tolerance mechanisms of acetic acid bacteria. The AP on HTA with 0.15% $CaCl_2$ addition at each of TC8, TC9 and TC10 was significantly ($p \leq 0.05$) higher than HTA with no $CaCl_2$ addition. Also when acid produced and ETA of TC8 with TC9 and TC10 were compared under all the conditions, the TC8 were significantly ($p \leq 0.05$) higher than TC9 and TC10 (TC8: acid produced 6.8 ± 0.2 g/L, ETA 1.24 ± 0.12 g/L/d; TC9:acid produced 4.3 ± 0.1 g/L, ETA 0.78 ± 0.08 g/L/d; TC10: acid produced 2.5 ± 0.1 g/L, ETA, 0.45 ± 0.04 g/L/d). However, these values of ETA were still significantly ($p \leq 0.05$) lower than with ETA at 30 °C.

Keywords: Primary adaptation, *Acetobacter pasteurianus*, high temperature acetification, calcium chloride

3.2 Introduction

Acetic acid bacteria (AAB) are aerobic strictly Gram-negative bacteria that belonging to the *Alphaproteobacteria* class. They are used especially in the food industry for commercial fermented beverage production (Yamada 2016; Matsushita and Matsutani, 2016; Komagata et al., 2014). Strains belonging to the genera *Acetobacter, and Komagataeibacter* are mainly used for vinegar production because they can produce suitable amounts of acetic acid (Gullo and Giudici, 2008; Zheng et al., 2018). Normally, *Acetobater aceti* and *A. pasteurianus* species are used for vinegar production, the optimum temperature is usually regulated to about 30 °C (Lu et al., 1999). However, the large-scale industrial fermentation is carried out in a closed fermentor (semi-continuous acetification system), which generates a lot of heat to oxidize alcohol into acetic acid (Matsutani et al., 2013). High temperature in the system affects to decrease acetification rate, disturbs cells including effect to long time for fermentation which is a problem in large-scale industrial fermentation (Denich et al., 2003).

Microorganisms are known for their capability to modify their behavior and physiological requirements based on their environmental conditions (Matsushita et al., 2016; Hill et al., 1995). Recent studies in vinegar production showed that *A. pasteurianus*, one of the most species used in producing vinegar, has the capability to conduct stable fermentations if adapted to environmental stressors of the fermentation conditions. In particular, *A. pasteurianus* was shown to be able to activate tolerant mechanisms against high acidity conditions and high temperatures and was considered a highly versatile microorganism (Qi et al., 2013; Phathanathavorn et al., 2019).

Previous studies have shown that the addition of calcium chloride ($CaCl_2$) could improve thermo-tolerance mechanisms of bacteria, reducing the negative effects of high temperature (Krusong et al., 2015b; Nedjimi and Daoud, 2009). Also, Nedjimi and Daoud (2009) reported that in higher plants, $CaCl_2$ can modify the membrane permeability of cells by the interaction with specific membrane-bound enzymes, stabilizing the effect of high temperature and acidity.

In this study, we used *A. pasteurianus* UMCC 2951 which was isolated from fully ripen pineapple fruit in Thailand and that shows strong ability to resist to acetic acid during fermentation. The aims of the study was to cultivate *A. pasteurianus* UMCC 2951 under high temperature acetification (HTA) at 40 ± 1 °C and to evaluate the effect of $CaCl_2$ and high acid concentration to provide higher acetic acid production under HTA.

3.3 Materials and methods

3.3.1 Microorganisms preparation and growth media

3.3.1.1 Starter culture of high acid tolerant strain Acetobacter pasteurianus UMCC 2951

Starter culture preparation of *A. pasteurianus* UMCC 2951, which is a high acid tolerant strain was isolated from fully ripen pineapple fruit and re-cultured over a 10 years period (Krusong et al., 2007). it was cultivated in a complex medium (Merck, Darmstadt, Germany) composed of (per liter of water) 50 g glucose, 5 g yeast extract, 0.2 g $Mg_2SO_4.7H_2O$ and 0.5 g $(NH_4)_2HPO_4$ under aeration at 4.5 L/min for 7 days at 30°C as described in earlier reports (Krusong et al., 2010). and cultivated at 30 °C up to 40 °C under elevated 2 °C in every 2 years before use.

3.3.1.2 Amylomyces sp.

This mold strain was used for saccharifying starch in rice to sugar. It was cultivated on Potato Dextrose Agar (PDA) slant at 30°C for 3 days. For preparation of mold bran, the *Amylomyces* sp. in PDA slant was added with 5 mL distilled water suspended for use.

Mold bran of this mold was prepared. Briefly, 5 g rough rice bran was mixed with 5 g fine rice bran after adding 10 mL of distilled water. After sterilized at 121°C for 30 min twice, rice bran was inoculated with suspended solution of *Amylomyces* spp., as above, and incubated at 30°C for 3 days before use in rice saccharification method.

3.3.1.3 Saccharomyces cerevisiae M30

This flocculate yeast which was provided from Laboratory of Yeast Technology, Department of Microbiology, Kasetsart University (Bangkok, Thailand) was used for rice wine production. It was cultivated on Potato Dextrose Agar (PDA) slant at 30°C for 1 day and kept at 4°C as stock culture. Meanwhile, it was re-cultivated in the same agar slant and incubated at 30°C for 1 day before use.

3.3.2 Preparation stock of rice wine for vinegar fermentation

15 kg steamed rice (for 100 L wine) was inoculated with mold bran *Amylomyces* spp. at the ratio of 1 kg stream rice: 25 g mold bran: 1 L of sterilized water and incubated at 30°C for 3 days. Then, it was transferred to 120 L tank for alcohol fermentation by yeast. *Saccharomyces cerevisiae* M30 from the previous step was cultivated in yeast malt (YM) broth for 20 h before use. After adding nutrients consisting (per liter of water) of 5 g yeast

extract, 5 g $(NH_4)_2HPO_4$ and 0.2 g $Mg_2SO_4.7H_2O$ and sugar 10% w/v then adjusted volume to 100 L by water, the 5% (v/v) yeast starter was inoculated. The fermentation was taken place for 7-14 days until the 10% alcohol content reached. The rice wine obtained was pasteurized at 72°C for 30 sec after cross-flow filtration and was used for acetification.

3.3.3 Fermentation media

Upland rice (*Oryza sativa*) wine and rice vinegar were prepared in the Laboratory of Fermentation Technology, King Mongkut's Institute of Technology Ladkrabang (Bangkok, Thailand) and used as substrate or acetification. The ethanol (ET) and acetic acid (AA) contents of the rice wine were 100 ± 0.5 g/L and 1.5 ± 0.1 g/L, respectively, and the content of AA in rice vinegar was 80 ± 1 g/L. The total concentration (TC) of fermentation medium which is the sum of ethanol (g/L) and acetic acid (g/L) was adjusted to 80 g/L (called as TC8) by standardizing with 35 ± 1 g/L ET and 45 ± 1 g/L AA. In addition, the AA in the high acid concentration of TC9 and TC10 were adjusted to AA as 55 ± 1 g/L and 65 ± 1 g/L, respectively, while the content of ET was constant at 35 ± 1 g/L.

3.3.4 Primary cultivation of *Acetobacter pasteurianus* UMCC 2951 at 30 °C and 40 °C

A 2 L bottle, with a working volume 1.5 L, was connected to an air centrifugal pump for generating oxygen at a flow rate 4 L/min in the process operation. The centrifugal pump was modified to simulate the internal Venturi injector bioreactor (Krusong et al., 2015a). Two acetification processes were operated, the first process at 30 ± 1 °C (the temperature generally used in acetification processes including commercial process) as control and the second process at 40 ± 1 °C (the high temperature acetification process abbreviated as HTA). *A. Pasteurianus* was cultured at either 30 and 40 °C during both the start-up (0.5 L) and the operational (1.5 L) phases following inoculation of the growth medium with 5% v/v starter culture. The culture medium was controlled at TC8 (35 ± 1 g/L ET and 45 ± 1 g/L AA), additional nutrients composing of (per liter of water) 1 g glucose, 0.5 g yeast extract, 0.2 g $Mg_2SO_4.7H_2O$ and 0.5 g $(NH_4)_2HPO_4$ were added, while HTA trial was incubated at 40 °C. The cycle of adaptation was changed when the ethanol content in the fermentation medium reached below 0.5%. The cycle was changed with a new cycle beginning with the discharge of 40% of the medium, and the remaining fresh medium topped up (Krusong et al., 2015b).

The fermentation period of 120 days was conducted for primary adaptation, and the values of AA production and residual ethanol for each cycle was measured.

3.3.5 Effect of $CaCl_2$ concentration on acetification of high initial acetic acid concentration at 40 °C

The effects of $CaCl_2$ on acetification were studied *in vitro* using a 2 L Duran bottle with 1.5 L medium for TC8 for primary adaptation. The comparative effects of six $CaCl_2$ concentrations at 0.00, 0.05, 0.10, 0.15, 0.20 and 0.25 (% w/v) on the acetification rate (ETA; expressed in terms of g/L/d). The ETA measures the rate of acid production and is calculated as the difference between the final and initial acidities during each acetification cycle were determined under HTA using the modified method described by Krusong et al. (2015b). The preparation of a new cycle of semi-continuous acetification was with added $CaCl_2$ into fresh medium and nine semi-continuous acetification cycles for each concentration. The values of acetic acid production and residual ethanol was measured for each cycle.

3.3.6 Adaptation of high temperature and high acidity on acetification process with optimal $CaCl_2$ by *Acetobacter pasteurianus*

The 5% v/v of *A. pasteurianus* UMCC 2951 culture broth from the primary cultivation TC8 of the HTA process was used as an inoculum for testing higher acid conditions in TC9 and TC10. The contents of nutrients, ET and AA were added to the optimum $CaCl_2$ concentration, mixed well and aerated. The acetification process was conducted at either 30 or 40 °C and nine semi-continuous acetification cycles were carried out for each concentration and the acid production, ETA and cell dry weight (CDW) were recorded.

3.3.7 Analytical methods

Acidity was measured using acid- base titration with 0.1 mol/L NaOH with phenolphthalein as the pH indicator and expressed as % (v/v) of acetic acid (Fregapane et al., 2001). The ethanol content was analyzed using gas chromatography (Agilent 685, US) with a capillary column 20M Carbowax on Chromosorb 0.2 μm and an FID detector (De Ory et al., 2002). The biomass, in terms of CDW in the acetification medium, was determined using a spectrophotometer (Genesys 10VIS) at 660 nm. The suitable dilution of samples was between 0.3 and 0.8 OD_{660nm} and used for measuring the CDW standard curve. To determine the standard curve of CDW, cells of *A. pasteurianus* UMCC 2951 were separated from the acetification medium by centrifugation at 8000xg for 5 min and then rinsed with a few drops of distilled water. Each of dilution serial samples were separated into two parts, the first part was measured by spectrophotometer at 660 nm and second part was weighed, dried in an oven at 105 °C overnight, then re-weighed. Calculation of in g/L was used to determine the data relationship with the spectrophotometer measurements in order to plot a standard curve of CDW (Krusong et al., 2015b).

3.3.8 Statistical analysis

The experiments, AAP, ETA and CDW were conducted in triplicate. All data were analyzed and statistical significance determined, using one-way analysis of variance (ANOVA) and a mean comparison test was carried out using the Tukey's test ($p \geq 0.05$). The SPSS Version 10.0 software was used for these analyses. Means of triplicate sample were reported together with their appropriate standard deviation (± SD).

3.4. Results and discussion

3.4.1 Effect of primary cultivation of *Acetobacter pasteurianus* at 40 °C on acetic acid production

The acetification performance at 40 °C, TC8 (80 g/L) by *A. pasteurianus* UMCC 2951 was tested by repeating several cycles at two operational temperatures (Fig. 3.1). This was done to allow to bacterial culture to adapt to the fermentation conditions. The acetification rate was lower at 40 °C respect to 30 °C. At 40 °C, the production of acetic acid was slightly lower (47-49 g/L) than that produced at 30 °C (50-52 g/L).

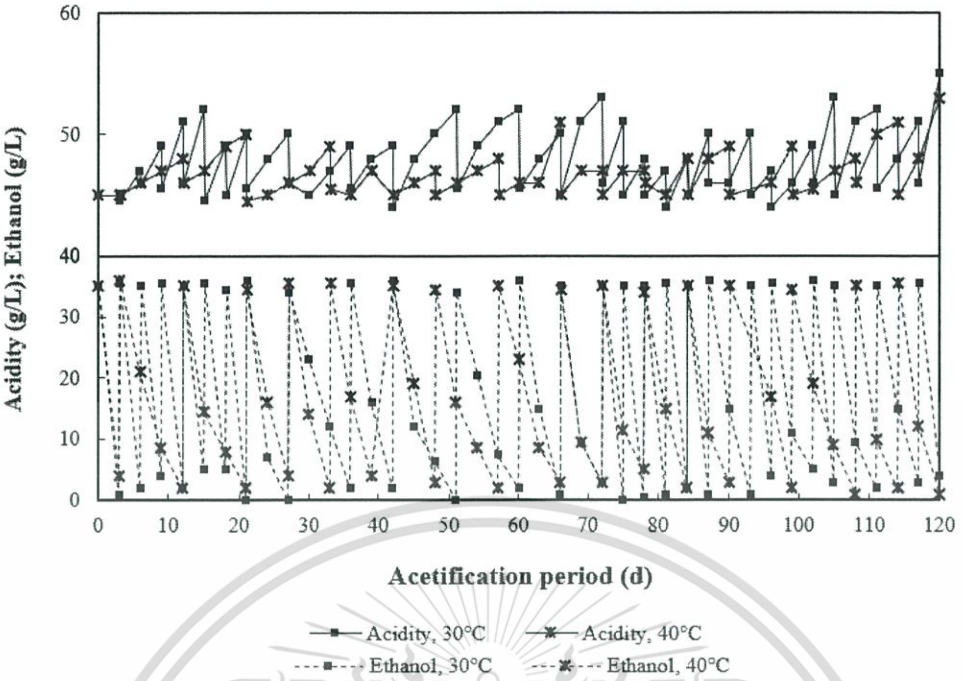

Figure 3.1 Comparative acetification performance of *Acetobacter pasteurianus* UMCC 2951 at 40 °C and 30 °C (as control), on target total concentration of 80 g/L (initial acetic acid and ethanol were adjusted to 45 ± 1 g/L and 35 ± 1 g/L, respectively) operated for 120 days.

Previous works highlighted the importance of selecting thermotolerant AAB for vinegar fermentations at high temperature. The suitability of thermotolerant Acetobacter strains, isolated from fruits and coconut water vinegar, was previously essayed in performing vinegar processes at the temperature range 37- 40 °C (Saeki et al., 1997 and Perumpuli et al., 2014). In particular, thermotolerant *Acetobacter* strains isolated from fruits were used to produce vinegar at 38-40 °C, producing an acetic acid amount comparable to that of mesophilic strains. Nevertheless, thermotolerant strains showed higher fermentation rate respect to mesophilic strains (Saeki et al., 1997). Other thermotolerant *Acetobacter* strains, collected from coconut water, produced 4% of acetic acid in shaking conditions at 37°C, suggesting their suitability as starter for vinegar production (Perumpuli et al., 2014). The production of acetic acid by *A. pasteurianus* UMCC 2951 at 40 °C was similar to a that described for a thermotolerant strain of *A. rancens* sub sp. pasteurianus, carried out in a 2-L jar fermenter (1L of medium), by Saeki et al. (1997).

Optimal fermentation in industrial field is the result of the synergic effect of the adoption of suitable managing parameters such as temperature, dosage of ethanol, oxygen supply and acidity monitor (Baena-Ruano et al., 2010). The management of these parameters allow the adaptation of AAB culture to the high stressing conditions of the acetification environment (Matsushita et al., 2016). Previous studies highlighted that AAB respond to high stringent fermentation conditions both by physiological and genetic mechanisms, as widely observed in *A. pasteurianus* species (Perumpuli et al., 2014).

3.4.2 Effect of $CaCl_2$ concentration on acetification of high initial acetic acid concentration in 2 L of bottles at 40 °C

To improve the thermo-tolerance property of *A. pasteurianus* UMCC 2951 under HTA processing at 40 °C, $CaCl_2$ was added to the fermentation medium at the start of each cycle. Results showed that the addition of 0.15% $CaCl_2$ was optimal in terms of acetic acid production (7.89 ± 0.09 g/L) (Table 3.1). In addition, comparing with out no $CaCl_2$ addition with 0.25% $CaCl_2$ addition a significant ($p \geq 0.05$) negative effect on the HTA process was observed. Krusong et al. (2015b) previously showed that $CaCl_2$ can be used to adapt strains of AAB to stabilize their viability under HTA processing at 36 ± 1 °C and confirmed that a concentration of 0.15 % (w/v) $CaCl_2$ was optimal. In this study, the addition of 0.15% $CaCl_2$ resulted in significantly ($p \leq 0.05$) increased acetic acid production and ETA (7.89 ± 0.09 g/L and 1.39 ± 0.01 g/L/d), respectively, of *A. pasteurianus* UMCC 2951 at 40 °C. Nevertheless, acetic acid production and ETA were lower than that from *A. aceti* WK, 26.0 ± 0.8 g/L and 8.67 + 0.5 g/L/d, respectively, under the condition of TC8 with 0.15% (w/v) $CaCl_2$ addition at 36 ± 1 °C (Krusong et al., 2015b).

Table 3.1 Comparative effect of $CaCl_2$ concentration on acetification by *Acetobacter pasteurianus* UMCC 2951 at 40 °C.

	$CaCl_2$ concentration (%)[1]					
	0	0.05	0.1	0.15	0.2	0.25
Total acid (g/L)*	$46.6^{e} \pm 0.1$	$49.7^{c} \pm 0.2$	$50.6^{b} \pm 0.2$	$53.9^{a} \pm 0.1$	$48.0^{d} \pm 0.2$	$46.0^{e} \pm 0.1$
AAP (d)	6	6	6	5.5	6	6
AP (g/L)*	$1.56^{e} \pm 0.07$	$3.67^{c} \pm 0.19$	$4.56^{b} \pm 0.18$	$7.89^{a} \pm 0.09$	$3.00^{d} \pm 0.18$	$1.00^{f} \pm 0.07$
ETA(g/L/d)*	$0.26^{e} \pm 0.01$	$0.61^{c} \pm 0.02$	$0.76^{b} \pm 0.02$	$1.39^{a} \pm 0.01$	$0.50^{d} \pm 0.02$	$0.17^{f} \pm 0.01$

[1] Means with the same column of each $CaCl_2$ concentration (%) were shown in terms of "Mean ± one standard deviation".

* Means with different letters within the same row were significantly different at $p \leq 0.05$. Abbreviations are: AAP (d), average acetification period in days; AP, acid production; ETA, acetification rate.

Instead, the addition of higher concentrations of $CaCl_2$ negatively affected cells, especially at 0.25% (w/v), causing a substantial decrease of ETA. Previous works have suggested that $CaCl_2$ addition could improve the thermo-tolerance mechanisms of bacteria, reducing the negative effects of high temperature, and that $CaCl_2$ is important for developing the cell walls of the bacteria to limit their permeability and give greater stability in stress conditions by changing the electrolyte conformation of peptidoglycan (Walter et al., 2002; Melcrová et al., 2016)

3.4.3 Comparative effect of optimal $CaCl_2$ concentration on high acetic acid acetification by *Acetobacter pasteurianus* UMCC 2951 at high temperature (40 °C) and 30 °C in 2 L of bottles

To test the ability of the *A. pasteurianus* UMCC 2951 to perform acetification at 40 °C, trials were conducted in a semi-continuous regime. The acetification process was handled at 40 °C at TC8 (45 ± 1 g/L AA and 35 ± 1 g/L ET), TC9 (55 ± 1 g/L AA and 35 ± 1 g/L ET) and TC10 (65 ± 1 g/L AA and 35 ± 1 g/L ET). Each of TC level was investigated using nine semi-continuous cycles and compared with trials conducted at 30 °C. The results (Table 3.2) showed that the acetic acid produced on HTA at 40 °C with 0.15% $CaCl_2$ addition at each of TC8, TC9 and TC10 was significantly ($p \leq 0.05$) higher than HTA with no $CaCl_2$ addition. Also when acid produced and ETA of TC8 with TC9 and TC10 were compared under all the conditions, the TC8 were significantly ($p \leq 0.05$) higher than TC9 and TC10 (TC8: acid produced 6.8 ± 0.2 g/L, ETA 1.24 ± 0.12 g/L/d; TC9:acid produced 4.3 ± 0.1 g/L, ETA 0.78 ± 0.08 g/L/d; TC10: acid produced 2.5 ± 0.1 g/L, ETA, 0.45 ± 0.04 g/L/d). However, these values of ETA were still significantly ($p \leq 0.05$) lower than with ETA at 30 °C. The addition of $CaCl_2$ improved the ability of *A. pasteurianus* UMCC 2951 to perform acetification in high initial acetic acid concentration (45-65 g/L) at 40 °C, which contrasts with the results of Saeki et al. (1997) who found that the initial acetic acid production of up to 4% was the upper limit for a thermotolerant strain.

Table 3.2 Comparative effect of optimal $CaCl_2$ concentration on acetification by *Acetobacter pasteurianus* UMCC 2951 at 40 °C and 30 °C (as control) in 2 liter of bottles with a working volume of 1.5 L.

		Acetic acid production^{1,*} (g/L)		
TC	30 °C		40 °C	
	Control (2.5d)	0.15 %$CaCl_2$ (2.5d)	Control (6d)	0.15 %$CaCl_2$ (5.5d)
TC8**	$6.8^{aA} \pm 0.1$	$6.9^{aA} \pm 0.2$	$3.5^{aB} \pm 0.1$	$6.8^{aA} \pm 0.2$
TC9**	$4.4^{bA} \pm 0.1$	$4.3^{bA} \pm 0.1$	$1.6^{bB} \pm 0.1$	$4.3^{bA} \pm 0.1$
TC10**	$2.6^{cA} \pm 0.1$	$2.7^{cA} \pm 0.2$	$0.8^{cB} \pm 0.1$	$2.5^{cA} \pm 0.1$
		Cell dry weight (g/L)		
TC	30 °C		40 °C	
	Control	0.15 %$CaCl_2$	Control	0.15 %$CaCl_2$
TC8	0.0041	0.0043	0.0028	0.0041
TC9	0.0030	0.0033	0.0019	0.0028
TC10	0.0020	0.0020	0.0015	0.0019
		Acetification rate^{1,*} (g/L/d)		
TC	30 °C		40 °C	
	Control	0.15 %$CaCl_2$	Control	0.15 %$CaCl_2$
TC8**	$2.72^{aA} \pm 0.11$	$2.76^{aA} \pm 0.17$	$0.58^{aC} \pm 0.02$	$1.24^{aB} \pm 0.12$
TC9**	$1.76^{bA} \pm 0.09$	$1.72^{bA} \pm 0.10$	$0.27^{cC} \pm 0.03$	$0.78^{bB} \pm 0.08$
TC10**	$1.04^{cA} \pm 0.06$	$1.08^{cA} \pm 0.05$	$0.13^{cC} \pm 0.01$	$0.45^{cB} \pm 0.04$

[1] Means with the same column of each acetic acid production (g/L) or acetification rate (g/L/d) were shown in terms of "Mean ± one standard deviation" .

* Means with different lower-case letters within the same column are significantly different ($p \leq 0.05$).

** Means with different upper-case letters within the same row are significantly different ($p \leq 0.05$). Abbreviations are; TC, total concentration.

In the current study, acetic acid production was not significant different when 30 °C and 40 °C acetification temperatures with $CaCl_2$ addition were compared, except for the ETA. However, when acetic acid concentration was increased, there was a significant difference in acetic acid production. The negative effects of both high temperatures combined with high acetic acid resulted in a decreasing of cell viability (De Ory et al., 1999). However, the addition of 0.15% $CaCl_2$ resulted in an increase in the CDW of *A. pasteurianus* UMCC 2951 cells (Table 3.2). The biomass, in terms of CDW, is usually reported as cells in acetification medium that contains both viable and non-viable AAB cells. CDW in the acetification medium of HTA with $CaCl_2$ addition was higher than that HTA without $CaCl_2$ addition, resulting directly in increased acetic acid production in the HTA process (Krusong et al., 2014, 2015b). The initial acetic acid in the acetification process is a key parameter-for the adaption of cells, and our study demonstrates that at lower concentration of TC8 the acetic acid production, CDW and ETA were higher than at TC9 and TC10.

3.5 Conclusion

In industrial vinegar field the ability of AAB strains to produce suitable amount of acetic acid at temperature above the optimal value is a challenge. This study investigated the performance of a microbial starter composed of the strain *A. pasteurianus* UMCC 2951 for conducting acetification processes under high initial acetic acid content and high temperature. $CaCl_2$ addition (0.15%) provided fermentations improved in HTA. Our study confirm that the fermentation of re-cultivated AAB under HTA for long period and the $CaCl_2$ addition could enhance the effective acetification performance at 40 °C.

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CHAPTER 4

Effect of adsorbed cell of *Acetobacter pasteurianus* on luffa sponge and $CaCl_2$ on acetification of high initial acetic acid concentration (HAAi) process in internal Venturi injector bioreactor at 40°C and reciprocating shaking bioreactor under no temperature control

4.1 Abstract

Comparison of the efficiency of thermo-tolerant strain of *A. pasteurianus* UMCC 2951 on acetic acid production at high temperature (40 °C) under high initial acetic acid concentration (HAAi) process in internal Venturi injector (iVi) bioreactor and in reciprocal shaking bioreactor under with out temperature control were investigated. Results in iVi bioreactor showed that the addition of 0.15% calcium chloride ($CaCl_2$) caused to decrease the negative effect of the increment of fermentation temperature from 30 °C to 40 °C on both acid production (AP) and acetification rate (ETA). Meanwhile, the addition of luffa sponge matrices (LSM; 50% w/v) could also improve the AP and ETA by increasing AAB cell biomass during fermentation. The combination of LSM (50% w/v) and 0.15% $CaCl_2$, called as LSM- $CaCl_2$ could provide the highest AP and ETA under High temperature acetification (HTA), but still slightly lower than 30°C. The AP of LSM- $CaCl_2$ at total concentration TC8, TC9 and TC10 were 29 ± 1, 21 ± 1, 15.33 ± 0.6 g/L while ETA were 3.96 ± 0.19, 2.18 ± 0.23, 1.44 ± 0.07 g/L/d, respectively, during the last three cycles. In case of reciprocal shaking bioreactor for quick acetification process, LSM (50% w/v) also affected on AP and ETA (consisting of at TC8 with AP 20.33 ± 1.5 g/L, ETA 1.83 ± 0.1 g/L/d, TC9 with AP 11.00 ± 1.0 g/L, ETA 0.72 ± 0.04 g/L/d, and at TC10 with AP 7.33 ± 0.6 g/L, ETA, 0.38 ± 0.04 g/L/d). The strategy used in this study confirmed that the use of acetic acid bacteria as microbial starters could be effective also at temperature above the optimal values, when acetification processes are managed through repeated semi-continuous cycles. Moreover, co-addition of $CaCl_2$ and LSM could enhance AP in acetification process and reduce cost associated with HTA. Also, quick process in reciprocating shaker is a sustainable process for small-scale vinegar production system requiring minimal set-up..

Keywords: *Acetobacter pasteurianus* UMCC 2951 strains, $CaCl_2$, Luffa sponge matrices, high temperature acetification, quick acetification process

4.2 Introduction

Acetic acid bacteria (AAB) are Gram negative and strictly aerobic bacteria which are able to produce acetic acid from ethanol. Previous studies have highlighted the occurrence of *Komagataeibacter* species, especially *K. europaeus,* in high-acidity (10-15%) vinegar production and *Acetobacter,* mainly *A. pasteurianus* and *A. aceti* in vinegars that reach acetic acid contents of 6-8% (Xia et al., 2015; Gullo et al., 2016; Trček et al., 2016) Their ability to oxidize carbon sources, such as ethanol to acetic acid, varies according to genera and species, due to the different stability of the membrane-bound enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) at high acetic acid content (Trcek et al., 2006; Wu et al., 2017)

Moreover, in industrial acetification, many parameters can affect the entire process and should be considered in terms of its optimization, including oxygen dissolution, pH, temperature and availability of nutrients. Industrial acetification is carried out mainly in submerged conditions operated by semi-continuous system processes. In this system, the air supply provides a continuous oxygen flow, giving the optimum environment for AAB growth and subsequently for the acetification process to begin, which results in increased acetic acid content (Qi et al., 2013; Krusong et al., 2014a). The optimal temperature used in the acetification process, in order to guarantee the growth of bacterial starter culture, is 30 °C (Islam et al., 2017). However, at an industrial scale, the bioreactor used for vinegar production generates a large amount of heat, which needs to be controlled using a cooling system. This is why temperature higher than optimal grow values affects the viability of the bacterial culture, which result in a decreasing of the acetification rate (Denich et al., 2003). Temperature control in industrial conditions leads to high energy consumption and consequently high production costs (De Ory et al., 2002; Charee. et al., 2020).

Many studies have shown that loofa (*Luffa cylinddrica*) sponge is an excellent immobilization carrier for microorganisms due to offset negative of aeration, and provide cell to high bacterial biomass (Chen et al., 2014; Krusong et al., 2007; 2010; 2014b; 2016). And the addition of $CaCl_2$ to improve thermotolerant properties of cell microorganism, decrease negative effect of high temperature and as a source of Ca^{2+} binding the enzyme substrate on the membrane and cell-wall call thickness (Nedjimi and Daoud, 2009; Zeng et al., 2010).

The aim of this study was to improve the thermo-tolerant properties of *Acetobacter pasteurianus* UMCC 2951 strains for high temperature acetification (HTA) process in internal Venturi injector bioreactor with re-cultivated under HTA and evaluate the effect of

$CaCl_2$ and LSM addition and to develop Quick acetification process in reciprocating shaker by using LSM immobilized thermo-tolerant strain under no temperature control.

4.3 Materials and methods

4.3.1 Microorganisms preparation and growth media

Starter culture preparation of *A. pasteurianus* UMCC 2591, which is a high acid tolerant strain isolated from fully ripen pineapple fruit and re-cultured over a 10 years period (Krusong et al., 2007). it was cultivated in complex medium (Merck, Darmstadt, Germany) composed of (per liter of water) 50 g glucose, 5 g yeast extract, 0.2 g $Mg_2SO_4.{}_7H_2O$ and 0.5 g $(NH_4)_2HPO_4$ under aeration at 4.5 L/min for 7 day at 30°C as described in earlier reports (Krusong et al., 2010). and cultivated at 30 °C up to 40 °C under elevated 2 °C in every 2 years before use.

4.3.2 Fermentation media

Upland rice (*Oryza sativa*) wine and rice vinegar were prepared in the Laboratory of Fermentation Technology, King Mongkut's Institute of Technology Ladkrabang (Bangkok, Thailand) and used as substrate for acetification. The ethanol (ET) and acetic acid (AA) contents of the rice wine were 100 ± 0.5 g/L and 1.5 ± 0.1 g/L, respectively, and the content of AA in rice vinegar was 80 ± 1 g/L. The total concentration (TC) of fermentation medium which is the sum of ethanol (g/L) and acetic acid (g/L) was adjusted to 80 g/L (called as TC8) by standardizing with 35 ± 1 g/L ET and 45 ± 1 g/L AA. In addition, the AA in the high acid concentration of TC9 and TC10 were adjusted to 55 ± 1 g/L and 65 ± 1 g/L, respectively, while the content of ET was constant at 35 ± 1 g/L.

4.3.3 Effect of luffa sponge matrix-adsorbed cells of *Acetobacter pasteurianus* UMCC 2591 on high initial acetic acid concentration (HAAi) acetification process in internal Venturi injector bioreactor at 40°C

Luffa sponge matrices (LSM) sized 2 x 2 x 2 cm were prepared by modified method from Krusong et al. (2016). Firstly, LSM was washed thoroughly in running tap water for 10 min, soaked in upland rice vinegar (URV) containing 5 %v/v acetic acid for 1 h and then washed twice in running tap water for 10 min each . After drying in a laminar flow cabinet for 30 min the LSM (amounts indicated) was weighed, placed in a 2 L Duran bottle and sterilized twice in a pressure vessel at 121°C for 30 min each.

The iVi bioreactor was previously described by Krusong et al. (2015a). The iVi bioreactor sized 100 L, a stainless steel tank 1.00 m high and 0.40 m internal diameter (max. working volume 75 L) used. The sterilized LSM was packed in stainless steel basket before placing into iVi bioreactor. The target conditions were adjusted to TC8, TC9 or TC10 in 25 L of acetification medium with 5 %(v/v) inoculum of the high acid-thermotolerant *A. pasteurianus* UMCC 2951. The condition of oxygen-transfer was operated by adjusting the air supply at 1 L/min in 25 L at the start-up phase and 4 L/min with a working volume of 75 L during the operation phase. Each of the nine subsequent acetification cycles were changed when the ethanol content in the acetification medium reached to 0.5% or below. A new cycle was started after discharged of 40% of acetification medium and the remaining was topped up to 75 L with fresh supplemented nutrients. The LSM adsorbed cells in acetification process was performed under high temperature 40°C and compered with HT with out LSM addition, and under 30°C acetification with out LSM addition. Nine acetification cycles was conducted. Each cycle was determined for acidity and ethanol contents, acetification period, ETA, biomass in terms of cell dry weight (CDW) both planktonic cells and adsorbed cells on LSM.

4.3.4 Effect of $CaCl_2$ on acetification of high initial acetic acid concentration (HAAi) process in internal Venturi injector bioreactor at 40°C.

A. pasteurianus UMCC 2951 samples, which had been adapted to HTA, were tested for the acetification performance with optimum $CaCl_2$ concentration in 100 L iVi bioreactor at 40 °C. The iVi bioreactor was previously described by Krusong et al. (2015a). The target conditions were adjusted to TC8, TC9 or TC10 in 25 L of acetification medium with 5 % (v/v) of the high acid-thermotolerant *A. pasteurianus* UMCC 2951 as the starter culture. The acetification process was conducted as same as that mentioned in section 4.3.3. The optimum $CaCl_2$ concentration was added to the nine cycles of semi-continuous acetification under HTA, and compered with HT with no 0.15% $CaCl_2$ addition and under 30 °C acetification with no 0.15% $CaCl_2$ addition. Each cycle was determined for acidity and ethanol content, acetification period, ETA, biomass in terms of CDW.

4.3.5 Acetification performance of *Acetobacter pasteurianus* UMCC 2951 under high temperature acetification with optimal $CaCl_2$ concentration and LSM adsorbed cells in 100 L internal Venturi injector bioreactor at 40°C.

A. pasteurianus UMCC 2951 samples were tested for the acetification performance with optimum $CaCl_2$ concentration and LSM (50% w/v) in 100 L iVi bioreactor at 40 °C. The target conditions were adjusted to TC8, and high acid was up to TC9 and TC10. Nine subsequent cycles of semi-continuous HTA was carried out. Each TC was determined adaptation period for start up the acetification process, acidity and ethanol content, acetification period, ETA, biomass in terms of cell dry CDW. In addition, LSM was taken at random at the end of the 3rd cycle for scanning electron microscopy (SEM) analysis to image absorbed cells.

4.3.6 Effect of luffa sponge matrices adsorbed cells of *Acetobacter pasteurianus* UMCC 2591 on acetification of high initial acetic acid concentration in reciprocal shaker with out temperature control

A. pasteurianus UMCC 2951 samples were tested in Quick acetification process with 50% w/v LSM in 20 L plastic fermentation tank on reciprocating shaker with no temperature control. The reciprocating shaker was previously described by Krusong et al. (2014b), 50% working volume and reciprocating shaker rate 1.0 Hz was used. The target conditions were adjusted to TC8, and high acid was up to TC9 and TC10, each TC was compared with no LSM addition. Nine semi-continuous cycles were conducted. Each TC cycle was determined adaptation period for start up the acetification process, acidity and ethanol content, acetification period, ETA, biomass in terms of CDW of both planktonic cells and adsorbed cells on LSM.

4.3.7 Analytical methods

Acidity was measured using acid-base titration with 0.1 mol/L NaOH with phenolphthalein as the pH indicator and expressed as % (v/v) of acetic acid (Fregapane et al., 2001). The ethanol content was analyzed using gas chromatography (Agilent 685, US) with a capillary column 20M Carbowax on Chromosorb 0.2 μm and an FID detector (De Ory et al., 2002). The biomass, in terms of CDW in the acetification medium, was determined using a spectrophotometer (Genesys 10VIS) at 660 nm. The suitable dilution of samples was between 0.3 and 0.8 OD_{660nm} and used for measuring the CDW standard curve. To determine the adsorbed *A. pasteurianus* UMCC 2591 cells, the AAB cell were removed from LSM by suspending in 0.05 mol/L sodium citrate buffer solution and shaking on reciprocating shaker at shaking rate 1·5 Hz for 1 h at ambient temperature. To determine the standard curve of CDW, cells of *A. pasteurianus* UMCC 2951 were separated from the acetification medium by centrifugation at 8000xg for 5 min and then rinsed with a few drops of distilled water. Each of dilution serial samples were separated into two parts, the first part was measured by spectrophotometer at 660 nm and second part was weighed, dried in an oven at 105 °C overnight, then re-weighed. Calculation of in g/L was used to determine the data relationship with the spectrophotometer measurements in order to plot a standard curve of CDW (Krusong et al., 2014b; 2015b).

4.3.8 Statistical analysis

The experiments were conducted in triplicate. All data were analyzed and statistical significance determined using one-way analysis of variance (ANOVA) and a mean comparison test was carried out using the Tukey's test ($p \leq 0.05$). The SPSS Version 17.0 software was used for these analyses. Means of triplicate sample were reported together with their appropriate standard deviation (± SD).

4.4 Results and discussion

4.4.1 Effect of adsorbed cell of *Acetobacter pasteurianus* on luffa sponge matrices and $CaCl_2$ addition on acetification of high initial acetic acid concentration process in internal Venturi injector bioreactor at 40 °C

The acetification performance by LSM-adsorbed cells of *A. pasteurianus* UMCC 2951 in high acetic acid conditions (TC8, TC9 and TC10) at HTA process (called as HTA8 for TC8, HTA9 for TC9 and HTA10 for TC10) was compared with acetification at 30 °C. The semi-continuous process was investigated at the start-up phase and nine operational cycles (Fig 4.1). The average acetic acid production, ETA and CDW were calculated in the last three semi-continuous cycles (Table 4.1), there are five condition; (1) HT with no $CaCl_2$ addition (HT-Control), (2) HT with $CaCl_2$ addition, (3) HT with LSM addition, (4) HT with $CaCl_2$ + LSM addition and (5) RT with no $CaCl_2$ and LSM addition (RT-Control). Where the acid production from samples of HTA8, HTA9 and HTA10 with no $CaCl_2$ addition was only 11, 9 and 4 g/L, respectively. However, the samples with 0.15% $CaCl_2$ or 50% LSM under HTA had significantly ($p \leq 0.05$) increased in AP, ETA and CDW.

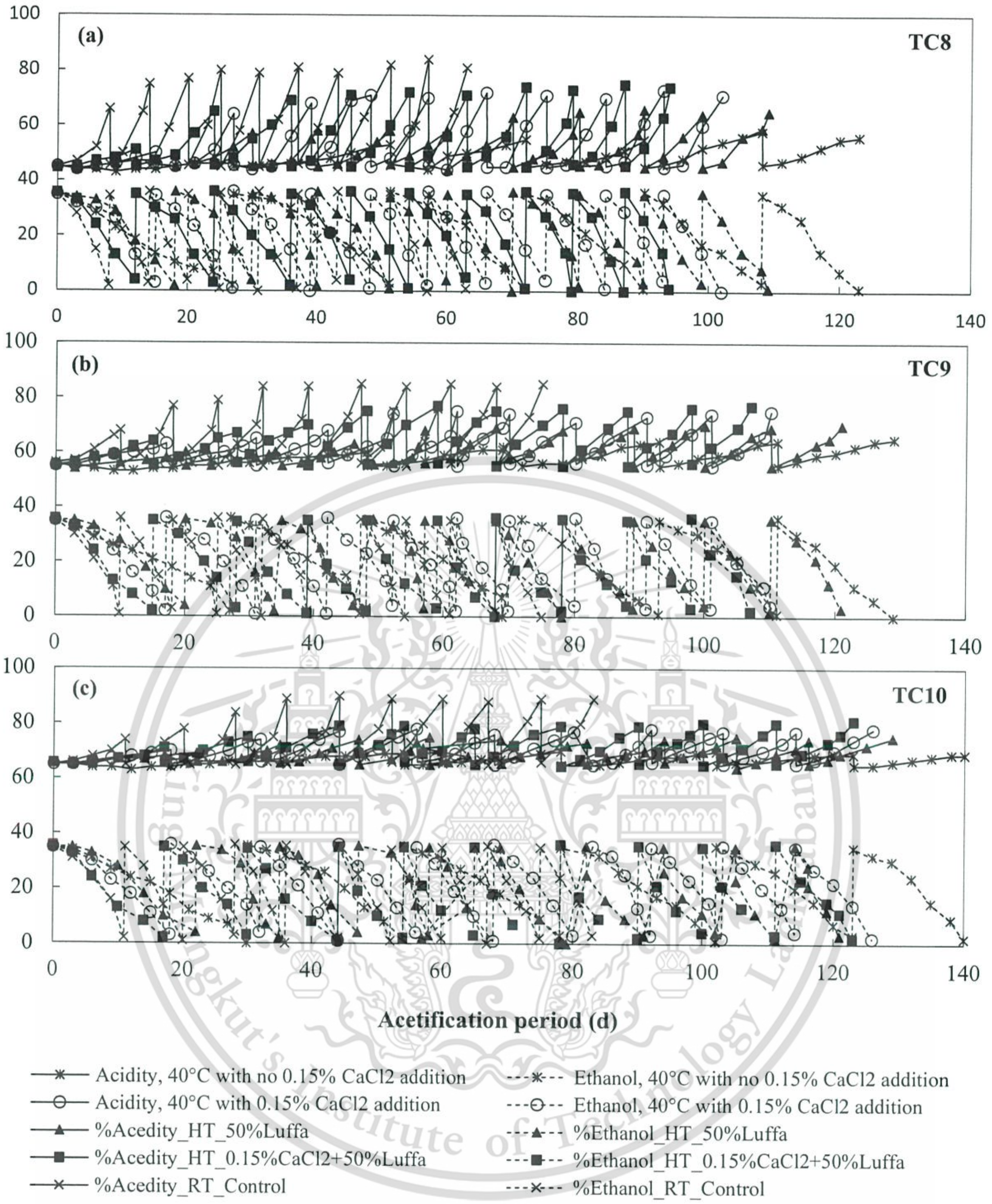

Figure 4.1 Comparative acetification performance by high acid, high temperature-tolerant *Acetobacter pasteurianus* UMCC 2951 at 40 °C and 30 °C on acetification on target total concentration of: (a) 80 g/L; (b) 90 g/L; and (c) 100 g/L (initial acidity content was adjusted to 45 ± 1 g/L, 55 ± 1 g/L and 65 ± 1 g/L, respectively, and ethanol content was constant at 35 ± 1 g/L. The semi-continuous process was investigated at the start-up phase, including 9 semi-continuous cycles except for 40°C with no 0.15% CaCl2 addition was 5 cycles. The process was conducted at 30 °C without 0.15% $CaCl_2$ addition (control); at 40 °C with and without 0.15% $CaCl_2$ addition, with 50% Luffa sponge and with 0.15% $CaCl_2$ + 50% Luffa sponge addition.

Table 4.1 Acetification performance of *Acetobacter pasteurianus* UMCC 2591 adsorbed on luffa sponge and optimal $CaCl_2$ addition under HAAi and HTA in ivi bioreactor.

	Acetification condition[1]				
	40°C				30 °C
	Control	0.15% (w/v) $CaCl_2$	50% (w/v) LSM	0.15% $CaCl_2$ + 50% LSM	Control
TC8					
Start-up phase (d)	27	15	15	12	8
AAP (d)	15	9	10	7	6
Final acidity* (g/L)	$56.33^{d} \pm 1.53$	$71.33^{b} \pm 1.53$	$65.00^{c} \pm 1.00$	$74.00^{b} \pm 1.00$	$82.33^{a} \pm 1.53$
AP* (g/L)	$11.33^{d} \pm 1.53$	$26.33^{b} \pm 1.53$	$20.00^{c} \pm 1.00$	$29.00^{b} \pm 1.00$	$37.33^{a} \pm 1.53$
ETA* (g/L/d)	$0.82^{d} \pm 0.13$	$2.93^{c} \pm 0.17$	$2.07^{b} \pm 0.06$	$3.96^{b} \pm 0.19$	$5.71^{a} \pm 0.97$
CDW* (g/L)					
Planktonic cells in FM	0.0209^{e}	0.0601^{b}	0.0252^{d}	0.0305^{c}	0.0652^{a}
Adsorbed cells on LSM	-	-	0.0332^{b}	0.0345^{a}	-
TC9					
Start-up phase (d)	28	17	20	15	10
AAP (d)	19	10	11	10	7
Final acidity* (g/L)	$64.33^{d} \pm 0.58$	$74.00^{b} \pm 1.00$	$69.67^{c} \pm 0.58$	$76.00^{b} \pm 1.00$	$84.67^{a} \pm 0.58$
AP* (g/L)	$9.33^{d} \pm 0.58$	$19.00^{b} \pm 1.00$	$14.67^{c} \pm 0.58$	$21.00^{b} \pm 1.00$	$29.67^{a} \pm 0.58$
ETA* (g/L/d)	$0.50^{d} \pm 0.05$	$1.92^{b} \pm 0.29$	$1.38^{c} \pm 0.02$	$2.18^{b} \pm 0.23$	$4.24^{a} \pm 0.08$
CDW* (g/L)					
Planktonic cells in FM	0.0161^{e}	0.0532^{b}	0.0209^{d}	0.0253^{c}	0.0597^{a}
Adsorbed cells on LSM	-	-	0.0250^{b}	0.0318^{a}	-
TC10					
Start-up phase (d)	30	18	22	17	11
AAP (d)	22	11	12	11	8
Final acidity* (g/L)	$69.33^{e} \pm 0.58$	$77.33^{c} \pm 0.58$	$75.00^{d} \pm 1.00$	$80.33^{b} \pm 0.58$	$88.67^{a} \pm 0.58$
AP* (g/L)	$4.33^{e} \pm 0.58$	$12.33^{c} \pm 0.58$	$10.00^{d} \pm 1.00$	$15.33^{b} \pm 0.58$	$23.67^{a} \pm 0.58$
ETA* (g/L/d)	$0.21^{e} \pm 0.02$	$1.09^{c} \pm 0.00$	$0.89^{d} \pm 0.05$	$1.44^{b} \pm 0.07$	$3.10^{a} \pm 0.17$
CDW* (g/L)					
Planktonic cells in FM	0.0126^{e}	0.0468^{b}	0.0188^{d}	0.0200^{c}	0.0481^{a}
Adsorbed cells on LSM	-	-	0.0239^{a}	0.0276^{a}	-

[1] Means with the same column were shown in terms of "Mean ± one standard deviation".

* Means with different letters within the same row were significantly different at $p \leq 0.05$.

Calculation of average acetification rate is based on last three acetification cycles. Abbreviations are: TC, total concentration; Start-up phase (d), adaptation start-up phase period in days; AAP (d), average acetification period in days; AP, acid production; ETA, acetification rate; CDW, cell dry weight; FM, fermentation medium; LSM, luffa sponge matrices.

Comparison of acetification performance between $CaCl_2$ addition and LSM addition, results showed that with $CaCl_2$ addition was slightly higher AA produced than with LSM at all TC8, TC9 and TC10. Moreover, the co-addition of optimum $CaCl_2$ and LSM under HTA caused consistently produce the highest acid but still slight lower than the processes conducted at 30 °C. The acid production in co-added $CaCl_2$ and LSM at TC8, TC9 and TC10 were 29 ± 1, 21 ± 1, 15.33 ± 0.6 g/L while ETA were 3.96 ± 0.19, 2.18 ± 0.23, 1.44 ± 0.07 g/L/d, respectively. The negative effects of both acetic acid and temperature on fermentation had strongly affect on acetification performances under high temperature acetification process at 40°C. With no $CaCl_2$ addition, the effect of increasing the temperature from 30 °C to 40 °C was to decrease the content of acetic acid production and also decreased cell AAB in fermentation medium and LSM.

There are many reasons that may account for the benefit of $CaCl_2$ on microbial cells, including that $CaCl_2$ can increase structural bilayer of cells membrane and can modify the membrane permeability by the interaction with specific membrane-bound enzymes and thus stabilize the membranes (Nedjimi and Daoud, 2009). Moreover, $CaCl_2$ could improve thermo-tolerance mechanisms of bacteria, reducing the negative effects of high temperature (Nedjimi and Daoud, 2009; Zeng et al., 2010; Krusong et al., 2015b).

Previous studies have shown that LSM is a natural material for industrial application, it is widely used in several fields, such as biotechnology, pharmaceutical engineering and industrial product (Choi et al., 2013; Shen et al., 2013; Krusong et al., 2014b; Chen et al., 2003, 2014, 2018; Cheng et al., 2020).

In our studies, LSM has been used for adsorbing AAB cell to increase biomass during acetification process. As shown in Fig. 4.2, the SEM image confirmed that LSM could enhance acetification performances by increasing cell biomass. The AP and ETA under LSM added was significantly higher than that without LSM. Also, it could be observed that adsorbed AAB cell biomass at 30 °C was higher than at 40 °C. which could support the higher acid production at 30 °C.

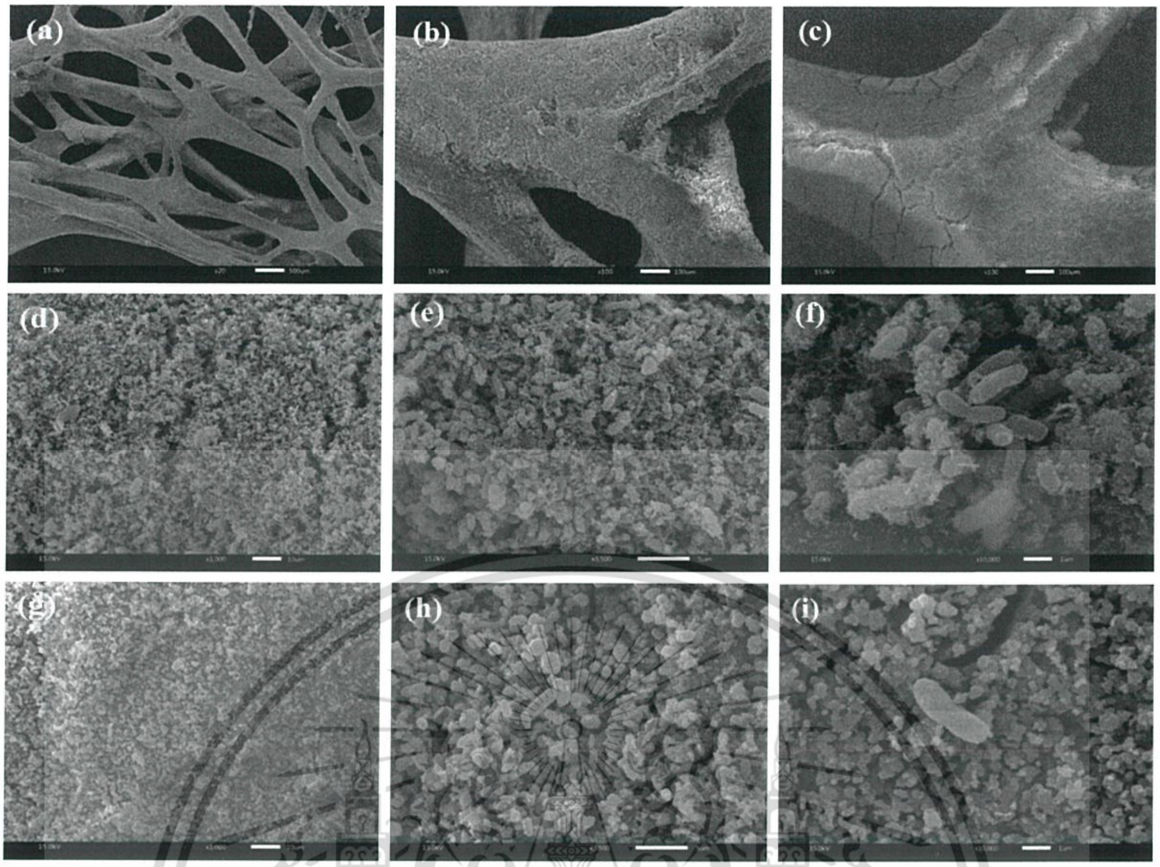

Figure 4.2 SEM images of adsorbed *Acetobacter pasteurianus* UMCC 2951 cells on loofa sponge matrices (LSM): (a) surface structure of LSM, at × 20 magnification; (b) surface of LSM under 30 °C, at × 100 magnification; (C) surface LSM under 40 °C, at × 100 magnification; (d-f) cells on surface LSM under 30°C, at × 1000, 3500, 10000 magnification, respectively; (g-i) cells on surface of LSM under 40 °C, at × 1000, 3500, 10000 magnification, respectively.

4.4.2 Effect of luffa sponge matrices-adsorbed cell of *Acetobacter pasteurianus* UMCC 2591 on acetification of high initial acetic acid concentration process in reciprocal shaker with out temperature control

The semi-continuous acetification by LSM adsorbed *A. pastrurianus* UMCC 2591 was carried out in 20 L plastic fermentation tank with 10 L working volume fermentation medium, 50% w/v LSM and 1.0 Hz reciprocating shaker rate on reciprocating shaker. The target conditions were adjusted to TC8 (45 ± 1 g/L AA and 35 ± 1 g/L ET), TC9 (55 ± 1 g/L AA and 35 ± 1 g/L ET) and TC10 (65 ± 1 g/L AA and 35 ± 1 g/L ET). Each of TC level was investigated using nine semi-continuous cycles with and without LSM at room temperature

(30 ± 2°C) with no temperature control. Results (Fig. 4.3) showed that the acetic acid produced with LSM addition at each of TC8, TC9 and TC10 was significantly ($p \leq 0.05$) higher than that control with no LSM. In addition, acid produced and ETA of TC8 were significantly ($p \leq 0.05$) higher than that at TC9 and TC10 (Table 4.2) (TC8, acid produced 20.33 ± 1.5 g/L, ETA 1.83 ± 0.1 g/L/d; TC9, acid produced 11.00 ± 1.0 g/L, ETA 0.72 ± 0.04 g/L/d; and TC10, acid produced 7.33 ± 0.6 g/L, ETA, 0.38 ± 0.04 g/L/d). In case of cell biomass, the highest amount of CDW in both planktonic cell in fermentation medium and adsorbed cell on LSM was found at TC8. Moreover, the content of CDW on LSM was higher than planktonic AAB cell. When acid was increased from TC8 to TC9 and TC10, the strong effect of high acetic acid content on population of planktonic AAB cells and LSM was occurred. The addition of 50% w/v LSM could enhance the biomass of *A. pasteurianus* UMCC 2951 to perform high acetification performance in quick acetification process.

Our results confirmed that AAB cell can be adsorbed on LSM and works well in reciprocating shaking condition. In addition, it can support the study of Krusong et al. (2014b) revealed that the 50% LSM-AAB give the highest ETA due to optimal acetification condition. Kocher et al. (2006) reported the use of bagasse, corn cobs, wood shavings and entrapped (calcium alginate) cells of *Acetobacter aceti* NRRL 746 on semi-continuous acetification. The results showed that all matrix can absorb AAB cells causing to the better fermentation.

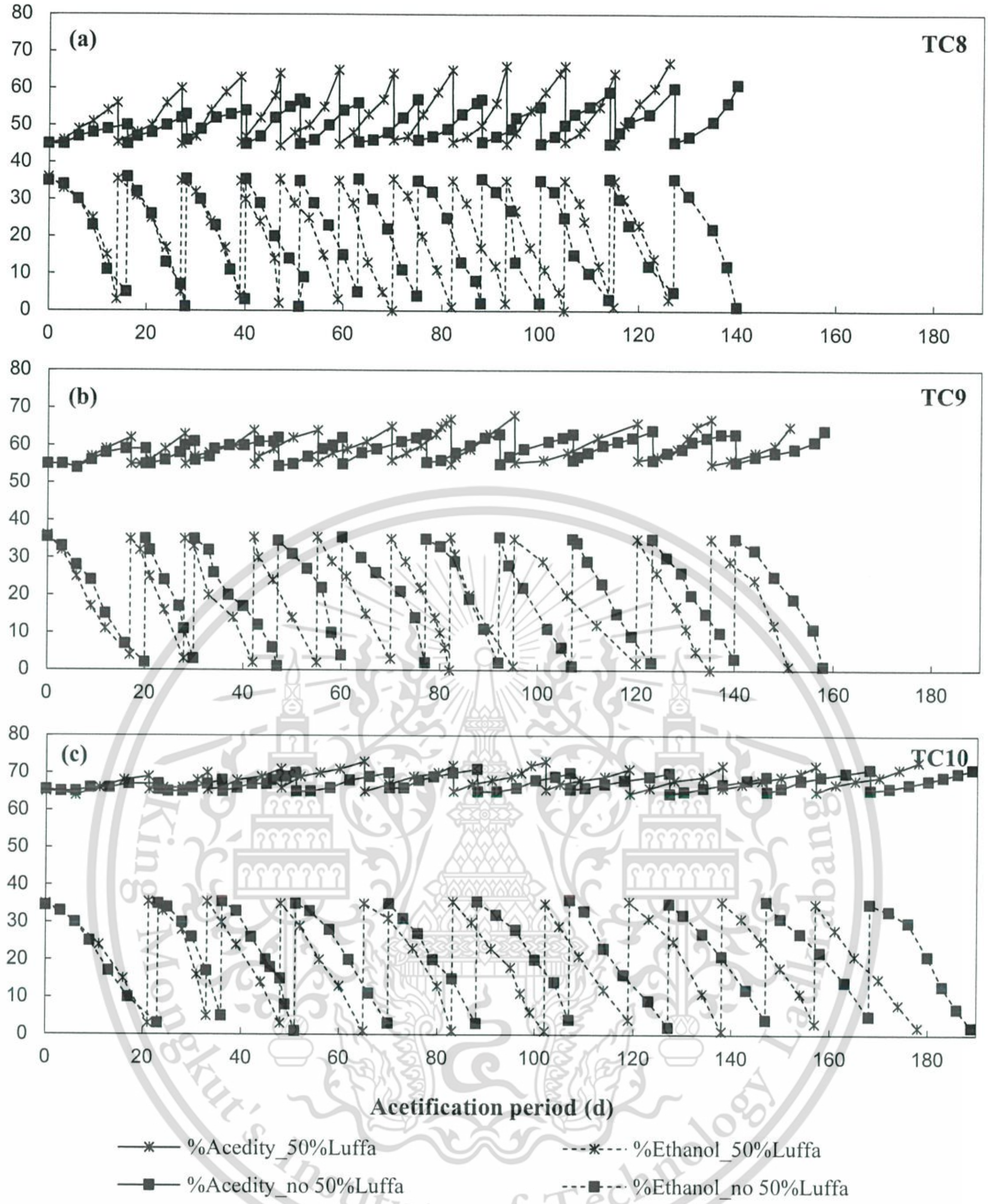

Figure 4.3 Comparative acetification performance of LSM adsorbed *Acetobacter pasteurianus* UMCC 2951 cell at 30 °C on Quick acetification process on target total concentration of: (a) 80 g/L; (b) 90 g/L; and (c) 100 g/L (initial acidity content was adjusted to 45 ± 1 g/L, 55 ± 1 g/L and 65 ± 1 g/L, respectively, and ethanol content was constant at 35 ± 1 g/L. The quick acetification process was investigated at the start-up phase and 9 consecutive cycles with and without 50% w/v Luffa sponge matrices in a 20 L reciprocating shaking bioreactor.

Table 4.2 Acetification performance of adsorbed cell of *Acetobacter pasteurianus* UMCC 2591 on luffa sponge matrices under HAAi in 10 L of fermentation medium in a 20 L tank. Reciprocating shaking rate was 10 Hz at ambient temperature.

	Acetification condition[1]	
	Control	50% (w/v) LSM
TC8		
Start-up phase (d)	16	14
AAP (d)	14	11
Final acidity* (g/L)	$60.00^{b} \pm 100$	$65.67^{a} \pm 1.53$
AP* (g/L)	$15.00^{b} \pm 1.00$	$20.33^{a} \pm 1.53$
ETA* (g/L/d)	$1.10^{b} \pm 0.09$	$1.83^{a} \pm 0.08$
CDW* (g/L)		
Planktonic cells in FM	0.0101^{e}	0.0098^{a}
Adsorbed cells on LSM	-	0.0105
TC9		
Start-up phase (d)	20	17
AAP (d)	18	15
Final acidity* (g/L)	$63.67^{d} \pm 0.58$	$66.00^{b} \pm 1.00$
AP* (g/L)	$63.67^{d} \pm 0.58$	$11.00^{b} \pm 1.00$
ETA* (g/L/d)	$0.48^{d} \pm 0.02$	$0.72^{b} \pm 0.04$
CDW* (g/L)		
Planktonic cells in FM	0.0085^{a}	0.0080^{a}
Adsorbed cells on LSM	-	0.0086
TC10		
Start-up phase (d)	23	21
AAP (d)	21	19
Final acidity* (g/L)	$70.00^{e} \pm 1.00$	$72.33^{c} \pm 0.58$
AP* (g/L)	$5.00^{e} \pm 1.00$	$7.33^{c} \pm 0.58$
ETA* (g/L/d)	$0.24^{e} \pm 0.04$	$0.38^{c} \pm 0.04$
CDW* (g/L)		
Planktonic cells in FM	0.0056^{a}	0.0051^{a}
Adsorbed cells on LSM	-	0.0068

[1] Means with the same column were shown in terms of "Mean ± one standard deviation".

* Means with different letters within the same row were significantly different at $p \geq 0.05$. Calculation of average acetification rate is based on last three acetification cycles. Abbreviations are: TC, total concentration; Start-up phase (d), adaptation start-up phase period in days; AAP (d), average acetification period in days; AP, acid production; ETA, acetification rate; CDW, cell dry weight; FM, fermentation medium; LSM, luffa sponge matrices.

4.5 Conclusion

This study investigated the performance of a microbial starter of high acid thermotolerant strain of *A. pasteurianus* UMCC 2951 for conducting acetification processes under high initial acetic acid content and high temperature in iVi bioreactor and can develop the suitable quick acetification process with no use cooling system.

The long adaptation cultivation cycles are necessary to provide stability property of acetic acid production. Co-addition of $CaCl_2$ (0.15%) and LSM (50%) can create the abundant cell biomass which provided the improvement of acetic acid production under HTA. The addition of $CaCl_2$ is recommended in order to improve the process, but only very small amounts were necessary. These levels of $CaCl_2$ did not affect the taste of the product or negatively affect the cost of production .Although cost analysis was not the object of this study, we believe that the supplementation of $CaCl_2$ does not negatively affect the economic feasibility of the designed industrial vinegar process. Indeed, it is important to highlight that the conventional industrial vinegar production is performed under very stringent conditions for the AAB culture, such as low amount of carbon source except for ethanol and other elements (vitamins, mineral salts) and high amount of acetic acid. For these reasons, high acidity vinegar production is generally performed adding nutrient supplements to stimulate the activity of the AAB culture. Moreover, according to previous works dealing with the beneficial effect of $CaCl_2$ on the effectiveness of the vinegar fermentation processes (Krusong el at., 2015b), we expect that $CaCl_2$ can replace the amount of added nutrients. Therefore, considering both the reduction of cooling costs due to the use of a thermotolerant AAB strain and the saving of nutrients, it is reasonable to suppose that costs of $CaCl_2$ can be lower than those of conventional vinegar processes. Overall these results could be a benefit for implementing vinegar productions at higher temperature respect to the optimal AAB growth, contributing to the reduction of productions costs.

For the application of high acid-thermotolerant AAB strain to quick acetification process in reciprocating shaker, the adsorbed AAB cells on LSM could improve the acetification performance due to an abundant amount of cell biomass AAB cell. This process significant reduced cost for small-scale vinegar production.

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CHAPTER 5

Effect of thermo-tolerance adaptation with optimum $CaCl_2$ and high acetic acid on changes of lipids, membrane-bound enzymes (alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH)), cell wall thickness and gene adaptation of *Acetobacter pasteurianus* high temperature (HT)-adapted strains

5.1 Abstract

Acetobacter pasteurianus UMCC 2951 (HT-adapted strain, 40°C) is a thermotolerant acetic acid bacteria. It was adapted from *A. pasteurianus* UMCC 2952 (wild type strain, 35 °C) and compared with *A. pasteurianus* UMCC 2953 (RT-Adapted strain, 30 °C). The result showed that, addition of 0.15% calcium chloride reduced the negative effects of 40 °C on both acid production and acetification rate. A strong decrease in fatty acids and phosphatidylethanolamine and increases in phosphatidylcholine and phosphatidylglycerol in cell membranes were found under high acid and high temperature acetification (HTA). In addition, transmission electron microscopic images revealed a more compact cell wall when calcium chloride was added to the cultivation medium. While, gene adaptation after adapted strain under high temperature. In this, three strain belonging *A. pasteurianus* species were analyzed. The comparative analysis 16s rRNA sequencing, showed that no substantial differences occurs in the genes set for all the three strain, but all three strains different in term of physiology and morphology properties in adapted cells.

Thus, it is suggested that the thermo adapted cells with $CaCl_2$ addition could become strong and resistant to high temperature acetification (HTA), and thus could be useful for large-industrial acetification.

Keywords: *Acetobacter pasteurianus* UMCC 2951 HT-adapted strains, $CaCl_2$, physical properties, high temperature acetification, genomic analysis

5.2 Introduction

Acetic acid bacteria (AAB) is a group of gram-negative, ellipsoidal to rod-shaped cell, it was classification in the family *Acetobacteraceae*. *Acetobacter* and *Gluconobacter* were determined as a main species of AAB. *Acetobacter* belongs to use for acetic acid fermentation cause of oxidize ethanol more strongly than glucose (Lu et al., 1999). *A. pasteurianus* is one of AAB strain have been used for industrial acetic acid fermentation. Their ability to oxidize ethanol to acetic acid, due to the different stability of the membrane-bound enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) at high acetic acid content (Trcek et al., 2006; Wu et al., 2017).

One of the most challenging in biology is environmental adaptation. Especially, temperature is one of the most important for bacterial as environmental stress. Microorganisms are known for their capability to modify their behavior and physiological requirements based on their environmental conditions (Hill et al., 1995). The adaptive response of microbial cells to a toxic environment (high acid-thermo resistance) can be by changes of membrane lipid composition in AAB cell, as has been previously suggested (Andres-Barrao et al., 2011). It had previously been reported that the combination of high acid-high temperature acetification created the strong negative effect on cells by changing their "morphology" after exposure to high acid at 40°C. (Trček et al., 2007; Krusong et al., 2014; 2015a; Zheng et al., 2017). Moreover, the AAB adaptation to high temperature resistant can reduce the cooling system and protect fermentation production from accidental failure management in large-industrial fermentation (Matsushita and Mastsutani, 2016; Matsushita et al., 2016).

In particular, *A. pasteurianus* was shown to be able to activate tolerant mechanisms against high acidity conditions and high temperatures and is considered a highly versatile microorganism (Qi et al., 2013; Phathanathavorn et al., 2019). The activation of tolerance mechanisms is the result of genetic mutations that occur with target genes. Matsutani et al. (2013) showed that *A. pasteurianus* SKU1108, a thermo-adapted strain, exhibited mutations against two proteins, MarR and amino acid transporter. They assumed that the two mutated proteins are involved in the thermo-tolerance activation mechanisms. Genomic mutations are fundamental phenomena in the evolution of microorganisms which factor high impact directly in environment. Many researchers reported that the mechanism of gene mutation in microbial cell was presented in many varieties of formation (Steiner and Sauer, 2001; Martin et al., 2005; Azuma et al., 2009).

There are many reasons that may account for the benefit of $CaCl_2$ on microbial cells, including that $CaCl_2$ can increase structural bilayer of cells membrane and can modify the membrane permeability by the interaction with specific membrane-bound enzymes and thus stabilize the membranes (Nedjimi and Daoud, 2009). Also, $CaCl_2$ has been shown to balance ions and the function of cytoplasmic membranes by transferring material giving self-protection and self-recovery from metabolic toxicity and stress and delaying cell senescence (Shen et al., 2008; Zeng et al., 2010). Moreover, Zeng et al. (2010) and Krusong et al. (2015b) reported that the addition of $CaCl_2$, during bacterial fermentation could result in modification of their phospholipids content, fatty acids composition and membrane-bound enzymes affecting the stability of the cells and consequently the fermentation process.

In this study, we used *A. pasteurianus* UMCC 2951 which was isolated from fully ripen pineapple fruit in Thailand and that shows strong ability to resist to acetic acid during fermentation. The aims of the study were to cultivate *A. pasteurianus* UMCC 2951 under high temperature acetification (HTA) at 40 ± 1 °C and to evaluate the effect of $CaCl_2$ and high acid concentration on cell wall thickness, changes in lipid and membrane-bound enzymes, especially ADH and ALDH, and also to comparison the effect of high temperature adaptation fermentation on gene mutation.

5.3 Materials and methods

5.3.1 Microorganisms preparation and growth media

A. pasteurianus UMCC 2953 (wild type strain; RTN), which was a high thermotolerant strain at 35 ± 1°C was isolated from fully ripen pineapple fruit in tropical region in Thailand. *A. pasteurianus* UMCC 2952 (adapted RT-strain; RT) and *A. pasteurianus* UMCC 2951 (adapted HT-strain; HT) were obtained from strains adaptation method which described in Fig. 5.1. All cultures were cultivated under aeration at 4.5 L/min at 30 ± 1°C (adapted RT-strain), 35 ± 1°C (wild type strain) and 40 ± 1°C (adapted HT-strain) for 7 days in complex medium (Merck, Darmstadt, Germany) composing per liter of water: glucose 50 g, yeast extract 5 g, $Mg_2SO_4.7H_2O$ 0.2 g and $(NH_4)_2HPO_4$ 0.5 g as described by (Krusong et al., 2007; 2010).

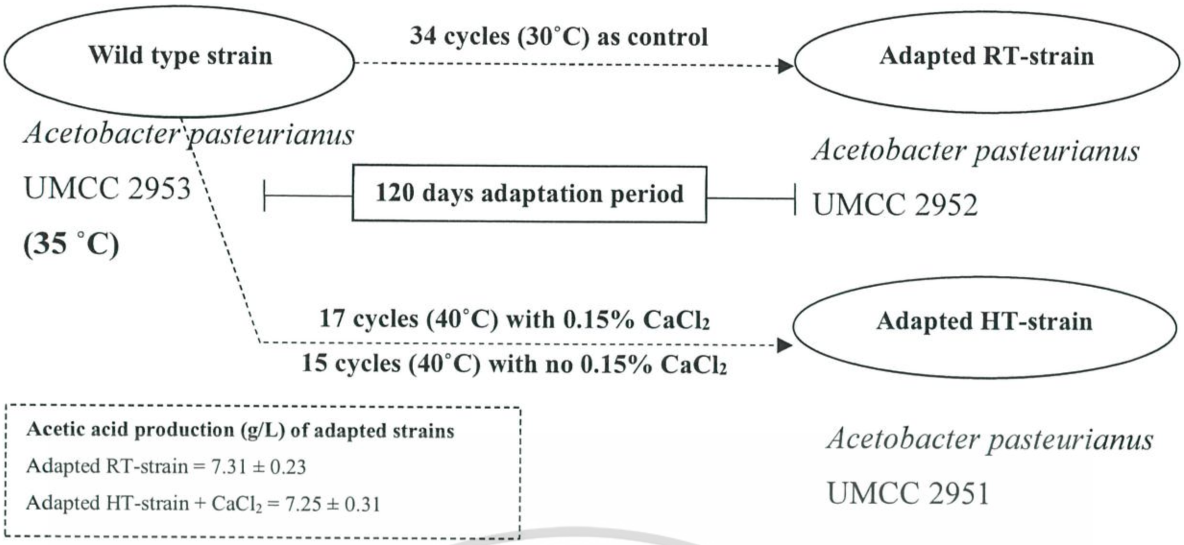

Figure 5.1 Schematic diagram of an adaptation of thermo-tolerant strains *Acetobacter pasteurianus* UMCC 2951 (adapted HT-strain) from *Acetobacter pasteurianus* UMCC 2953 (wild type strain; RTN) for high temperature acetification at 40 °C (with or without $CaCl_2$ addition). The acetification was compared with *Acetobacter pasteurianus* UMCC 2952 (adapted RT-strain) at 30 °C. The adaptation experiment was re-cultivated fermentation in 2 L of bottles with a working volume of 1.5 L, acetification target total concentration was 80 g/L (initial acidity content was adjusted to 45 ± 1 g/L and ethanol content was constant at 35 ± 1 g/L and operated for 120 days.

5.3.2 Fermentation media

Upland rice (Oryza sativa) wine from previous step and rice vinegar were prepared in the Laboratory of Fermentation Technology, King Mongkut's Institute of Technology Ladkrabang (Bangkok, Thailand) and used as the raw materials for acetification. The ethanol (ET) and acetic acid (AA) contents of the rice wine were 100 ± 0.5 g/L and 1.5 ± 0.1 g/L, respectively, and the content of AA in rice vinegar was 80 ± 1 g/L. The total concentration (TC) which is the sum of ethanol (g/L) and acetic acid (g/L) was adjusted to 80 g/L (called as TC8) by standardizing with 35 ± 1 g/L ET and 45 ± 1 g/L AA. In addition, the AA in the high acid concentration of TC9 and TC10 were adjusted to AA as 55 ± 1 g/L and 65 ± 1 g/L, respectively, while the content of ET was constant at 35 ± 1 g/L.

5.3.3 Evaluation effect of $CaCl_2$ and high acetic acid on lipids, membrane-bound enzymes and cell wall thickness at high temperature acetification at 40 °C

Cells of *A. pasteurianus* UMCC 2951 was obtained from a previous adaptation study, it was sampled at the third cycle from each TC acetification medium. The preparation method for determining lipids and membrane-bound enzymes were modified from (Qi et al. 2013). Briefly, cells from medium acetification were centrifuged at 8000xg for 10 min and washed twice with 50 mmol/L potassium phosphate buffer (pH 7). The washed cells were re-suspended in the same buffer. The harvested cells were kept at -20 °C until analysis. Cell suspensions and the supernatant were used for measurement of the activities of the membrane-bound enzymes ADH and ALDH and phospholipids and fatty acids on the two membrane-bound enzymes.

The cells of *A. pasteurianus* UMCC 2951 acetification medium at the 3rd cycle were observed by a transmission electron (microscope JEOL JEM-2100, Tokyo, Japan). Cells from fermentation broth was centrifuged at 8,000xg for 5 min at 4 °C. Then, cells were rinsed with a few drops of sterile distilled water and re-centrifuged at the same condition. Cells was kept at 4 °C then, were vacuum dried and sputter coated with gold before inspection and photography.

5.3.4 Effect of high temperature on gene thermo-tolerant strain of *Acetobacter pasteurianus* HT-adapted strain and compared with Wild-type strain and RT-adapted strain

All strain (HT-adapted, RT-adapted and RTN-wild type strain) identity was determined by 16S rRNA gene sequencing. Genomic DNA extraction, amplification and sequencing of 16S rRNA gene was performed by Macrogen Inc. company, according to their protocols. Then, the sequence was trimmed, removing low quality bases, using Phred v 0.071220.c and assembled using Pharp v 1.090518 (Ewing and Green, 1998). The dataset was structured by downloading a total of 10 16S rRNA sequences of *Acetobater* strains from NCBI 16S rRNA databases, selecting the strains isolated from food matrices. The 16S rRNA sequences were aligned using Muscle v3.8.31 (Edgar, 2004). Resulting alignment was imported in MegaX (Kumar et al., 2018) and trimmed in order to obtain sequences with the same length. Trimmed alignment was used to generate a maximum-likelihood (ML) phylogenetic tree, applying the Tamura-Nei evolutionary model (Tamura and Nei, 1993), setting a discrete gamma distribution to model evolutionary rate differences among sites.

5.3.5 Analytical method

The acidity and alcohol content were measured by following method of Krusong et al. (2015a).

The membrane-bound enzyme, ADH and ALDH, cell suspensions and supernatants from the cell preparation were treated ultrasonically at 750 W 20 kHz, for 15 s .The cell debris were then removed by centrifugation at 10000 x g for 30 min and the supernatant obtained was used to determine the activity of ADH and ALDH calorimetrically using potassium ferricyanide as an electron acceptor at 660 nm as described by Adachi et al. (1978). One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of ethanol in 1 min .The protein levels were measured using the modified Lowry method (Lowry et al., 1951) with bovine serum albumin as the protein standard. The activities of ADH and ALDH were reported for cells under HTA (40 °C) of each TC and compared with acetification at 30 °C.

For the analysis of phospholipids and fatty acids on membrane-bound enzymes, the harvested cells from the cell preparation were extracted using chloroform:methanol at a ratio of 2:1 (v/v) from the modified method described by Folch et al. (1957). The total lipid residue, after solvent evaporation, was dissolved in hexane and, using a silica column (10x100m) eluted with 50 % methanol for subsequent separation of phospholipids using the modified method described by Jernejc et al. (1989). Then, each of phospholipid was determined using two-dimensional thin-layer chromatography (TLC) on silica gel plates following the method of Bartowsky and Henschke (2008). Firstly, the composition of the solvent development phase using a chloroform:methanol:25 % ammonia mixture (65:35:5, v/v) and the secondly, the mobile, phase using a chloroform:acetone: methanol:acetic acid:water mixture (50:20:10:10:5, v/v).

Analysis of total fatty acids, as fatty acid methyl esters (FAMEs) were analyzed using gas chromatography (Agilent 6850 series II Networked GC System, Agilent Technologies, USA) equipped with a flame ionization detector (FID). The fatty acid methyl esters were separated using a HP-ULTRA2 capillary column (30m long, 0.25μm film thickness). The conditions used were :injection temperature 250 °C, detector temperature 220 °C, oven temperature 220 °C, with nitrogen as the makeup gas, a column flow rate of 2 mL/min and a run time 50 min .The peak areas were compared with a fatty acid methyl ester standard using the modified method of Wirasnita et al. (2013).

5.3.6 Statistical analysis

The experiments were conducted in triplicate. All data were analyzed and statistical significance determined, using one-way analysis of variance (ANOVA) and a mean comparison test was carried out using the Tukey's test ($p \geq 0.05$). The SPSS Version 17.0 software was used for these analyses. Means of triplicate sample were reported together with their appropriate standard deviation (± SD).

5.4 Results and discussion

5.4.1 Effect of optimal $CaCl_2$ on lipids, membrane-bound enzymes and cell wall thickness of *Acetobacter pasteurianus* UMCC 2951 under high temperature acetification at 40 °C

The effect of 0.15% $CaCl_2$ on changes of phospholipids in cell membrane of *A. pasteurianus* UMCC 2951 under high acid-thermo acetification at 40 °C (HTA) was investigated and compared with at 30 °C (as control). The three major phospholipids in cell membranes were dominants-phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) (Table 5.1). The amount of PC, PG and PE at 30 °C acetification of each TC were higher when compared with HTA with no additional $CaCl_2$. Furthermore, HTA reduced the amount of three phospholipids in the cell membrane. However, the addition of 0.15% $CaCl_2$ in the acetification medium enhanced the stability of lipids in cell membranes when the acetic acid level was increased from TC8 to TC9 and TC10 from an initial level of 45 g/L to 65 g/L. The incremental contents of both PC (TC8, 40.4%; TC9, 41.9% and TC10, 45%) and PG (TC8, 16.5%; TC9, 18% and TC10, 18.7%) were examined as the initial acetic acids in the medium increased. The results showed that there was a reduction of PE when acetic acid in the medium increased (TC8, 15.3%; TC9, 12.1% and TC10, 10%), but there appeared to be a negative effect of high acetic acid on PE.

It had been previously shown that the composition of phospholipids in membranes of *A. pasteurianus* UMCC 2951 and *A. aceti* WK (Krusong et al., 2015b) varied at 30 °C. The content of both PC and PE were higher, 40.5% and 16.5%, respectively, but lower PE (15.4%) were found in *A. pasteurianus* UMCC 2951 compared to PC (38.8%), PG (14.7%) and PE (16.3%) in *A. aceti* WK. In *A. aceti* WK strain cultured at TC8 condition, the content of these three phospholipids was shown similarly to be decreased when grown at higher temperature, while the reduction was recovered by the addition of $CaCl_2$ (Krusong et al., 2015b). The adaptive response of microbial cells to a toxic environment (high acid-thermo resistance) can be by changes of membrane lipid composition in AAB cell, as has been previously suggested (Andres-Barrao et al., 2011). In our study, we also conducted the analysis of cell membranes by TLC, which showed that the main phospholipids were PC, PG and PE, which were the same as reported by Nakano and Ebisuya (2016); Trček et al. (2007). Moreover, the major phospholipids found in our *A. pasteurianus* strain were similar to those found in *Komagataeibacter europaeus* and strains of the species *Gluconobacter* and *Gluconacetobacter* (Hanada et al., 2001).

The negative effects of both acetic acid and temperature on fermentation also made change of the individual composition of phospholipids, where PC and PG increased while PE decreased when the acetic acid level was adjusted to increase from TC8 to TC9 and TC10 at high temperature acetification (HTA8, HTA9, and HTA10). With no $CaCl_2$ addition, the effect of increasing the temperature from 30 °C to 40 °C was to decrease the content of phospholipids, but phospholipids in cell membrane were slightly increased when 0.15% $CaCl_2$ was added. There are many reasons that may account for the benefit of $CaCl_2$ on microbial cells, including that $CaCl_2$ can increase structural bilayer of cells membrane and can modify the membrane permeability by the interaction with specific membrane-bound enzymes and thus stabilize the membranes Nedjimi and Daoud (2009). Also, $CaCl_2$ has been shown to balance ions and the function of cytoplasmic membranes by transferring material giving self-protection and self-recovery from metabolic toxicity and stress and delaying cell senescence (Zeng et al., 2010; Shen et al., 2008).

In our study, the major fatty acids in the cell membrane were cis-vaccenic acid, palmitic acid, 2-hydroxypalmitic acid, myristic acid and 2-hydroxymyristic acid (Table 5.1). These results are consistent with previous studies, reporting cis-vaccenic acid being the major fatty acid in PG in almost all AAB, representing about 80 % (Hanada et al., 2001). The highest content of total fatty acids was found in samples processed at 30 °C with TC8 (64.9%). The co-effect of both high acetic acid and high temperature resulted in a strong decrease of fatty acid especially at HTA with no $CaCl_2$ addition. Alternatively, the addition of 0.15% $CaCl_2$ could reversibly increase the amount of total fatty acids in cell membranes. The total fatty acid content of *A. pasteurianus* UMCC 2951 cells decreased slightly during fermentation at 40 °C in high acetic acid conditions, but total fatty acid increased slightly when $CaCl_2$ was added into the fermentation medium.

Table 5.1 Effect of $CaCl_2$ on changes of phospholipids, fatty acids and enzyme activities of ADH and ALDH in *Acetobacter pasturianus* UMCC 2951 cells during acetification at 40 °C and 30 °C.

Phospholipid compounds		Phospholipid (%)								
		TC8			TC9			TC10		
		30 °C	40 °C	HTA-C	30 °C	40 °C	HTA-C	30 °C	40 °C	HTA-C
Phosphatidylcholine **(PC)**		40.5	40.0	40.4	42.2	40.9	41.9	45.5	44.7	45.0
Phosphatidylglycerol **(PG)**		16.5	16.0	16.5	18.2	17.3	18.0	18.9	18.0	18.7
Phosphatidylethanolamine **(PE)**		15.4	15.0	15.3	12.0	11.8	12.1	10.1	9.5	10.0
Peak name	Fatty acid	Fatty acid (%)								
		TC8			TC9			TC10		
		30 °C	40 °C	HTA-C	30 °C	40 °C	HTA-C	30 °C	40 °C	HTA-C
18:1 w7c	cis-Vaccenic acid	64.9	59.7	62.9	59.7	55.5	56.6	54.5	51.2	53.1
16:00	Palmitic acid	11.0	8.1	9.2	16.4	13.8	14.2	21.7	16.9	17.5
16:0 2OH	2-Hydroxypalmitic acid	2.9	2.6	2.8	2.9	2.1	2.5	1.0	0.8	0.8
14:0 2OH	Myristic acid	2.5	2.0	2.4	1.7	1.1	1.5	1.1	1.0	1.0
14:0 2OH	2-Hydroxymyristic acid	6.5	5.9	6.1	4.9	2.4	2.8	1.8	0.3	0.9
Membrane-bound enzyme		Enzyme (Unit/mg)[1]								
		TC8			TC9			TC10		
		30 °C	40 °C	HTA-C	30 °C	40 °C	HTA-C	30 °C	40 °C	HTA-C
Alcohol dehydrogenase **(ADH)**		10.19	4.87	8.36	5.63	1.32	4.13	1.31	0.41	1.00
Aldehyde dehydrogenase **(NADH)**		19.93	11.85	17.18	6.99	3.08	6.49	2.02	0.87	1.70

[1] One unit of enzyme activity is defined as the amount of enzyme catalyzing the oxidation of 1 µmol of ethanol in 1 min. Acetification at 40 °C and 30 °C were conducted with no $CaCl_2$ addition comparing with acetification at 40 °C with $CaCl_2$ addition (HTA-C). Abbreviations: TC, total concentration; HTA- C, high temperature acetification with 0.15% $CaCl_2$ addition.

Previously, it has been shown that the addition of $CaCl_2$ at 0.15% (w/v) to the culture media for *A. aceti* WK resulted in an increase in cis-vaccenic acid content, palmitic acid and myristic acid at 36 ± 1 °C (Krusong et al., 2015b), which were higher than that at 30 °C. Also, the increment of these three fatty acids was found in *A. pasteurianus* UMCC 2951 at 40 °C with 0.15% (w/v) $CaCl_2$ addition, which was higher than cultures without $CaCl_2$ addition. But all fatty acids levels were lower than those found at 30 °C, which was different to *A. aceti* WK. With the addition of 0.15% (w/v) $CaCl_2$, the increase in cis-vaccenic acid content subsequently results in increased PG. This was mentioned by Trček et al. (2007), who found that PG was involved in cell wall integrity, which means that the integrity of both *A. aceti* WK and *A. pasteurianus* UMCC 2951 cells were also improved, allowing them to better resist the surroundings of high acidity in the HTA process.

The effect of $CaCl_2$ on membrane-bound enzymes (ADH and ALDH), showed the activity of the enzyme in cells at 30 °C, with all TC especially TC8 (ADH, 10.19 and ALDH, 19.93 unit/mg), which was higher than that at HTA and high temperature acetification with $CaCl_2$ addition (HTA-C). The reduction of these enzyme activities at higher temperature and its recovery with $CaCl_2$ was also observed in *A. aceti* WK grown at 36 °C (at TC8 condition) (Krusong et al., 2015b). Also, the increment of acetic acid content from TC8 to TC10 at high temperature (called HTA8 to HTA10, respectively) caused a strong negative effect on the activity of both ADH and ALDH. However, the addition of $CaCl_2$ in the fermentation medium was shown to protect the activity of ADH and ALDH. Moreover, the high temperature acetification conditions (HTA and HTA-C) resulted in the reduction of both enzymes when compared with at 30 °C condition. At the TC8, HTA with no $CaCl_2$ addition the enzyme activity was reduced by 52.3% for ADH and 40.5% for ALDH when compared with at 30 °C. Moreover, when the acetic acid level was increased from TC8 to TC9 and TC10 at HTA the activity of ADH were reduced slightly to 87.1% at TC9, 95.9% at TC10, while ALDH was reduced to 84.6% at TC9 and 95.0% at TC10 when compared with TC8 at 30 °C. Qi et al. (2013) reported similarity results on the activity of enzymes in cytoplasmic membrane of *A. pasteurianus* FS1, which showed that the activity of two enzymes decreased as the acidity was increased. When the acidity in the medium was 70 g/L, the activity decreased to 0.21 unit/mg for ADH and 0.65 unit/mg for ALDH with the same acetic acid content (in the same TC), When the activity of both enzymes, with the addition of 0.15% $CaCl_2$, at 40 °C it was found that acetification was still lower than at 30 °C. Zeng et al.

(2010) reported that Ca^+ improves the stability of cell membrane by balancing the ions through the transfer of material for maintaining the membrane.

Comparison of the effect of $CaCl_2$ on cell wall thickness in cells of *A. pasteurianus* UMCC 2951 under high acid-high temperature acetification was investigated by observing TEM image. The different sizes and layers of dense cell walls are shown in Fig 5.2

Added 0.15% $CaCl_2$ with treatments of 40 °C (HTA-C); TC8, TC9 and TC10 (Fig 5.2g, h and i) resulted in higher density in the cell walls of 40 °C with no $CaCl_2$ for all the TC levels (Fig 5.2d, e and f). Also, when the total concentration of acetic acid was increased from TC8 to TC9 and TC10 the cell wall thickness decreased for all of TC levels compared to fermentation at 30 °C, 40 °C and HTA-C. The cell wall thickness of *A. pasteurianus* UMCC 2951 at acetification at 40 °C with 0.15% $CaCl_2$ addition (Fig 5.2g, h and i) resulted in a more compact layer in cell walls than sample without $CaCl_2$ (Fig 5.2a, b, c, d, e and f).

Also, when acidity was increased to TC9 (Fig 5.2b, e and h) and TC10 (Fig 5.2c, f and i), cell wall thickness was less than at TC8 (Fig 5.2a, d and g). It had previously been reported that the combination of high acid-high temperature acetification created the strong negative effect on cells by changing their morphology after exposure to high acid at 40 °C (Trček et al., 2007; Krusong et al., 2014; 2015a; Zheng et al., 2017).

The similar cell wall morphology change was observed in *A. aceti* WK grown at higher temperature with $CaCl_2$ (TC8 condition) where the cell wall becomes more dense and thinner (Krusong et al., 2015b).

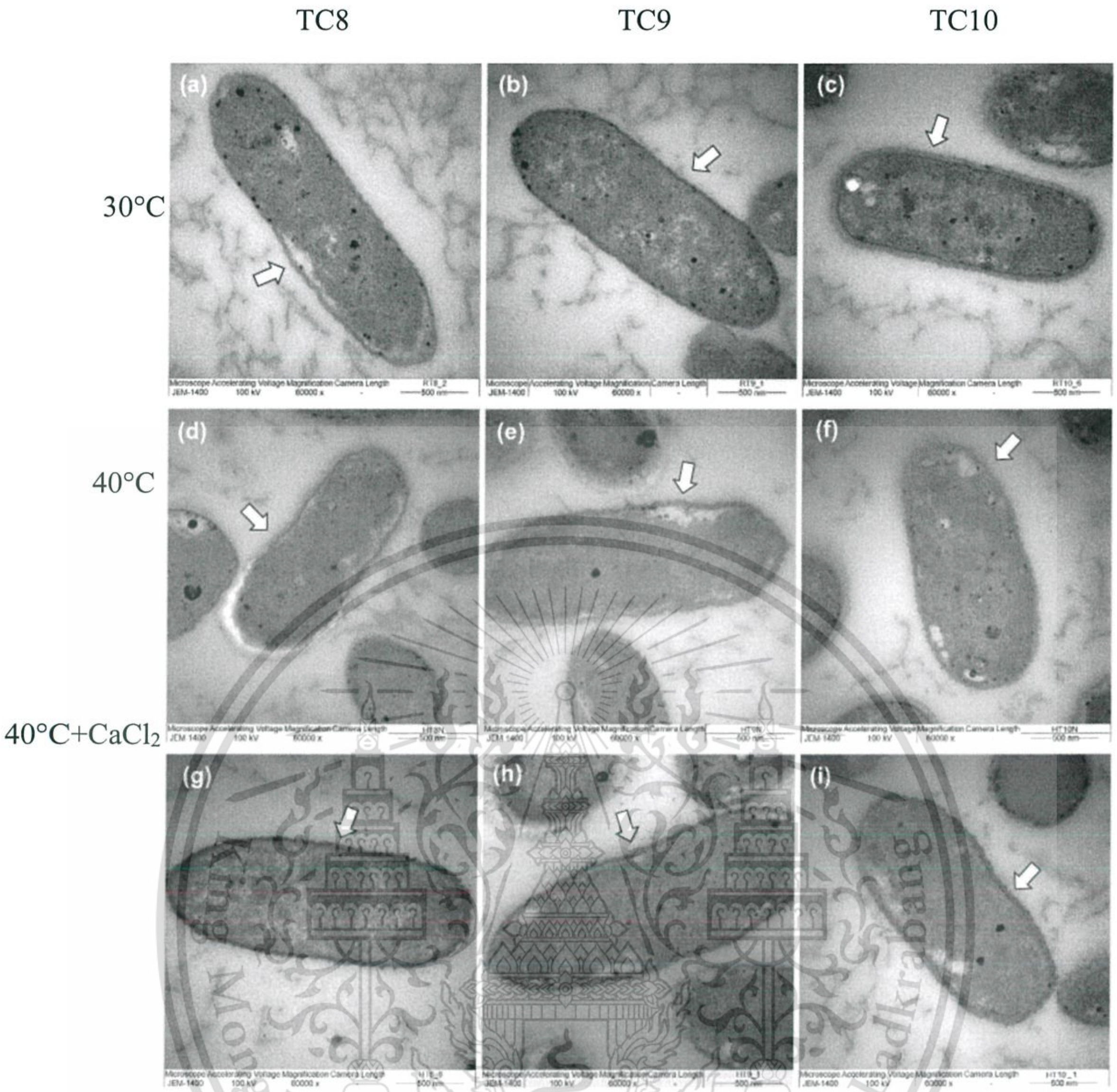

Figure 5.2 TEM image (size 500 nm bar length) of *Acetobacter pasteurianus* UMCC 2591 under high temperature acetification (HTA) at 40 °C compared with acetification at 30 °C. The condition of: acetification at 30 °C with no $CaCl_2$ addition: (a) TC8, (b) TC9, and (c) TC10; acetification at 40 °C with no 0.15% $CaCl_2$ addition: (d) TC8, (e) TC9, and (f) TC10; acetification at 40 °C with 0.15% $CaCl_2$ addition: (g) TC8, (h) TC9, and (i) TC10.

5.4.2 Effect of high temperature on gene thermo-tolerant strain of *Acetobacter pasteurianus* HT-adapted strain and compared with wild-type strain and RT-adapted strain

5.4.2.1 History of strains adaptation to high temperature acetification at 40 °C

To adaptation thermotolerant strain, *A. pasteurianus* UMCC 2591 (adapted HT-strain) and *A. pasteurianus* UMCC 2592 (adapted RT-strain) were adapted from *A. pasteurianus* UMCC 2593 (wild type strain; RTN) under high temperature acetification process (40 °C) and compared with commercial temperature acetification (30 °C) (Fig. 5.1). The acetification period was for 120 days in terms of repeating the cultivation under each condition fermentation. As a result, a thermotolerant *A. pasteurianus* UMCC 2591 (adapted HT-strain) with or without 0.15% $CaCl_2$ addition was conducted by repeated 17 cycles and 15 cycles, respectively. While comparison with *A. pasteurianus* UMCC 2592 (adapted RT-strain), the acetification performance was lower than that fermentation at 30 °C by repeated 34 cycle. And when focusing on acetic acid production (AAP) of each condition, *A. pasteurianus* UMCC 2591 (adapted HT-strain) with 0.15% $CaCl_2$ addition and *A. pasteurianus* UMCC 2592 (adapted RT-strain) were not significant different (7.25 ± 0.31 g/L and 7.31 ± 0.23 g/L, respectively) except for *A. pasteurianus* UMCC 2591 (adapted HT-strain) without 0.15% w/v $CaCl_2$ addition (3.70 ± 0.28 g/L).

5.4.2.2 Phylogenetic tree analysis

In order to identify HT, RT and RTN strains, the resulted phylogenetic tree (Fig. 5.3) was characterized by 16S rRNA gene sequence. HT, RT and RTN strains were localized in *A. pasteurianus,* with highest identical similarity (99 %). All the strains belonging to *A. pasteurianus* species and subspecies. The most near strain to HT, RT and RTN was *A. pasteurianus subsp. Pasteurianus* (gi:NR_118169). All the strains included in the *A. pasteurianus* clade, were isolated from different vinegar fermentation process (Xia et al., 2020).

The analysis of HT, RT and RTN represent a starting point in order to study the thermo-tolerance biological mechanisms in *Acetobacter* species. Actually a wide amount of data are available in literature regarding the thermo-tolerance mechanisms from genetic point of view. (Azuma et al., 2009; Murata et al., 2011; Matsushita et al., 2016). Thanks to the new development of sequencing platform, sequencing technology and bioinformatics algorithms, it is possible to compare a large amount of sequencing data. So, in the next future, a genome-wide association analysis (GWAS) will be performed comparing a dataset of *Acetobacter* strains, known to be able to grow at high temperature.

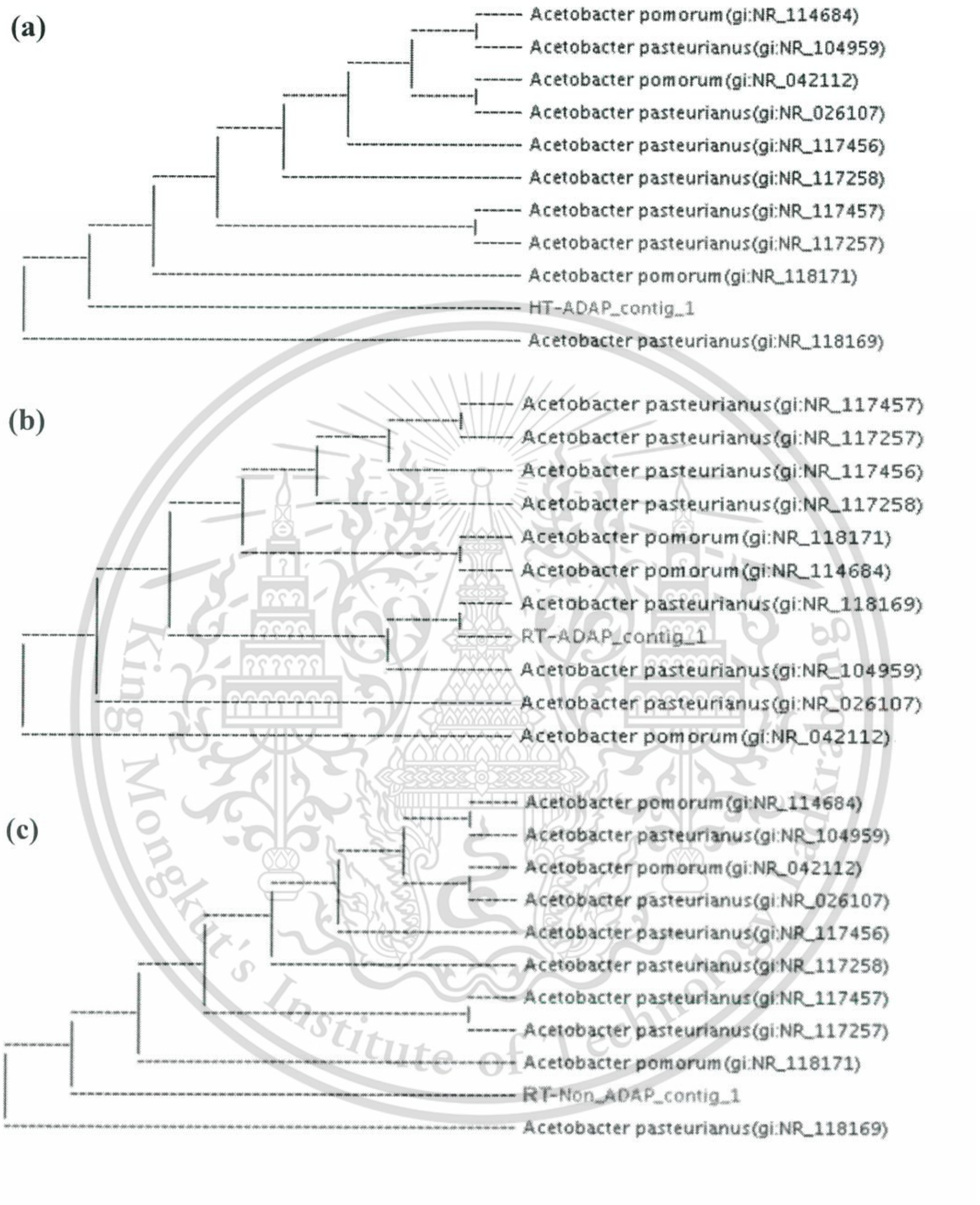

Figure 5.3 Phylogenetic tree obtained by 16s rRNA analysis: (a); HT-adapted strain; (b); RT-adapted strain; RTN-wild type strain.

5.5 Conclusion

In industrial vinegar production efficiency, the ability of AAB strains to produce suitable amount of acetic acid at temperature above the optimal value is a challenge. This study investigated the effect of $CaCl_2$ and long cultivated adaptation of *A. pasteurianus* UMCC 2951 under high initial acetic acid content and high temperature acetification. Results showed that long adaptation cultivation cycles are necessary to provide stable acetic acid productions. $CaCl_2$ addition (0.15%) improved fermentations and its effects were correlated to the content of phospholipids, fatty acids and to the activities of ADH and ALDH. Moreover, since one of the major lack in controlled acetification processes is the shift from microscale to large scale when using microbial starter, the exact implementation and handling of microbial culture is a prerequisite for large scale use of AAB starters, as confirmed by this study in which the microbial starter was tested at small and at prototype scale.

For gene adaptation after adapted strain under high temperature. In this, three strain belonging *A. pasteurianus* species were analyzed. The comparative analysis 16s rRNA sequencing, showed that no substantial differences occurs in the genes set for all the three strain. However, all three strains may differences in terms of physical and morphology properties in cell-adapted strain. Future analysis of gene mutation will be addressed in order to understand the biological mechanisms of the adaptation at high temperature. Based on the literature, it is well documented that in the adaptation mechanisms, mutations occur in targeted gene sequences, with completely different biological function. In the next step, a GWAS analysis could be used in order to associate phenotypic traits to gene presence/absence. A study on targeted genes will be used in order to understand if mutation occur.

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CHAPTER 6

Vinegar processing methods on volatile organic components and their effects on controlling *Aspergillus flavus* in dried chili peppers

6.1 Abstract

Determination of antifungal volatile organic components (VOCs) produced during acetification under high temperature (40°C)-high acid conditions of upland rice vinegar (URV-HT) was conducted using GC-MS. Six major VOCs were found; acetic acid (AA), ethyl acetate, isoamyl acetate, isoamyl alcohol, phenethyl acetate and phenethyl alcohol with AA (50.48 ± 2.12 mg/L) and ethyl acetate (31.43 ± 1.01 mg/L) at the higher levels, but these levels were lower than those found during acetification at 30°C (URV-RT). Subsequently, URV-HT and other major components were tested, *in vitro,* for their inhibition on mycelial growth and conidia germination (10^6 conidia/mL) of *Aspergillus flavus*. Exposure to URV-HT (5% v/v AA) for 15 min (0.27 ± 0.01 mmol/L AA) effectively inhibited mycelial growth, but up to 90 min (1.98 ± 0.02 mmol/L AA) was needed for complete inhibition of conidia germination. Simultaneously, complete inhibition of mycelial growth by exposure to isoamyl alcohol (5% v/v) for 10 min (0.37 ± 0.02 mmol/L) was determined but 15 min (0.51 ± 0.01 mmol/L) exposure was required in order to have a strong impact on conidia germination. Isoamyl alcohol vapor was then tested on conidia germination and aflatoxin production of *Asp. flavus* on dried chili peppers, and complete inhibition was achieved after exposure for 40 min (1.21 ± 0.03 mmol/L) and no *Asp. flavus* was found during 90 days subsequent storage. Our results showed that UVR-HT and some of its VOCs were effective as antifungal agents, which are suitable for safe application in foods and feeds to control some microorganisms.

Keywords: Upland rice vinegar, high temperature acetification, vapor-phase, isoamyl alcohol, *Aspergillus flavus*, dried chili pepper

6.2 Introduction

Food and animal feed contamination with *Aspergillus* species, particularly *Asp. flavus,* results in economic losses and health hazards that can be lethal. *Asp. flavus* can infect crops both pre-harvest and post-harvest, including almonds, wheat, rice, peanuts, maize, soybeans, sunflower seeds, cotton, chilies, coconuts as well as milk (Kamika and Takoy, 2011; Pornpukdeewattana et al., 2017). Infections can produce aflatoxigenic substances that are carcinogenic, mutagenic, teratogenic and immunosuppressive, which can have major harmful effects on both humans and animals worldwide (Wild and Montesano, 2009; Baquiao et al., 2016). Aflatoxin B1 has been shown to be produced at low spore inoculum and is a proven human carcinogen that is classified as a Group I carcinogen to humans by the International Agency for Research (Pelaez et al., 2012).

Dried chili peppers (*Capsicum annuum* L.) are a common ingredient of food that is used to enhance taste, flavor and aroma, 80% of which is produced in warm regions with half of the supply, during the past decade, grown in Thailand, India and China (FAOSTAT, 2017). Dried chilies can be contaminated with *Aspergillus* species depending on biological factors and on storage environmental, especially when large volumes are stored, where it may be difficult to control temperature and humidity to the recognized standard, resulting in high levels of aflatoxin. Aflatoxin contamination was reported to be the most important problem of dried chili peppers in warm regions (Santos et al., 2011; Gong et al., 2019). The European Union regulates the limit of total aflatoxins at 10 ppm and aflatoxin B1 at 5 ppm in spices (European Spice Association, 2004). Every year the dried chili peppers shipments from several nations to the EU are rejected because of aflatoxin contamination (RASFF, 2015). For example, Singh and Cotty (2017) reported that *Asp. flavus* contamination on dried chili peppers was detected on 100% sample from Nigeria (55 sample of total: mean 5.68×10^4 CFU/g) and 40% from USA (169 sample of total: 1.87×10^3 CFU/g).

Application of volatile organic components (VOCs) has been used to control infections of crops for many years. VOCs containing acetic acid (AA), alcohol, aldehydes, acetates and esters were shown to have antibacterial having antifungal properties (Krusong et al., 2015b; Pornpukdeewattana et al., 2017). Vinegar is a source of VOCs, whose type and level of VOCs depend on many factors including microorganisms used (yeast usually strain of *Saccharomyces cerevisiae* and acetic acid bacteria, especially *Acetobacter pasteurianus* and *A. aceti*), type of raw material (fruits and vegetables such as rice, grapes, pineapples) and the acetification process (static fermentation, generator process or submerged fermentation in specifically designed fermentor) (Krusong et al., 2010; Morales et al., 2002). A semi-continuous process is usually used in the industrial production of vinegar, which can produce high AA content (De Ory et al., 2002; Qi et al., 2013; Krusong, et al., 2015a). Considerable interest has been shown in the development of the acetification in order to decrease energy consumption by processing at lower temperatures using selected and well-adapted strains of microorganisms (Matsutani et al., 2013 and Krusong et al., 2015a). Tran et al. (2018) showed that fermentation at high temperature can change some chemical and physical properties of vinegar including color and aroma, but the effects of temperature on VOCs in vinegar is unclear.

Bjornsdottir et al. (2006) and Krusong et al. (2015b) described the properties of VOCs as natural low molecular weight substances with high vapor pressure and low polarity that provide high activity to inhibit fungi and bacteria. Several investigators have reported that many species of fungi and bacteria on fruits and animal feeds, including maize seeds, tomatoes, grapes, sweet cherries and strawberries, can be protected by treatment with vinegar in either the liquid or vapor phases (Sholberg et al., 2000; Tzortzakis, 2010; Pornpukdeewattana et al., 2017) or with specific VOCs (Ando et al., 2012; Siri-udom et al., 2017).

This study investigated antifungal properties of volatile organic components produced during the high temperature/high acetification processing of upland rice vinegar and the inhibitory impact of its main components on mycelial growth, conidia germination and aflatoxin production of *Asp. flavus* on dried chili peppers.

6.3 Material and Methods

6.3.1 Materials

50 samples of dried chili peppers were purchased from local market in the Ladkrabang area of Bangkok in Thailand. Vinegar of Upland rice that had been produced by fermentation at either 40 ± 1 °C (URV-HT) or 30 ± 1 °C (URV-RT) at the Fermentation Technology Laboratory, Faculty of Food Industry, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. Each sample had an AA content of 5 ± 0.1% (v/v). Pure acetic acid (PAA) was purchased from Merck KGaA (Darmstadt, Germany). The volatile components of the URV were: acetic acid ethyl ester, 1- butanol,3- methyl- acetate, isoamylalcohol, phenylethyl acetate and benzeneethanol. The analytical grade of each of these chemicals was purchased from Sigma-Aldrich, Thailand. The culture medium used for

conidia and viability assessment was Potato Dextrose Agar (PDA) purchased from Merck, Germany. A vapor exposure plastic box (0.25x0.30x0.25 m) with a slide cover for protecting the increase of pressure was used to apply the vapor treatments as described by Krusong et al. (2015b).

6.3.2 Sample preparation and Microorganism

After determining initial *Asp. flavus* contamination (Pitt and Hocking, 1985), dried chili peppers samples were further dried in a hot air oven at 40°C to below 13% moisture content, cooled in a desiccator, sealed in plastic bags and stored at room temperature (about 30 °C). A stock culture of *Asp. flavus* AIKL 4024, screened previously from contaminated maize that had been kept on PDA slants containing 50% glycerol at 3-5 °C (Pornpukdeewattana et al., 2017), was provided from the microbial culture collection of Faculty of Food Industry, KMITL, Thailand. To prepare conidia inoculum, *Asp. flavus* was cultured on the slants for 7 days at 30 ± 2 °C and then dispersed by sterile distilled water plus 0.1% (v/v) Tween 80. Conidia concentrations in the suspensions were counted using a haemacytometer and diluted to achieve a final concentration of 10^6 conidia/mL.

To prepare the inoculated dried chili peppers by *Asp. flavus* conidia for studying the inhibition of vapor phase VOCs of URV, dried chili pepper samples were sterilized with ultraviolet radiation (UV) for periods of 0, 5, 10, 15 or 20 min. From which the most suitable UV treatment was selected for further use after no microbial contaminants, including *Asp. flavus,* were observed.

6.3.3 Analytical method

The determination of VOCs of URV-HT and URV-RT was conducted using GC-MS. The solid phase micro-extraction (SPME) of samples were conducted using the modified procedure of Vas and Rekey (2004) where 5 mL of each URV-HT or URV-RT sample were placed in a 25 mL glass bottle with 20 mL headspace volume. Before sealing with a septum cap (Stableflex PDMS/DVB for SPME fiber size 60 mm; Supelco Inc., Bellefonte, PA, USA), 3 g NaCl was added and extracted for 30 min at 37 °C. The components were analyzed using GC-MS (Thermo Scientific Trace GC Ultra coupled to an ISQ Single Quadrupole Mass Spectrometer, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) with a Stabiwax column (30m length, 0.25 mm film, and 0.25 mm ID). The 250 °C inlet temperature and 75 mL/min splitless injection were controlled. 1.2 mL/min flow rate of helium gas was used as carrier. After introducing prepared sample, the temperature was maintained for 5 min at 40 °C. Then, increments of temperature at 5 °C/min up to 230 °C was carried out, and held constant for 5 min. An electron ionization mode for MS determination was operated with a 230 °C ion source temperature, 35e300 amu scan mass range, and at 240 °C MS transfer line with 0 min solvent delay time. The identification of main VOCs conducted, depended on retention times, mass spectra fragmentation patterns and compared with the Wiley, 275. L data library for the GC-MS system. The quantitative data of an identified components was achieved by measuring the relative peak areas compared to that of the internal standard (ISTD 2-pentanol, 4-methyl).

For ELISA aflatoxin analysis, the samples were conducted using the Verotex® aflatoxin detection kit from Neogen Bio-Scientific Technology (Shanghai) Co., Ltd., China following the modified procedure of Sidhu et al. (2009). Briefly, 5 g of each ground dried chili peppers were mixed with 25 ml of methanol (70%) for 3 min using an homogenizer and the supernatant was filtered using a Whatman No.1 filter paper. 100 mL of each filtrate was diluted with 600 mL dilution buffer and then, 50 mL of the diluted sample was transferred through an immunoaffinity column. After removal of the aflatoxin fraction by 0.5 mL methanol, the total aflatoxin content was determined using the ELISA method. The reaction mixture was measured at 650 nm absorbance using a Stat Fax Reader Model 321.

6.3.4 *In vitro* susceptibility of vapor- phase URV-HT, major volatile organic components and pure acetic acid on *Aspergillus flavus*

In vitro susceptibility of *Asp. flavus* to the vapor-phase of URV-HT, pure acetic acid (PAA) or major VOCs in URV-HT (consisting of ethyl acetate, isoamyl acetate, isoamyl alcohol, phenethyl acetate and phenethyl alcohol) was conducted in a vapor exposure box. 5% (v/v) concentration liquid solution of each compound was prepared and used in the treatment. The high conidia content of *Asp. flavus* (10^6 conidia/mL) were inoculated onto PDA using the spread plate technique. Mycelial growth of *Asp. flavus* was further tested using the point inoculation method on PDA with tartaric acid 10% w/v (modified method of Miedaner et al., 2003). During the vaporized treatment, the plates, without covering, were placed on a sterile stainless steel rack in the box. The evaporated vapor phase was supplied by pumping the ambient cleaned air, at 4 L/min flow rate, into 500 mL of 5% liquid URV-HT or PAA or main VOCs in URV-HT, in a 1,000 mL sterile closed bottle. The delivery of each treatment from the headspace of the bottle waspumped into the box at the same rate of 4 L/min (Krusong et al., 2015b). Seven treated periods were tested: 0, 15, 30, 45, 60, 75 and 90 min. After exposure, all plates were covered and incubated at 30 ± 2 °C for 2 days for conidia germination and 5 days for mycelia growth and the percentage reduction in survival of conidia and mycelia growth were recorded. The diameter of mycelia growth was measured using Vernier calipers and the proportional reduction of mycelial growth and reduction of conidia were expressed in relation to the exposure of vapor-phase URV-HT, PAA and major VOCs of URV-HT. Three replicates were used for each treatment.

6.3.5 Inhibitory effect of vapor- phase selected volatile organic components on *Aspergillus flavus* in dried chili peppers

According to the results on the major VOCs in URV-HT, the selected VOCs which provided the highest inhibitory effect on *Asp. flavus* was used for further inhibitory susceptibility on *Asp. flavus*. Each 25 g of prepared dried chili peppers were dipped in 10 mL high conidia suspension of *Asp. flavus* (10^6 conidia/mL) in a sterile plastic bag, mixed gently for 2 min and then dried in a laminar flow for 30 min (modified from Krusong et al., 2015b). The moisture content of each sample was determined after inoculation. Seven treated durations were examined: 0, 10, 20, 30, 40, 50 and 60 min. After exposure, all samples were immediately examined for survival evidence of the conidia of *Asp. flavus* on PDA medium after incubation at 30 ± 2 °C for 7 days. The reduction percentage of survival conidia were recorded using three replications. Meanwhile, total aflatoxin in samples of dried chili peppers were detected by ELISA analysis as mentioned in the analytical method with three replicates per batch.

6.3.6 Evaluation the inhibitory effect of the selected major VOCs of URV-HT on *Aspergillus flavus* conidia and total aflatoxin in dried chili peppers during storage at room temperature

The evaluation the effect of the selected major VOCs on *Asp. flavus* conidia and total aflatoxin in dried chili peppers during storage were determined. The optimum vapor-phase exposure time was used that had been obtained from the previous steps described above. After exposure, all samples were incubated at room temperature (about 30°C), and the survival evidence of *Asp. flavus* conidia and total aflatoxin content was determined after 0, 15, 30, 45, 60, 75 and 90 days. The reduction percentage of survival conidia and the content of total aflatoxin were recorded from three replications.

6.3.7 Statistical analyses

Data from three replicates was analyzed using one-way analysis of variance (ANOVA). According to the significant differences at $p \leq 0.05$, the mean comparison were determined using a Tukey's test. Statistical analysis was performed using the SPSS program version 17.0 for windows.

6.4 Results and discussion

6.4.1 Effect of high temperature on major volatile organic components (VOCs) in upland rice vinegar on acetification at 40 °C and 30 °C.

The quantification of the main volatile organic components (VOCs) in upland rice vinegar on its acetification at 40 °C (URV-HT) and at 30 °C (URV-RT) were determined using GC-MS (Table 6.1). Six major VOCs were found, acetic acid ethyl ester (ethyl acetate), 1-butanol, 3-methyl-acetate (isoamyl acetate), isoamyl alcohol, AA, phenylethyl acetate (phenethyl acetate) and benzene ethanol (phenethyl alcohol) from both URV-HT and URV-RT (Table 6.2). The high concentration of VOCs in both URV were AA (HT; 50.48 ± 2.12 mg/L, RT; 56.32 ± 2.18 mg/L), ethyl acetate (HT; 31.43 ± 1.01 mg/L, RT; 36.90 ± 2.01 mg/L) and phenethyl alcohol (HT; 5.37 ± 0.23 mg/L, RT; 5.45 ± 0.18 mg/L). In addition, the concentration of these three VOCs in URV-HT were significantly ($p \leq 0.05$) lower than that found in URV-RT.

The calibration parameter of the GC-MS analysis showed the matrix-marched calibration curves, obtained by using five concentrations for determining the linearity (Table 6.1), showed that the linearity was effectively within range of tested concentrations, as R^2 was >0.99, which is above the general standard of 0.98 (Zhu et al., 2015). The limits of detection (LOD) and quantification (LOQ) were calculated from the ratio of peak areas to the average noise before and after each peak. Generally, the LODs of most VOCs were < 0.001 ppm except for acetic acid ethyl ester, acetic acid and benzene ethanol, which was assumed to be due to their high concentration compared with the other components (Andujar-Ortiz et al., 2009). Also, the fiber extraction was previously shown to have low efficiency in GC-MS (Guerrero et al., 2007). The relative standard deviations (RSD) of the area ratios of all identified VOCs were calculated from five identical samples of spiked vinegar. The RSD levels were < 10% for all components, which confirmed the acceptable precision of the method. The accuracy of the analytical procedures was estimated by calculation of the recoveries of spiked range sample, which were generally 100% for both addition level (Zhu et al., 2015). Results showed that the recoveries all of components were near 100%, confirming that the method could be used for determination of VOCs concentration in vinegar. The main mechanism for VOCs changes during the acetification process are that the proportion of alcohol groups decreased as the concentration of acetic acid increased until the maximum concentration of iso-type alcohol was less than 0.3% (Furukawaand and Kuramitsu, 2004).

Table 6.1 Calibration parameters of GC-MS method for determination of main volatile organic components in upland rice vinegar on acetification process at 40°C (URV-HT) and 30 °C (URV-RT)

Compounds	Rt	Linear Rang (ppm)	R^{2} [a]	LOD[b] (ppm)	LOQ[c] (ppm)	RSD[d] (mean value, %, n =10)	Spiking level (ppm)	Recovery (mean value, %, n = 6)
Acetic acid, ethyl ester (Ethyl acetate)	2.874	1-100	0.995	0.105	0.348	3.30	25 75	100 98
1-Butanol,3-mehyl-, acetate (Isoamyl acetate)	8.543	0.01-1	0.992	0.001	0.004	8.89	0.2 0.8	100 101
Isoamyl alcohol	11.472	0.01-1	0.999	0.001	0.003	2.27	0.2 0.8	100 97
Acetic acid	18.013	1-100	0.999	0.052	0.172	3.45	25 75	100 98
b-Phenylethyl acetate (Phenethyl acetate)	26.992	0.1-2.5	0.994	0.0002	0.0008	4.26	0.75 2	106 106
Benzene ethanol (Phenethyl alcohol)	29.040	1-100	0.997	0.148	0.493	6.44	25 75	116 108

[a]R^{2}, Squared regression coefficient.

[b]LOD, Limit of detection.

[c]LOQ, Limit of quantification.

[d]RSD, Relative standard deviation.

The major VOCs in upland rice vinegar were acetic acid ethyl ester, isoamyl acetate, isoamyl alcohol, phenethyl acetate, phenethyl alcohol (Table 6.2) as previously reported by Lee et al. (2012) and Pornpukdeewattana et al. (2017). Normally, commercial acetification is conducted at 30 °C and the main impact factors affecting quality and quantitative of vinegar during acetification are oxygen supply as well as temperature (De Ory et al., 2002; Baena-Ruano et al., 2010; Krusong et al., 2015b). In our study, we determined the changes of VOCs in URV during acetification at 40 °C (Table 6.2), which showed that high temperature reduced the concentration of acetic acid ethyl ester and phenethyl alcohol. These results confirm those of Guan et al. (2014) who also showed that high temperature had a negative effect on VOCs in vinegar.

Table 6.2 The quantitation of main volatile organic components in upland rice vinegar on acetification process at 40°C and 30°C using gas chromatography-mass spectrometry.

Component Rt	Compound Name	Cas#	Formula	Concentration (mg/L) *	
				HT	RT
2.874	Acetic acid, -ethyl ester (Ethyl acetate)	141-78-6	$C_4H_8O_2$	$31.43^{a} \pm 1.01$	$36.90^{b} \pm 2.01$
8.543	1-Butanol, 3-mehyl-, acetate (Isoamyl acetate)	123-92-2	$C_7H_{14}O_2$	$0.035^{a} \pm 0.003$	$0.033^{a} \pm 0.002$
11.472	Isoamyl alcohol	123-51-3	$C_5H_{12}O$	$0.05^{a} \pm 0.01$	$0.05^{a} \pm 0.01$
18.013	Acetic acid	64-19-7	$C_2H_4O_2$	$50.48^{a} \pm 2.12$	$56.32^{b} \pm 2.18$
26.992	b-Phenylethyl acetate (Phenethyl acetate)	103-45-7	$C_{10}H_{12}O_2$	$0.54^{a} \pm 0.02$	$0.61^{b} \pm 0.01$
29.040	Benzene ethanol (Phenethyl alcohol)	60-12-8	$C_8H_{10}O$	$5.37^{a} \pm 0.23$	$5.45^{a} \pm 0.18$

* Means ± standard deviation of triplicate data ; means with different letters within the same row are significantly different)*p* (*0.05* ≥based on Tukey's test. Abbreviations are: Rt, retention time; HT, 40°C; RT, 30°C.

6.4.2 In vitro susceptibility of vapor-phase upland rice vinegar (40 °C; URV-HT), pure acetic acid (PAA) and major volatile organic components in upland rice vinegar (VOCs-URV-HT) on *Aspergillus flavus*

There were significant reductions of *Asp. flavus* conidia and mycelial growth after they were exposed to vapor-phased URV and major VOCs in URV, especially when the exposure period was increased (Fig. 6.1). After 15 min of exposure, vapor-phased isoamyl alcohol (0.42 ± 0.02 mmol/L) had the strongest inhibitory effect on both conidia germination and mycelial growth. Also, vapor-phased URV-HT (0.33 ± 0.02 mmol/L AA) and vapor-phased PAA (0.34 ± 0.02 mmol/L AA) after 15 min exposure, inhibited mycelial growth and conidia formation by 10.5 % and 11.1 %, respectively.

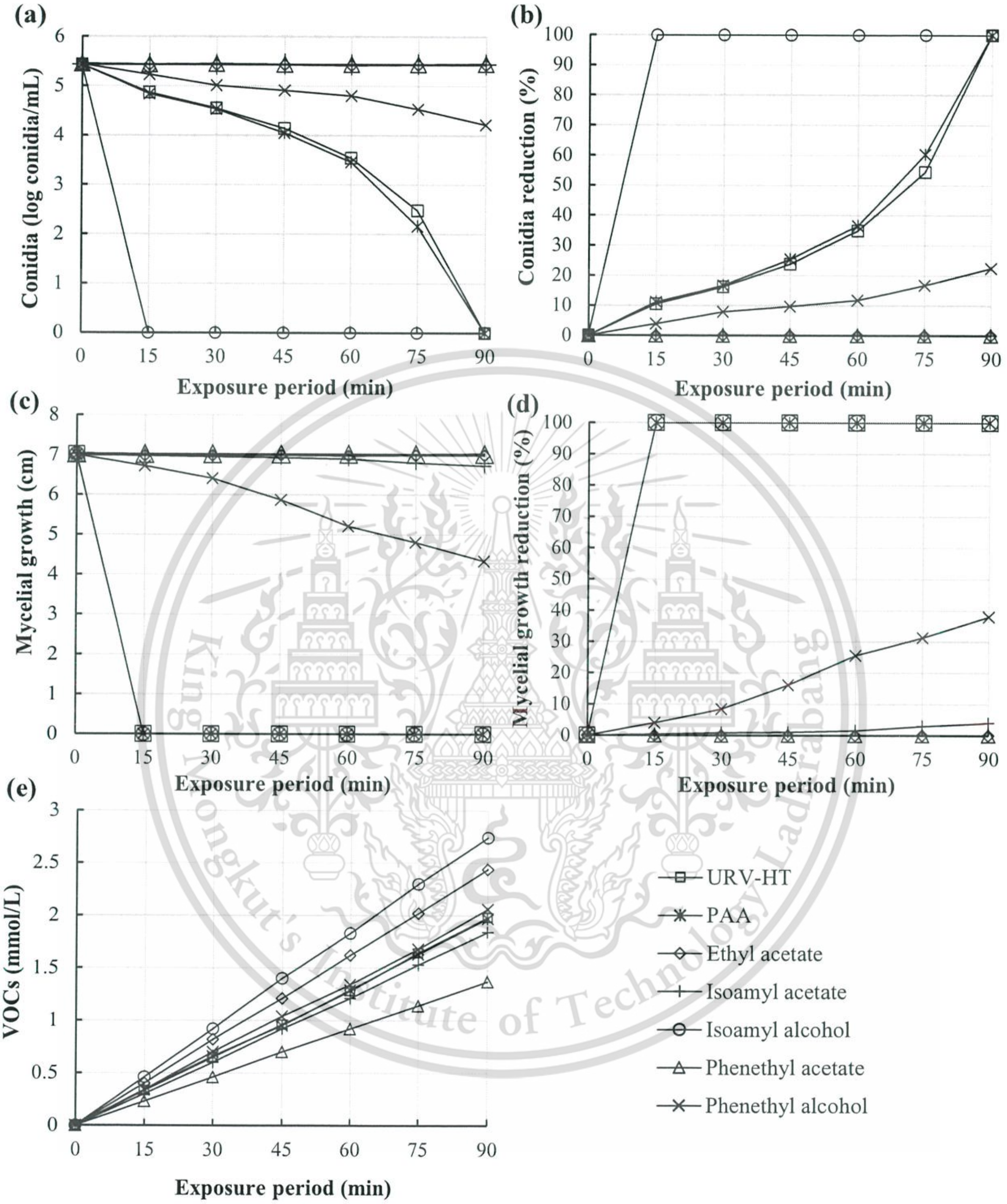

Figure 6.1 Effect of vapor phase main VOCs of upland rice vinegar on *in vitro* inhibition of conidia and mycelial growth of *Aspergillus flavus* cultivated on PDA plate: (a) conidia after exposure; (b) conidia reduction; (c) mycelial growth after exposure; (d) mycelial growth reduction; (e) VOCs contents. Plate were incubated at 30°C for 2 days for conidia germination and 5 days for mycelia growth.

Complete conidial inhibition was obtained after 90 min exposure with vapour-phased URV-HT (1.98 ± 0.02 mmol/L AA) and vapor-phased PAA (1.97 ± 0.01 mmol/L AA). After exposure to the vapor-phase of phenethyl alcohol for 90 min (2.06 ± 0.02 mmol/L) conidia germination and mycelial growth was inhibited by 22.4 % and 38.0 %, respectively. However, exposure by 5 % (v/v) of other VOCs showed only slight effects on conidia germination and mycelial growth of *Asp. flavus.*

In vitro comparative effects of vapor-phased URV-HT, PAA and isoamyl alcohol on mycelial growth of *Asp. flavus* after incubating at 30 °C for 5 days were studied. All treatments showed a strongly inhibitory effect on mycelial growth of *Asp. flavus* on PDA plates (Fig. 6.2). 10 min exposure to isoamyl alcohol vapor (0.37 ± 0.02 mmol/L) had the strongest effect giving complete inhibition of mycelial growth. In a comparison between vapor-phased URV-HT (0.27 ± 0.01 mmol/L AA) and vapor -phased PAA (0.24 ± 0.03 mmol/L AA) after 10 min exposure, PAA gave a reduction of 8.3 % while with URV-HT the reduction was only 5.0 %. However, when the exposure period was 15 min, both showed the complete inhibition of mycelial growth of *Asp. flavus.*

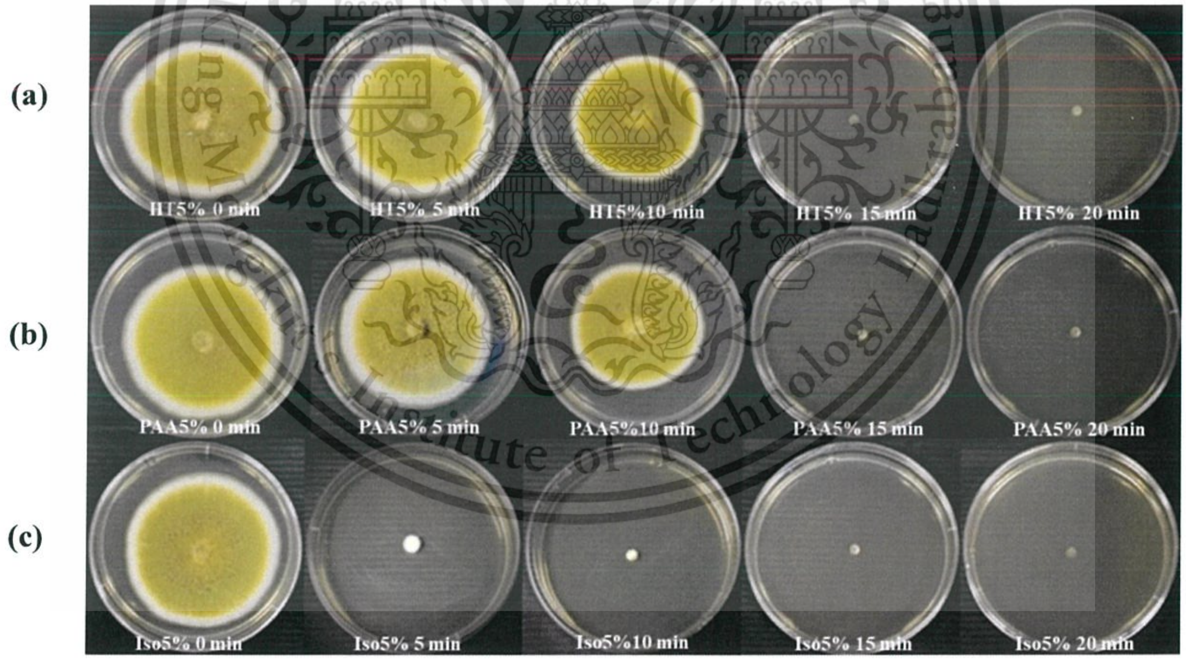

Figure 6.2 *In vitro* comparative effect of vapor-phased upland rice vinegar at 40°C (URV-HT), pure acetic acid (PAA) and isoamyl alcohol on mycelial growth of *Aspergillus flavus* after incubating at 30 °C for 5 days: (a) HT, 5% acetic acid; (b) PAA, 5% acetic acid; (c) Isoamyl alcohol, 5%.

Many investigations have been reported that VOCs have been used as the antifungal and antibacterial agents to improve food microbiology safety (Mercier and Jiménez, 2004; Xu et al., 2013; Krusong et al., 2015b). Organic compounds, especially weak acids or short-chain (low molecular weight) acids in an undissociated form, can easily diffuse through microbial cells resulting in their death (Buchanan et al., 2004). Many researchers have reported that vinegar can be applied for postharvest control of microbial contamination on fruits and vegetables (Rhee et al., 2003 and Wu, 2015) and Krusong et al. (2015b) showed that exposing the bacterium *Klebsiella pneumoniae* (high inoculum 4 log CFU/mL)to the vapor-phase of vinegar containing 8 % v/v of AA for 50 min gave complete control on fresh coriander. Also, exposure for 5 h to the vapor-phase of vinegar containing 8 % v/v of AA can inhibit mycelial growth and aflatoxin formation of *Asp. flavus* in maize (Pornpukdeewattana et al., 2017). In this study, there was a significant reduction of mycelial growth and conidia germination of *Asp. flavus* after exposure to vapor-phased URV-HT (5% v/v AA), PAA (5 % v/v AA) or isoamyl alcohol (5% v/v) (Fig. 6.1). Also, when focusing on vapor-phased URV-HT and PAA for the same exposure period, the vapor-phase of PAA of was slightly more effective than the vapor-phase of HT-URV from the lower concentration of AA in URV-HT compared to PAA. However, it was previously shown that other VOCs in URV-HT resulted in an increase in the antifungal properties of URV-HT (Al-Dalali et al., 2019).

6.4.3 Inhibitory effect of vapor-phased selected volatile organic compounds on *Aspergillus flavus* in dried chili peppers

Of the 50 samples dried chili peppers tested, there were *Asp. flavus* contamination on 21. Therefore, to ensure no *Asp. flavus* or other microorganism contamination on dried chili peppers before use, all the samples were treated with UV radiation. Results showed that the average amount of *Asp. flavus* contamination was 3.74 ± 0.02 log conidia/g, but after exposure to UV radiation for 15 min there was complete inhibition of *Asp. flavus* (Table 6.3) and these samples were used in further studies.

This confirmed that the sample used in the experiments had no contamination with *Asp. flavus* before testing with vapor-phased VOCs and that URV was well prepared. This effect of UV radiation confirms that UV is a suitable non-thermal processing method for microorganism control, which may be related to UV radiation strongly effecting breaking DNA transcription and replication and eventually causing cell death (Gabriel, 2012).

Table 6.3 The effect of UV radiation on *Aspergillus flavus* contamination in dried chili pepper samples

Time (min)	Survival** (log conidia/g)	Reduction (%)
0*	$3.74^{a} \pm 0.02$	0
5	$1.71^{b} \pm 0.01$	54.3
10	$0.69^{c} \pm 0.01$	81.6
15	ND^{d}	100
20	ND^{d}	100

* Means of contaminated conidia of *Asp. flavus* on 21 samples (of 50 total samples) of dried chili peppers.

** Mean ± standard deviation of triplicate data, means with different letters within the same column are significantly different)p (0.05≥based on Tukey's test.

Due to the strong effect of vapor-phased isoamyl alcohol as an antifungal agent on *Asp. flavus*, its use to inhibition *Asp. flavus* conidia in dried chili peppers were investigated by exposing dried chili peppers for up to 60 min with high *Asp. flavus* conidia (5.01 ± 0.02 log conidia/g) (Table 6.4). This treatment resulted in complete elimination of conidia germination after exposure to vapor-phased isoamyl alcohol for 40 min (1.21 ± 0.03 mmol/L) and subsequent incubation for 7 days. Also, the effect of vapor-phased isoamyl alcohol on total aflatoxin showed a reduction from 123.21 ± 0. 34 ppb to 20.71 ± 0. 52 ppb after 30 min exposure and no aflatoxin was detected after 40 min exposure.

Thus, the URV-HT can be recommended for application as a bio-control agent for controlling quality and safety of food produces (Alawlaqi and Alharbi Asmaa, 2014 and Sholberg et al., 2000). As the results show (Fig. 6.1), the vapor-phased isoamyl alcohol, one of the major VOCs of URV-HT, was strongest affected on mycelial growth (5 min exposure; Fig. 6.2) and conidia germination (15 min exposure; Fig. 6.3) of *Asp. flavus*. On the other hands, vapor-phased phenethyl alcohol for the same exposure period inhibited conidia germination and mycelial growth, but at concentrations lower than isoamyl alcohol, PAA and URV-HT. Furthermore, other VOCs compounds showed only slightly inhibitory effect on mycelial growth and conidia germination.

Table 6.4 Inhibitory effect of vapor-phased isoamyl alcohol on conidia germination and aflatoxin formation by *Aspergillus flavus* on surface of dried chili peppers. After exposure, the survival conidia and total aflatoxin in sample were determined after incubation at 30 ± 2 °C for 7 days.

Isoamyl alcohol 5% (v/v)		High inoculation conidia of *Asp. flavus* on dried chili peppers		Total Aflatoxin
Exposure period (min)	Content in vapor phase* (mmol/L)	Survival** (log conidia/g)	Reduction (%)	(ppb)
0	0	6.01^{a} ± 0.02	0	123.21 ^{a} ± 0.34
10	0.30 ± 0.01	4.09^{b} ± 0.03	31.9	85.45 ^{b} ± 0.45
20	0.62 ± 0.02	2.18^{c} ± 0.02	63.7	46.90 ^{c} ± 0.33
30	0.91 ± 0.02	1.04^{d} ± 0.02	82.7	20.71 ^{d} ± 0.52
40	1.21 ± 0.03	0 ^{e}	100	ND ^{e}
50	1.53 ± 0.02	0 ^{e}	100	ND ^{e}
60	1.84 ± 0.02	0 ^{e}	100	ND ^{e}

Using 5%(v/v) isoamyl alcohol as source .

*Content of isoamyl alcohol in vapor phase was calculated depended on the average weight loss of the solution used at the rate of 4 L/min during exposure period and molecular weight of isoamyl alcohol (88) in the 37.5L volume of the vapor exposure box (Krusong et al., 2015b).

** Mean ± standard deviation of triplicate data, means with different letters within the same column are significantly different ($p \leq 0.05$) based on Tukey's test.

6.4.4 Evaluation the inhibitory effect of the selected major VOCs of URV-HT on *Aspergillus flavus* conidia and total aflatoxin on dried chili peppers during storage at room temperature

The effect of vapor-phased isoamyl alcohol at the optimal exposure time (40 min) obtaining from the previous study were used for inhibiting *Asp. flavus* conidia and total aflatoxin formation on dried chili peppers during storage at room temperature for up to 90 days. Results showed that after exposure for 40 min and subsequent storage at room temperature (30 °C), the vapor-phased isoamyl alcohol completely inhibited *Asp. flavus* conidia (initial conidia inoculation at 5.02 ± 0.1 log conidia/g) (Fig. 6.3). Also, no aflatoxins was detected on the dried chili peppers during storage.

Results of isoamyl alcohol of URV-HT as a bio-fumigant to inhibit conidia germination of inoculated *Asp. flavus* on dried chili peppers during storage (Table 6.4) showed the complete inhibition of conidia germination and aflatoxin production after treatment for 40 min of vapor-phased isoamyl alcohol after storage for 7 days at 30 °C, and none developed even after 90 days storage (Fig. 6.3).

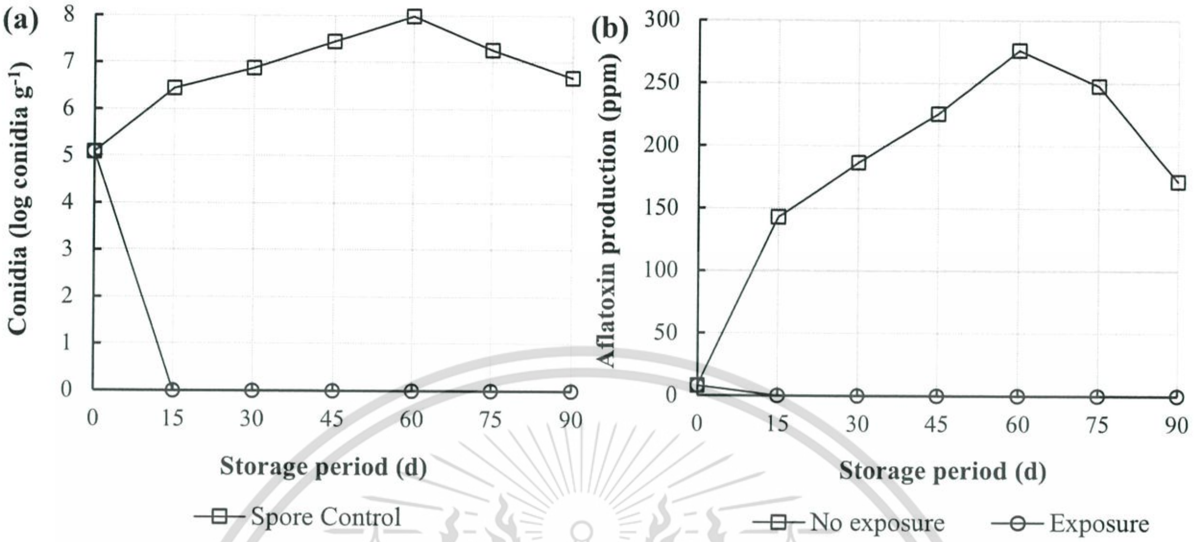

Figure 6.3 Inhibitory effect of vapor-phased isoamyl alcohol (5% v/v) on conidia and aflatoxin production by *Aspergillus flavus* on dried chili peppers after exposure for 40 min and storing at 30 °C for 90 days: (a) conidia survivor; (b) aflatoxin production.

Ethyl acetate, isobutyl acetate, isoamyl acetate, isoamyl alcohol, phenethyl acetate, phenethyl alcohol, 2-pentanone from VOCs have previously been shown to be effective antifungal substances (Ando et al., 2012; Morath, et al., 2012; Gong et al., 2019) and 1-butanol, 3-methyl-acetate (Mitchell et al., 2010). Isoamyl alcohol, isoamyl acetate and phenethyl alcohol have also have been shown to have fungicidal properties and been used to inhibit conidia germination of *Asp. brasiliensis*, *Penicillium crysogenum*, *Botryotinia fuckeliana* and *Fusarium oxyspolum* (Ando et al., 2012). Also, heptanal and amyl alcohol produced by *Trichoderma* spp. have also been identified as antifungal compounds (Humphris et al.,2002).

The efficiency of antifungal property of each main VOCs from HRV-HT was different depending on the group and form of each component. In the liquid form, acetic acid was the most effective VOCs in inhibiting conidia germination of *Asp. flavus*. The alcohol group such as phenethyl alcohol and isoamyl alcohol was less effective supporting confirming the results reported by Ando et al. (2012). But, in the vapor form, isoamyl alcohol at 5 % (v/v) showed the highest inhibition of mycelial growth and conidia germination of *Asp. flavus* on dried chili peppers among main VOCs of URV-HT tested, and could be applied using only a short exposure period as previously reported by Inouye et al. (2001). Ando et al. (2012) also reported similar results on the antifungal activity of isoamyl alcohol, and also found that it had a stronger effect than isoamyl acetate and phenethyl alcohol. Maruzzella et al. (1961) found that the antifungal effects of VOCs were primarily from the organic acids, aldehydes, alcohols, ether, ketones, esters and lactone that they contained, from high to low impact. This could explain why the VOCs of ethyl ester, isoamyl acetate and phenethyl acetate were not effective in inhibition of conidia germination when high conidia levels were inoculated.

The mode of action of VOCs as antifungal agents, have been described as severe damage on dehydrated surfaces, shrinking cell bodies on cell-walls, interference of the membrane function and nutrient transport, inhibition of enzyme activity and overall metabolic (Blackburn and McClure, 2002). Additionally, changing genes expression in aflatoxin biosynthesis pathways has also been shown to reduce transcript levels after exposure to volatile components (Al-Amiery et al., 2012; Gong et al., 2019).

6.5 Conclusions

Volatile organic compounds of URV-HT, especially acetic acid and isoamyl alcohol, can inhibit mycelial growth, conidia germination and aflatoxin production of *Asp. flavus*. Hence, it is concluded that URV-HT can provide a novel bio-fumigant for protecting human food and animal feed from pathogens and mycotoxin production during storage.

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CHAPTER 7

Inhibition of *Pantoea agglomerans* contamination of fresh-cut jackfruit by exposure to weak organic acid vapors

7.1 Abstract

Fresh-cut jackfruit, purchased from markets in urban areas of Bangkok, were examined for bacterial contamination. *Pantoea agglomerans* was the predominant contaminant bacterium occurring in 90% of the 30 samples tested ; many with high loads. Inhibition of surface contamination of *P. agglomerans* by vinegar, lactic acid or citric acid, either as liquid or vapor, were tested *in vitro*. For liquid-phase exposure, vinegar had a higher inhibitory effect than the other two, both of which that gave only slight inhibition. Exposure to vinegar vapor containing 3.36 mmol/L acetic acid for 90 min gave complete inhibition of *P. agglomerans*. In order to test this vaporized vinegar treatment on the quality of jackfruit, non-inoculated samples, with or without vinegar treatment, were stored for 7 days at 5 ± 2 °C and then tested by a sensory evaluation panel. Results showed that in all the attributes tested, color, texture and overall acceptability, vaporized vinegar treated sample were preferred to non-treated samples. It was concluded that exposure of fresh-cut jackfruit to vaporized vinegar is a simple method of controlling surface bacterial contamination without detrimentally affecting their acceptability.

Keywords: Vaporized phase; Acids; *Pantoea agglomerans*; Fresh-cut jackfruit

7.2 Introduction

The market for prepared and packaged ready-to-eat fresh fruit has been progressively increasing over recent years, but it has been shown that they can be carriers of microorganisms (Qadri et al., 2015) that may cause diarrhoea, abdominal cramps, vomiting or even death (Harris et al., 2003). It is a challenge to find suitable methods of ensuring the safety of fresh-cut fruit, without affecting their acceptability.

Jackfruit (*Artocarpus heterophyllus*) is one such fruit that is grown in many Asian countries (Elevitch and Manner, 2006). It has a low calorific value and purgative attributes and contains antioxidants, dietary fiber and is rich in vitamins and minerals (Baliga et al., 2011).

During the preparation of fresh-cut jackfruit, microbial contaminants on the surface of a skin and hands of workers may be transferred onto the fruit. These contaminants may release hydrolytic enzymes that degraded the polymers on the fruit releasing constituents and water for use as nutrients for their growth (Barth et al., 2009). Ragaert et al. (2007) reported that the most frequently identified bacteria in fresh-cut fruit and vegetables were *Pantoea agglomerans* and *Pseudomonas fluorescens*, both of which produce pectinolytic enzymes. An example of this deteriorative effect of pectinolytic enzyme release is bronzing, caused by the pathogens *Pantoea stewartii* subsp. *stewartii* and *P. agglomerans*, which can detrimentally affect the consumer preference (Dutkiewicz et al., 2016; Zulperi et al., 2017; Abidin et al., 2018).

Among the simple, safe methods of controlling bacterial contamination on fresh-cut fruits are weak organic acids including citric, lactic and acetic acids that have been approved by USDA-FSIS (1996). These weak acids are relative safety for human use (Ricke, 2003) and have been extensively used pre-harvest and post-harvest in agriculture as well as in the food and drinks industry for their antimicrobial properties. Dickson and Anderson (1992) reported that reductions in levels of bacterial contamination were directly proportional to acid concentration and temperature and can be more effective if applied in combinations. Vinegar vapor contains acetic acid and has been shown to have antimicrobial properties when applied in various food products including apples, apricots, coriander, lettuce, mangoes, strawberries, tomatoes and maize grains (Sholberg et al., 2000; Meatherall et al., 2009; Tzortzakis, 2010; Krusong et al., 2012; Krusong et al., 2015a; Krusong et al., 2015b; Pornpukdeewattana et al., 2017; Suwapanich et al., 2019).

The present study surveyed bacterial contamination on fresh-cut jackfruit sold in local Thai markets, using *P. agglomerans* and total aerobic bacteria as an examples, and the effects of on fresh-cut jackfruit sold in local Thai markets vinegar (acetic acid), lactic acid and citric acid vapors on control of *P. agglomerans* inoculated into fresh-cut jackfruit and its effects on their sensory qualities.

7.3 Materials and methods

7.3.1 Materials

Thirty samples of fresh-cut jackfruit were purchased randomly from local markets of Bangkok between May to November 2019 for the *P. agglomerans* contamination survey. For the control tests of *P. agglomerans*, firm yellowish flesh fresh-cut jackfruit with an intense sweet taste were selected. Vinegar, made from upland rice wine, was obtained from Laboratory of Fermentation Technology, Faculty of Food Industry, King Mongkut's Institute of Technology Ladkrabang, Bangkok. Analytical grade acetic acid and citric acid were purchased from Merck, Germany.

7.3.2 Detection of total aerobic bacteria and *P. agglomerans* in fresh-cut jackfruit on local Thai markets

Samples of fresh-cut jackfruit were stored at 5 ± 2 °C for not more than 2 h before determination of total aerobic bacteria count and *P. agglomerans* level on their surfaces. Also, the surfaces of fresh-cut fruit were explored for possible habitats for bacteria.

7.3.3 Sample preparation for microbiological tests

Sample of the fresh-cut jackfruit were trimmed, washed in sterile distilled water for 5 min, soaked in per-acetic acid (50 ppm) for 5 min and finally washed again for 5 min with sterile distilled water and dried aseptically for 5 min in a laminar airflow. The samples were aseptically packed in sterile plastic containers and kept at 5 ± 2 °C until use.

For microbiological analysis, fresh-cut jackfruit samples were aseptically ground and homogenized. Then 25 g of ground samples were aseptically mixed with 225 mL sterilized peptone water (1% peptone; saline peptone water) in a sterile stomacher bag (Model BA 7021, Seward, UK), and homogenized in the stomacher bag for 2 min. Serial dilutions of each sample suspension with SPW was prepared for analysis of total aerobic bacteria count using trypticase soy agar (TSA) (Himedia, India) using a spreading technique. The *P. agglomerans* in the samples were then assessed using a modified method of Mardaneh and Dallal (2013) where 10 mL of each suspension sample was placed into 90 mL Enterobacteriaceae enrichment broth, mixed well and incubated for 18-24 h at 37 °C. A loopful of the diluted suspension was spread onto duplicate violet red bile glucose agar (VRBGA) plates (Himedia, India) and again incubated for 18-24 h at 37 °C. A total of 5 typical pink or red-violet colonies, with or without halos, 0.5 mm or more in diameter, were picked up from each VRBGA plate and streaked on the same plating medium to obtain pure cultures. Oxidase-negative and glucose fermenting positive isolates were streaked on TSA for 48 h at 37 °C. In addition, the species of each isolate was identified using a Bio type-100 system (Biomérieux, Marcy-L'Etoile, France) using Biotype medium 1. The turbidity or color change, which follow growth in single carbon source after culturing for either 48 h or 96 h at 30 °C, was observed and scored as a positive test. Then an API 20E strip was used, according to the instructions of the Biomérieux manufacturer. Tests were carried out, at least in duplicate, to investigate the analytical profiles after culture for 24 h and 48 h at 37 °C. The Apiweb identification software, the API 20E database (version 4.1) with the analytical profile index, was used for bacterial identification (Delétoile et al., 2009). Additionally, the identity of each selected isolate was confirmed using nucleotide sequencing by National Centre for Genetic Engineering and Biotechnology, Thailand. Pure cultures of selected *P. agglomerans* isolates were maintained on TSA slants at 5 ± 2 °C until use.

7.3.4 Effect of liquid and vapor forms of vinegar, lactic acid and citric acid on growth of *P. agglomerans* in vitro

Inoculum of selected *P. agglomerans* isolates were prepared by spreading them on TSA plates, leaving them for 24 h at 37 °C and, then, transferring several colonies into TSB. After cultivation of suspended culture, the dilution, with SPW, was prepared to achieve McFarland 1.0 (approximately 7 Log CFU/mL) using a spectrophotometer (UV-1601 Shimadzu, Japan), which was used as the inoculum.

The *in vitro* inhibitory effect of *P. agglomerans* by liquid vinegar, lactic acid or citric acid was conducted using the agar disc diffusion method modified from Chaudhry and Tariq (2006). A 20 μL inoculum was transferred into 5 mL sloppy TSA, then spread on a 10 mL TSA plate and allowed to set at 30 °C for 20 min. The sterilized paper disc was aseptically soaked in 1 mL of each solution (vinegar with 10% v/v acetic acid, 10% v/v lactic acid or 10% w/v citric acid). The soaked paper discs were placed aseptically on an inoculated TSA plate and the inhibition zone was measured after incubation for 24 h at 37 °C.

The inhibitory effect of vinegar vapor, lactic acid vapor or citric acid vapor on *P. agglomerans* was also investigated *in vitro* using a plastic vaporized exposure box, which measured 25 x 30 x 25 cm (Fig. 7.3). The sliding cover and vents was designed to protect the pressure building up during vaporization. The vapor was generated by pumping filtered air at 4 L/min into 500 mL, with each acid solution placed in a one litre glass bottle at ambient temperature (30 ± 2 °C). The vapor from the head space of the bottle was pumped into the box at 4 L/min. The calculation of vaporization rate was based on the rate of weight loss of the liquid in the bottle, which was on average 0.042 ± 0.002 g/min. Additionally, the acid content in each solution was calculated according from the weight loss and the molecular weight of the acid (acetic acid 60.05, lactic acid 90.08 and citric acid 192.12). For testing the inhibitory impact on *P. agglomerans*, 20 μL inoculum was spread on a 10 mL TSA plate and left to solidify at ambient temperature for 20 min. Then, the inoculated plate, with no cover, was placed aseptically on a sterilized perforated stainless-steel rack in the box for exposure of its vapor. Five exposure periods were tested: 0, 30, 60, 90 and 120 min. All sample plates were incubated for 24 h at 37 °C, then assessed for evidence of survival of *P. agglomerans* and the percentage inhibition was measured. The organic acid that provided highest inhibition of *P. agglomerans* was selected for further study.

7.3.5 Impact of vaporized selected acid on fresh-cut jackfruit artificially inoculated with *P. agglomerans*

Fresh-cut jackfruit samples were inoculated with high levels of *P. agglomerans* inoculum (6 Log CFU/mL). A volume of 100 mL inoculum was aseptically poured into each sterile plastic bag, together with jackfruit samples and then drained to waste. The flesh was further aseptically dried for 30 min in a laminar air flow. After inoculation the jackfruit samples were exposed to the vapor of one of the three acids. Five durations of vapor exposure were used at 80 ± 2% RH for 0, 1, 2, 3 or 4 h at 5 ± 2 °C. In order to restrict the moisture loss from samples during treatment, vaporized water was simultaneously pumped across the samples at the same rate as the acid vapor resulting in the maintenance of 80 ± 2% RH, which was measured by a thermo-hygrometer (Model TH-302, Diichi, Japan). After treatment, the inoculated samples were examined for evidence of survival *P. agglomerans* cultured on TSA medium, using the spread plate technique, after incubating for 24 h at 37 °C. Each sample was examined under SEM in order to observe the effect of the vaporized acids on the cells of *P. agglomerans* on the surface of sample, as described below.

7.3.6 Evaluation of the sensory qualities of fresh-cut jackfruit and bacterial contamination after treatment with vinegar, lactic acid or citric acid vapors

Non-inoculated fresh-cut jackfruits were well prepared as mentioned above and treated with the acid vapors either by soaking in 6 mm (diameter) filter paper discs or being placed in a vaporized exposure box for 7 days and then subjected to sensory evaluation. In order to protect the fruit from the smell and taste of the vinegar, jackfruit-flavored vinegar was developed by soaking 200 g fresh-cut jackfruit for 15 days in 800 mL liquid vinegar using the method described by Krusong et al, (2015b). The acidity of jackfruit-flavored vinegar was analyzed before use. After exposure of the jackfruit samples to the vapor, they were stored at 5 ± 2 °C and 80 ± %2 RH for up to 7 days, and examined daily for evidence of decay and for levels of *P. agglomerans* and total aerobic bacteria count. Selected samples were then subjected to sensory evaluation by a taste panel using the following procedure. Twenty members were selected from the Faculty of Food Industry and each panelist received three samples and distilled water to cleanse their palate after testing each sample. They were asked to score each sample for appearance, color, aroma, taste, texture and overall acceptability (Offia-Oluan and Ekwunife, 2015). Each attribute was independently evaluated using a five-point hedonic scale (Gerbi et al., 1997) where 1 = extremely disliked, 2 = disliked, 3 = neutral, 4 = liked, 5 = extremely liked (modified from Kappel et al., 1995).

7.3.7 Scanning electron microscope (SEM)

Samples were prepared by vacuum drying and sputter coated with gold. The photographic images from the SEM were taken using a JEOL JSM-5410LV camera (JEOL, Tokyo).

7.3.8 Statistical analyses

The data from three replicates was analyzed using one-way analysis of variance (ANOVA). A mean comparison test was performed using the Tukey's Test. SPSS Version 17.0 for Windows pocket program was used for these analyses.

7.4 Results and discussion

7.4.1 The detection of total aerobic bacteria and *P. agglomerans* in fresh-cut jackfruit on local Thai markets

Minimally processed fresh-cut fruits, as well as other fresh produce, are naturally contaminated by microorganisms from various sources such as fertilizer preparation, farm circumstances, pre-harvest and post-harvest processes (Beuchat, 1996 and Heard, 1999) and hygienic practices during fresh-cut processing. A diversification of microbiota is commonly found in fresh-cut produce. Nevertheless, most contamination in vegetables is associated with gram-negative bacteria, while yeast and molds are linked with fresh fruits (Burnett and Beuchat, 2000 and Tournas, 2005). On the contrary, our study show that plant pathogenic bacteria were dominant in fresh-cut jackfruit sold in the local Thai market where no practical bacterial protection is applied.

The operations during preparation of fresh-cut fruits or vegetables, consisting of cutting, shredding and slicing, damage cellular structure and tissues, resulting in the leakage of nutrients and cellular fluids (Heard, 2002; Yousuf et al., 2020) which a facilitate microbial attack due to the microbiota transfer from surfaces utensils and hands to the surface of the produce. Meanwhile, the nutrients of fresh-cut produce can support the growth of the microorganisms. The multiplication of bacterial contaminants may significant increase due to the conditions in the market stalls, such as temperature (approximately 30 to 34 °C) and protracted selling periods. High total aerobic bacterial counts, in the range of 5.1 ± 0.15 to 6.9 ± 0.15 Log CFU/g, were found in all the 30 samples of fresh-cut jackfruit analyzed (Fig. 7.1). According to their characteristics on VRBGA culture plates, many of these colonies were Enterobacteriaceae. After subjecting each sample to the Standard Rapid ID 32E Biochemical Reaction Profile Test of Biomérieux, some were identified as *Pantoea agglomerans*, which was confirmed using nucleotide sequencing by National Centre for Genetic Engineering and Biotechnology, Thailand. Results from the 16S rRNA sequence of selected strains showed that the highest similarity (98%) was with *P. agglomerans* (results not shown).

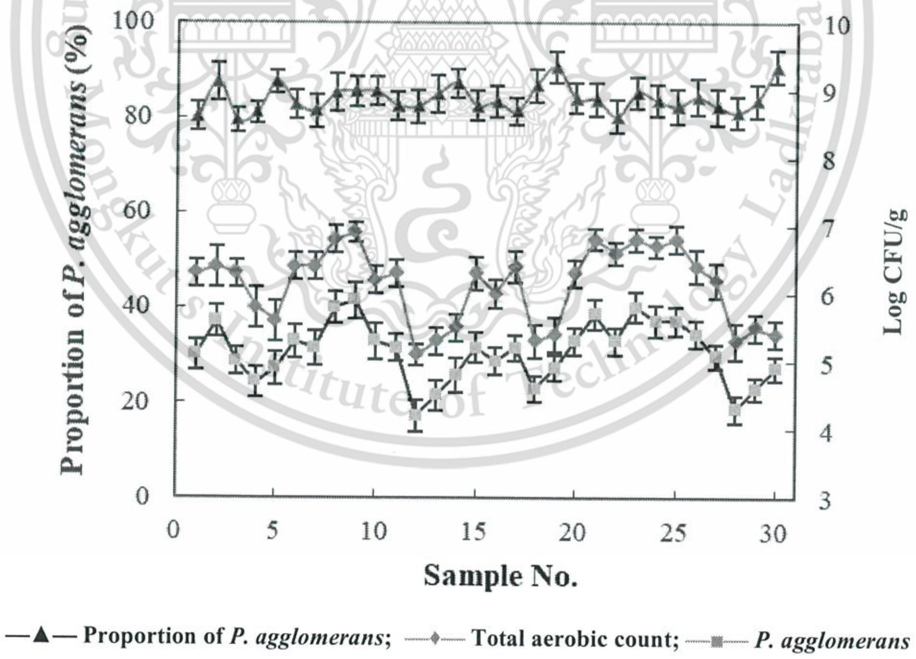

Figure 7.1 Total aerobic count, identified *P. agglomerans* and its proportion determined in 30 samples of fresh-cut jackfruit. Error bars mean ± one standard deviation.

The high levels of *P. agglomerans* were found between 4.2 ± 0.24 and 5.9 ± 0.26 Log CFU/g while the proportion of *P. agglomerans* contamination was in the range of 79.4 - 90.7% (Fig. 7.1). These results imply that there is a high possibility that shortening the marketing period of fresh-cut jackfruit to that which commonly occurs in the local market would result in lower waste and lower economic losses.

P. agglomerans, a non-spore former Gram-negative aerobic bacterium, is commonly found in water, soil and sewage as well as on growing and harvested plant material, which means it can be common on fruit and vegetables (Eickhoff et al., 1966; Liberto et al., 2009; Cruz et al., 2007; Octavia and Lan, 2014). Fortunately, it is not an obligate infectious agent in humans (Dutkiewicz et al., 2016). However, Abidin et al. (2018) reported that *P. agglomerans* can be found on fresh jackfruit in Malaysia where it adversely affects their quality and therefore consumer preference. Our results (Fig 7.1) confirm that bacteria, especially *P. agglomerans* is a predominant contaminant in fresh-cut jackfruit in Thailand.

The SEM analyses (Fig. 7.2) showed that the wave-like ridged surfaces of fresh-cut jackfruit (Fig. 7.2a) were a place for bacteria to accumulate and grow. Additionally, the surfaces were contaminated with bacterial cells before they were exposed to organic acid vapor in the vaporized exposure box (Fig. 7.2b). During the cutting process in the preparation of fresh-cut jackfruit, the microbiota on the surface of the hands may be transferred onto the fruit. These bacteria can produce extracellular lytic enzymes that hydrolyze polymers in the fruit that releases intracellular nutrients and water resulting growth of microbial contaminants (Barth et al., 2009). As a result, the quality of the jackfruit is affected. The SEM images of fresh-cut jackfruit surfaces (Fig. 7.2a-b) show that they have rough and wave-like ridges, which can provide good harbors for bacteria and other microorganisms. This roughness suggests that the flesh of jackfruit needs special treatment rather than just a quick wash under tap water in order to inhibit microbial contaminants. Consequently, fresh-cut jackfruit may become a risk for consumer's health due to the biofilm of microbial contaminants, which can be formed on the flesh surfaces (Morris et al., 1997).

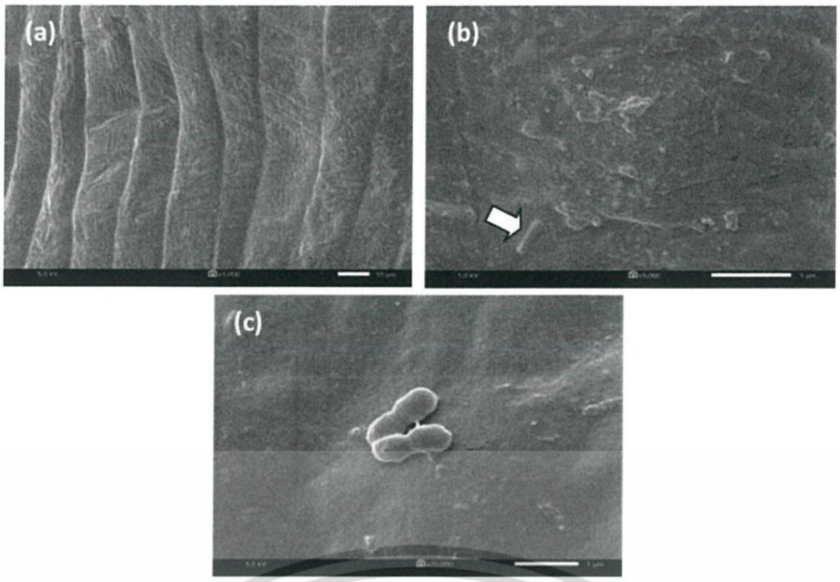

Figure 7.2 Photographed SEM image on flesh surfaces of fresh-cut jackfruit: (a) surface with wave-like ridges (×1,000), (b) bacterial contaminants on flesh surface (×5,000) (arrowed), (c) wrecked cell wall of inoculated *P. agglumerans* on fresh-cut jackfruit after exposure to vaporized vinegar (×20,000).

7.4.2 Effect of liquid and vapor forms of vinegar, lactic acid and citric acid on growth of *P. agglomerans* in vitro

Effective measures to prevent the microbial spoilage of fresh-cut fruit include weak organic acids, especially short-chain weak acids, have been shown to be effective inhibitors (Davidson and Juneja, 1990; Buchanan et al., 2004; Sengun and Karapinar, 2004; Barth et al., 2009). The antimicrobial properties of weak organic acids depends on various factors, including their un-dissociated or protonated form, which can destroy microbial membranes, inhibit of enzymes, pass through microbial membranes into inner cells resulting in cell death (Brul and Croote, 1999; Bjornsdottir et al., 2006), interfere with nutrient transport and impact on their metabolic activity (Blackburn and McClure, 2002). There are also many reports that show that weak organic acids, including of acetic, citric, lactic and propionic acids, have been applied in food products to inhibit pathogens (Nazer et al., 2005; Mani-López et al., 2011). Also, the application of weak organic acids such as acetic, citric and lactic acids, at concentrations up to 2.5%, has been allowed by USDA-FSIS (1996). However, reductions of the bacterial levels on fruit surfaces has been shown to be directly proportional to acid concentration, individual or combinations of acids, and temperature of acid solution (Dickson and Anderson, 1992).

The *in vitro* inhibitory effect of liquid vinegar on *P. agglomerans* using the modified agar disc diffusion method showed that the highest inhibition zone was just visible and was significantly higher inhibition ($p \leq 0.05$) for vinegar than lactic acid or citric acid with distinct evidence of inhibition observed in plates exposed to vinegar (Table 7.1). The *in vitro* inhibitory effect of diffused liquid vinegar (acetic acid), lactic and citric acids using the modified agar disc diffusion method (Table 7.1) showed that vinegar was the most effective with lactic acid next and citric acid the least effective. This result supports previous work on weak organic acids, where acetic acid was more efficient than others tested (Wu et al., 2000 and Rhee et al., 2003). The reason why acetic acid was more effective than lactic and citric acids may be due to the higher incomplete disassociation, since its antimicrobial properties are based on the percentage of un-dissociated molecules, which varies with concentration and with pH. Un-dissociated molecules (less so, dissociated ones) can pass readily through microbial membranes, and thus cause death of the bacterial cells (Casal et al., 1996).

Table 7.1 Inhibition of *P. agglomerans* by URV[†] (10% v/v acetic acid), citric acid (10% w/v) and lactic acid (10% v/v) by the modified agar disc diffusion method on trypticase soy agar.

Organic acid	Formula	MW[‡]	Inhibition zone* (cm)
Vinegar[†] (acetic acid)	$C_2H_4O_2$	60.05	$2.3^{a} \pm 0.12$
Lactic acid	$C_3H_6O_3$	90.08	$1.6^{b} \pm 0.08$
Citric acid	$C_6H_8O_7$	192.12	$1.1^{c} \pm 0.11$
Sterile distilled water	H_2O	18.01	0^{d}

[†]V, vinegar made from upland rice

* Mean ± one standard deviation. Mean comparison using Tukey's test, means with different letters in the same column are significantly different ($p \leq 0.05$).

[‡]MW, Molecular weight

In vitro results for *P. agglomerans* (5.8 ± 0.2 Log CFU/mL, on TSA plates) exposed to the three acid vapors in the 18.75 L in the vaporized exposure box are shown in Fig. 7.3. There were significant reductions ($p \leq 0.05$) of approximate 1 log colony number after a 30 min exposure to vinegar (Table 7.2). After 90 min exposure to 3.78 g vaporized vinegar, equalling to 3.36 mmol/L acetic acid content, there was complete inhibition of the *P. agglomerans* colonies. A reduction of 1 log colony was found after exposure to lactic acid vapor for 90 min (at 3.78 g lactic vapor used equal to 2.24 mmol/L lactic acid) or with citric acid vapor for 120 min (at 5.04 g citric acid vapor used equal to 1.4 mmol/L citric acid), which indicates that vaporized vinegar had more potential in inhibition of *in vitro P. agglomerans* growth on TSA plate than both lactic acid and citric acid vapors. This effect may be due to the higher protonated or un-dissociated forms of acetic acid in vinegar vapor than lactic acid and citric acid vapors, since it has been shown that acetic acid vapor can diffuse into the microbial cells causing their death (Brul and Croote, 1999; Bjornsdottir et al., 2006). Blackburn and McClure (2002) show that this killing of cells is due to enzyme inhibition, interference with nutrient transport, membrane damage as well as its an overall impact on the metabolic activity of the cells. Vinegar vapor was therefore selected for further study.

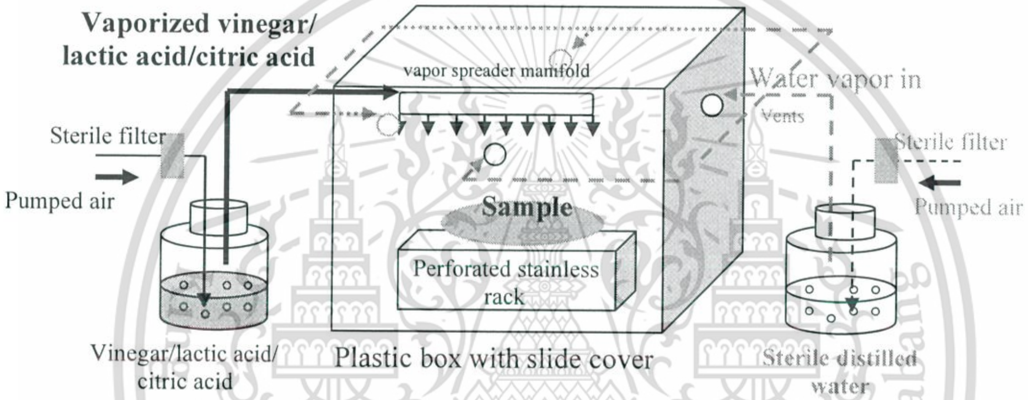

Figure 7.3 Diagram of vaporized exposure box used for fumigating samples by vinegar (10% v/v acetic acid), lactic acid (10% v/v) or citric acid (10% w/v) with/without vaporized water.

Weak organic acids in a vaporized form, especially vaporized acetic acid either alone or generated from vinegar, has been shown to be an effective antimicrobial agent for fumigation of many fresh produce (Sholberg and Gaunce, 1996; Sholberg et al., 2000; Krusong et al., 2015a; Krusong et al., 2015b). Acetic acid vapor appear destroys surface-borne microorganism and has been applied to surface sterilize some fruit and vegetables (Sholberg and Gaunce, 1996). Results showed that *P. agglomerans* completely destroyed *in vitro* after fumigation for 90 min with vaporized vinegar (Table 7.2). A longer exposure period was more effective in inhibiting *P. agglomerans* on TSA plates *in vitro*, but this effect may depend on the strength of the colony of *P. agglomerans* in its tolerance to diffused acetic acid.

The effects of vaporized lactic and citric acids on *in vitro* susceptibility of *P. agglomerans* was slight compared to vaporized vinegar with the same period of exposure, which might be due to the low level of lactic or citric acids in the vaporized form. It was found that if vaporized lactic acid or citric acid were to be used, the exposure period must be very long, which would not be practical for commercial application to fresh-cut fruits. However, these results indicate that vinegar, with a shorted effective exposure time, has practical potential for controlling *P. agglomerans* on fresh-cut jackfruit.

Table 7.2 Inhibitory effect of vaporized vinegar, citric acid and lactic acid on *P. agglomerans* colonies spread on TSA at 37°C.

Treatment Period (min)	Vaporized acid used* (g)	Vaporized acid content (mmol/L)**			*P. agglomerans* count*** (Log CFU/mL)			Proportion of inhibition		
		V	LA	CA	V	LA	CA	V	LA	CAs
0	0	0	0	0	$5.8^{aA}\pm0.2$	$5.8^{aA}\pm0.2$	$5.8^{aA}\pm0.1$	0.0	0.0	0.0
30	1.26	1.12	0.75	0.35	$5^{bB}\pm0.1$	$5.8^{aA}\pm0.2$	$5.8^{aA}\pm0.1$	15.0	0.0	0.0
60	2.52	2.24	1.48	0.7	$2.9^{cC}\pm0.1$	$5.2^{bB}\pm0.2$	$5.6^{aA}\pm0.2$	46.7	6.7	1.7
90	3.78	3.36	2.24	1.05	ND	$4.7^{cB}\pm0.2$	$5.1^{bA}\pm0.1$	100	15.0	10.0
120	5.04	4.48	2.99	1.4	ND	$4.3^{dBS}\pm0.2$	$4.8^{cA}\pm0.2$	100	23.3	16.7

Vinegar containing 10% (v/v) AA, lactic acid (10% v/v) and citric acid (10% w/v) were used.

* Vaporized acid used was calculated from weight loss of the acid used as 0.42 ± 0.003 g/min.

** Vaporized acid content was calculated on the average weight loss of the acid solution used at the rate of 4 L/min during treatment period and the molecular weight of each acid (acetic acid, 60.05; citric acid, 192.12; and lactic acid, 90.08) in the volume of 18.75 L of vaporized exposure box.

*** Mean ± one standard deviation. Mean comparison using Tukey's test, means with different both lowercase letters in the same column and capital letters in the same row are significantly different ($p \leq 0.05$).

Abbreviation: V, vinegar; AA, acetic acid; LA, lactic acid; CA, citric acid; TSA, Trypticase soy agar.

7.4.3 Impact of vaporized selected acid on fresh-cut jackfruit artificially inoculated with *P. agglomerans*

High levels of *P. agglomerans* contamination (4.2 ± 0.24 to 5.9 ± 0.26 Log CFU/g) were found on fresh-cut jackfruit in the survey (Fig. 7.1), it was decided to inoculate high level inoculum (6 Log CFU/g) of *P. agglomerans* in the fresh-cut jackfruit trials. After inoculation with 5.8 ± 0.1 Log CFU/mL *P. agglomerans*, treatment for 1 h with vaporized vinegar (2.52 g acetic acid equal to 2.24 mmol/L acetic acid content) reduced the population of *P. applomerans* by 1 Log to 18.96% inhibition (Table 7.3). However, more than a 3-Log reduction was observed after exposure for 5 h to 10.08 g acetic acid (equal to 8.96 mmol/L acetic acid content) in ambient URV vapor. The long exposure period was a major concern. It may be due to the colony characteristic of *P. agglomerans* which could protect the penetration of acetic acid molecules into the cells. Another possibility is the acid dilution effect of humidifying air flow through the exposure box (Fig. 7.3). It meant that a proportion of the acetic acid was vented to waste. Thus, the humid air supply was a dilution of the acetic acid vapor and this likely required extension of the exposure period.

Table 7.3 Inhibitory effect of vaporized vinegar on artificially inoculated *P. agglomerans* on surface of fresh-cut jackfruit. After treatment, samples were spread on TSA at 37 °C.

Treatment Period (h)	Vaporized vinegar used* (g)	AA Content in vapor (mmol/L)**	*P. agglomerans* count*** (Log CFU/mL)	Proportion of inhibition (%)
0	0	0	$5.8^{a}\pm0.1$	0.0
1	2.52	2.24	$4.7^{b}\pm0.2$	18.96
2	5.04	4.48	$3.4^{c}\pm0.2$	41.38
3	7.56	6.72	$2.7^{d}\pm0.3$	53.45
4	10.08	8.96	$2.1^{e}\pm0.2$	63.79

Vinegar containing 10% (v/v) AA was used.

* Vaporized acid used was calculated from weight loss of the acid used as 0.42 ± 0.003 g/min.

** AA content in vapor used was calculated on the average weight loss of the acid solution used at the rate of 4 L/min during treatment period and

*** Mean ± one standard deviation. Mean comparison using Tukey's test, means with different letters in the same column are significantly different ($p \leq 0.05$). Abbreviation: AA, acetic acid; TSA, Trypticase soy agar.

SEM images of the flesh of fresh-cut jackfruit artificially inoculated with *P. agglomerans* (Fig. 7.2c) showed the wrecked cell walls of inoculated *P. agglumerans* on fresh-cut jackfruit after exposure to vaporized vinegar. It may be due to the change of cell wall compositions by acetic acid molecules in vapor after penetration to the cell walls of *P. agglumerans* that may have resulted in the death of cells.

The efficacy of the vaporized vinegar treatment was affected by the initial microbial load (Table 7.3) as well as the dilution effect of humidified air on the acetic acid level in the vinegar vapor, which made it necessary to extend the exposure treatment. Sholberg and Gaunce (1996) suggest that high relative humidity is necessary for inactivation of fungal mycelia and spores by vaporized vinegar or acetic acid. However, in order to maintain the freshness of the fresh-cut fruit, the exposure treatment was carried out at 5 ± 2 °C and 80 ± %2 RH.

The decrease of the load of *P. agglomerans* after exposure to vaporized vinegar was associated with the acetic acid alone, but it has been hypothesized that other components of the vinegar could have antimicrobial properties (Pornpukdeewattana et al., 2017; Krusong et al., 2020; Teerarak et al., 2020). Therefore, it is possible that both the acetic acid and non-acetic acid components of vinegar from upland rice could have a synergistic impact on microbial levels on fresh-cut produce, as was shown in inoculated fresh-cut jackfruit (Table 7.3). Depending on the exposure time, vaporized vinegar contained levels of the un-dissociated form of acetic acid, which is more effective in microorganism control. Also, the vinegar in a gaseous form can easily and rapidly diffuse and penetrate into cells more effectively than the liquid form.

7.4.4 The sensory qualities of fresh-cut jackfruit and bacterial contamination after treatment with vinegar, lactic acid or citric acid vapors

For the sensory evolution, jackfruit-flavored vinegar was used because of the concern that the vinegar could affect the taste and smell of the fresh-cut jackfruit. No change of acidity in the flavored vinegar was found, which means that there is no change in the acetic acid content of the solution that was used for vaporization.

Vaporized vinegar was used for treatment of fresh-cut jackfruit for sensory evaluation each day during storage for 7 days at 5 ± 2 °C under 80 ± %2 RH. The appearance of treated samples was more acceptable than non-treated samples during storage (Fig. 7.4). The attributes used by the taste panel of both treated and non-treated samples show that appearance, color, texture and overall acceptability of URV vapor treated samples had higher scores than non-treated samples except the taste and aroma (Table 7.4). However, the score of taste and aroma were still acceptable, which was accounted for by the effect of jackfruit flavor in the jackfruit-flavored vinegar. Treatment of fresh-cut jackfruit with URV resulted in higher acceptance than non-treated samples during storage (Fig. 4 and Table 4) indicating that URV treatment can prolong their safe shelf-life.

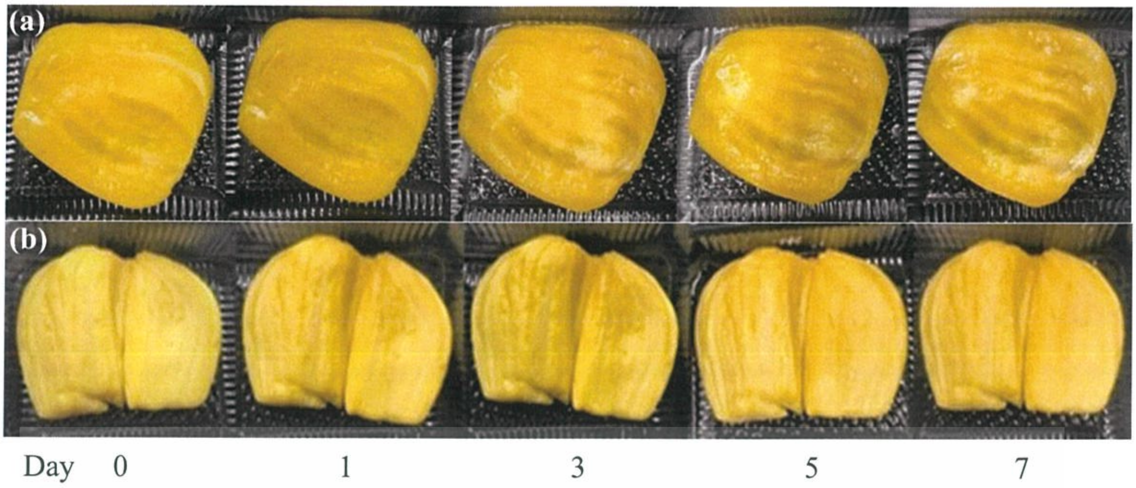

Figure 7.4 Appearance of fresh-cut jackfruit during storage for 7 days at 5 ± 2 °C : (a) no treatment, as control; (b) vaporized vinegar (10% v/v acetic acid) treatment for 4 h at 5 ± 2 °C with vaporized water (80 ± 2% relative humidity). Vinegar made from upland rice was used. Fresh-cut jackfruit samples without *P. agglumerans* inoculation were used.

Table 7.4 Sensory evaluation during storage (for 7 days at 5 ± 2 °C) of fresh-cut jackfruit with and without vaporized vinegar (10% v/v acetic acid) treatment for 4 h at 5 ± 2 °C accompanying with vaporized water (80 ± 2% relative humidity).

Storage period (d)	Sample	Appearance*	Color*	Aroma*	Taste*	Texture*	Overall * acceptability
0	C	$4.65^{a}\pm0.5$	$4.51^{a}\pm0.7$	$4.3^{a}\pm0.8$	$4.05^{a}\pm1.1$	$4.23^{b}\pm1.3$	$4.23^{a}\pm1.1$
	URV	$4.62^{a}\pm0.7$	$4.45^{a}\pm0.4$	$4.18^{b}\pm0.7$	$3.99^{a}\pm0.8$	$4.43^{a}\pm1.1$	$4.11^{b}\pm0.5$
1	C	$4.21^{d}\pm0.7$	$4.23^{b}\pm0.5$	$4.11^{b}\pm0.9$	$4.01^{a}\pm0.9$	$3.87^{d}\pm1.5$	$4.18^{a}\pm1.2$
	Vinegar	$4.48^{b}\pm0.5$	$4.21^{b}\pm0.8$	$4.03^{c}\pm0.3$	$3.73^{c}\pm1.1$	$4.28^{b}\pm0.9$	$4.03^{c}\pm0.7$
3	C	$3.92^{f}\pm0.9$	$4.16^{c}\pm0.8$	$3.92^{c}\pm0.7$	$3.93^{b}\pm1.2$	$3.33^{g}\pm1.4$	$3.73^{e}\pm1.1$
	Vinegar	$4.3^{c}\pm0.5$	$4.13^{c}\pm0.9$	$3.77^{d}\pm0.8$	$3.67^{c}\pm1.5$	$4.02^{c}\pm0.8$	$3.81^{d}\pm1.5$
5	C	$3.41^{h}\pm0.9$	$3.63^{f}\pm0.9$	$3.73^{d}\pm0.5$	$3.87^{b}\pm1.3$	$2.83^{h}\pm1.5$	$2.93^{g}\pm0.5$
	Vinegar	$4.08^{e}\pm0.5$	$3.83^{d}\pm0.8$	$3.65^{e}\pm0.3$	$3.27^{e}\pm1.2$	$3.73^{e}\pm1.1$	$3.73^{e}\pm1.1$
7	C	$2.98^{i}\pm1.1$	$3.16^{g}\pm0.7$	$3.33^{g}\pm0.9$	$3.53^{d}\pm1.5$	$2.53^{i}\pm1.5$	$2.73^{h}\pm1.5$
	Vinegar	$3.72^{g}\pm0.5$	$3.7^{e}\pm0.9$	$3.47^{f}\pm1.2$	$3.31^{e}\pm1.1$	$3.43^{f}\pm1.1$	$3.21^{f}\pm1.4$

* Mean ± one standard deviation. Mean comparison using Tukey's test, means with different letters in the same column are significantly different ($p \leq 0.05$). Abbreviation: C, control, no vaporized vinegar treatment

Total aerobic count and *P. agglumerans* showed significant increments of total aerobic count in sample without vapor treatment, but there was a slight number of aerobic bacteria in treated sample after 3 days of storage (Table 7.5). However, no *P. agglumerans* was detected in either samples with or without URV treatment. This result supports the benefit in prolonging shelf-life of fresh-cut jackfruit by vaporized vinegar treatment.

Table 7.5 Determination of *P. agglomerans* and total aerobic count during storage (for 7 days at 5 ± 2 °C) of fresh-cut jackfruit with and without vaporized vinegar (10% v/v acetic acid) treatment for 4 h at 5 ± 2 °C accompanying with vaporized water (80 ± 2% relative humidity).

Storage period (d)	No vaporized vinegar*		Vaporized vinegar*	
	Total aerobic count (Log CFU/mL)	PA (Log CFU/mL)	Total aerobic count (Log CFU/mL)	PA (Log CFU/mL)
0	$1.8^{e}\pm0.2$	ND	ND	ND
1	$2.7^{d}\pm0.3$	ND	ND	ND
3	$3.5^{c}\pm0.1$	ND	$1.3^{e}\pm0.1$	ND
5	$4.9^{b}\pm0.2$	ND	$2.4^{e}\pm0.3$	ND
7	$5.7^{a}\pm0.3$	ND	$3.6^{e}\pm0.4$	ND

* Mean ± one standard deviation. Mean comparison using Tukey's test, means with different letters in the same column are significantly different ($p \leq 0.05$). Abbreviation: PA, *P. agglomerans*; ND, not detected.

7.5 Conclusion

Fumigation with vinegar vapor controlled *P. agglumerans* development of inoculated fresh-cut jackfruit during storage. This is because it allows acetic acid molecules, which are dissociated in solution, to become un-dissociated and more potent in the gaseous phase. His change in phase allows the gas molecules to diffuse more rapidly onto the fruit surfaces so penetrating to places that are less accessible to liquids, especially the wave-like ridges on the fruit surface. It was concluded that, vaporized vinegar can safely prolong the safe shelf-life of fresh-cut jackfruit and had no adverse effects on their organoleptic quality and consumer acceptance and can be used as a simple and economical method for commercial production.

7.6 References

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CHAPTER 8

SUMMARY AND RECOMMENDATIONS

8.1. Summary

Acetic acid bacteria as microbial starters could be effective also at temperature above the optimal values, when acetification processes are managed through repeated semi-continuous cycles. Also, AAB cells under the long cultivation with stress environment could adapt physically to activate at high temperature. $CaCl_2$ addition could improve thermo-tolerance mechanisms properties of acetic acid bacteria. The adsorbed AAB cells on LSM could improve the acetification performance due to an abundant amount of cell biomass AAB cell. Volatile organic components of upland rice vinegar could inhibitory impact on mycelial growth, conidia germination and aflatoxin production of *Asp. flavus* on dried chili peppers. Also, vaporized vinegar is a simple method of controlling surface bacterial contamination on fresh-cut jackfruit.

8.2. Recommendations

8.2.1 The thermo adapted cells with $CaCl_2$ addition could become strong and resistant to high temperature acetification (HTA).

8.2.2 The AAB adapted cell could be useful for large-industrial acetification.

8.2.3 Quick process in reciprocating shaker with LSM is a sustainable process for small-scale vinegar production system requiring minimal set-up.

8.2.4 For gene adaptation, in this study showed that all three strains were different in terms of physiology and morphology properties. Future analysis of gene mutation will be addressed in order to understand the biological mechanisms of the adaptation at high temperature. Based on the literature, it is well documented that in the adaptation mechanisms, mutations occur in targeted gene sequences, with completely different biological function. In the next step, a GWAS analysis could be used in order to associate phenotypic traits to gene presence/absence. A study on targeted genes will be used in order to understand if mutation occur.

8.2.5 VOCs of vinegar were effective as antimicrobial agents, which are suitable for safe application in foods and feeds to control some microorganisms.

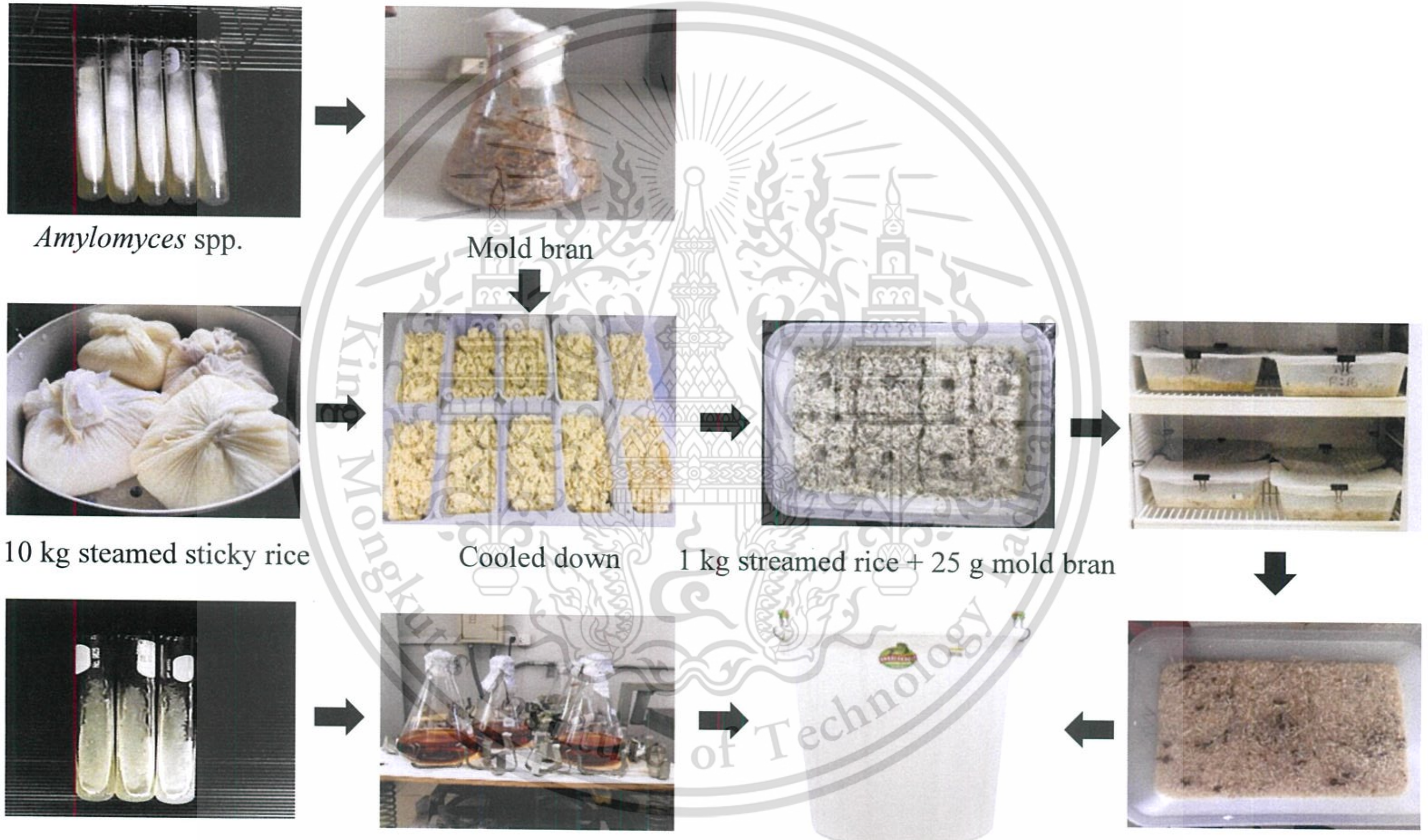
8.2.6 The advantages of VOCs of vinegar are applied easily to dried fruits and vegetable which contain low Aw, especially feed and cereals.

8.2.7 The disadvantages of VOCs of vinegar may cause the negative affect on surface of exposed product by interfering and changing taste and aroma, especially fresh-cut fruits.

APPENDIX

Appendix A

Appendix A1: Rice wine preparation

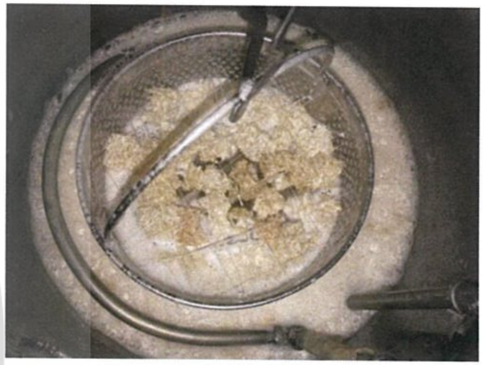


Appendix A2: Acetification process

0.15% $CaCl_2$

Primary adaptation at 40 °C in 2 L of bottle

Controlled at 30 °C in 2 L of bottle



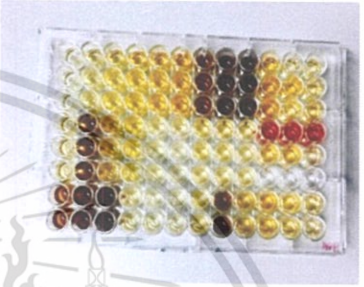
Luffa sponge matrices

100 L iVi bioreactor and cooling unit located at Labolatory of Fermentation Technology, Division of Fermentation Technology, Faculty of Food Industry, KMITL

Appendix A3: Cells preparation and analytical method

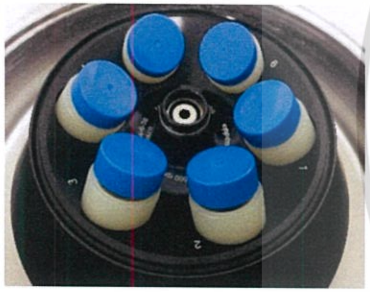
Vinegar sample

treated ultrasonic at 750 W 20 kHz, for 15 s



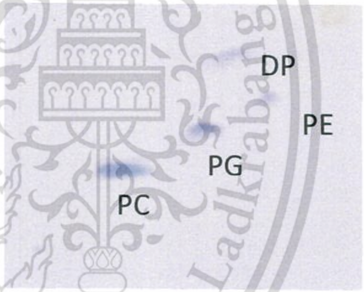
ADH and ALDH test kits

Calorimetrically by Microplate reader



Centrifugation at 8000xg for 5 min, 4 °C

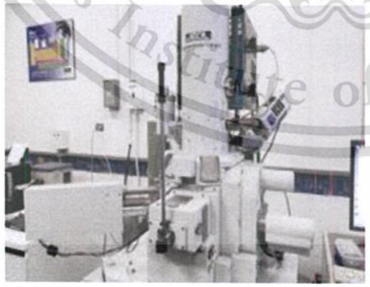
Cells was kept at 4 °C



TLC for phospholipid

GC-FID for fatty acid

AAB cell in LSM

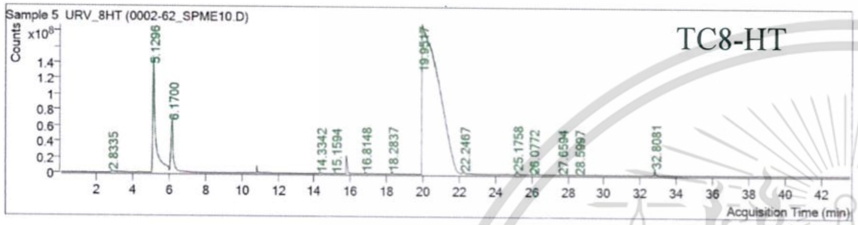


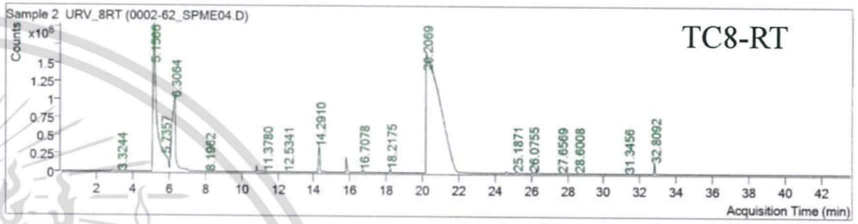
SEM analysis

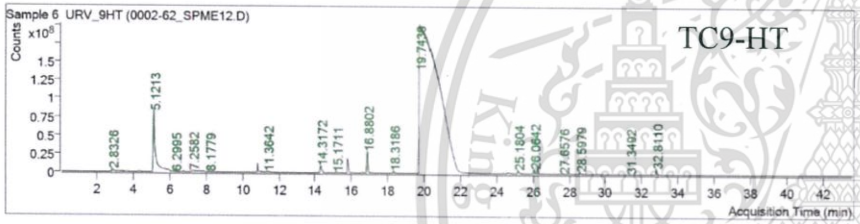
AAB strain sample

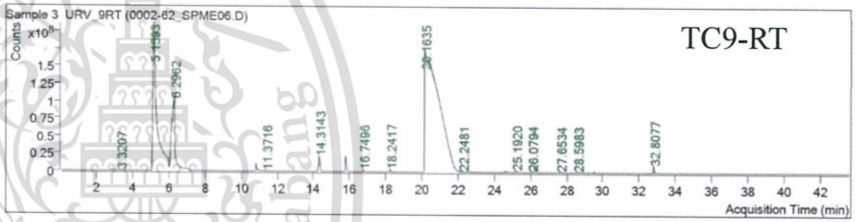
TEM analysis

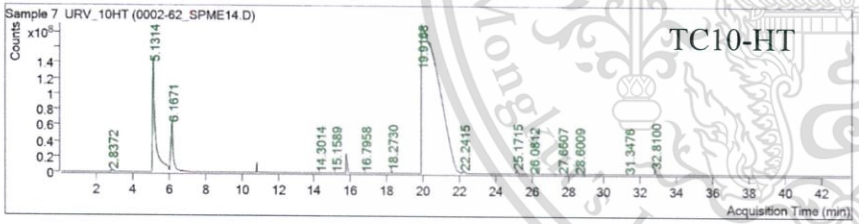
Appendix A4: Chromatography of vinegar by GC-MS

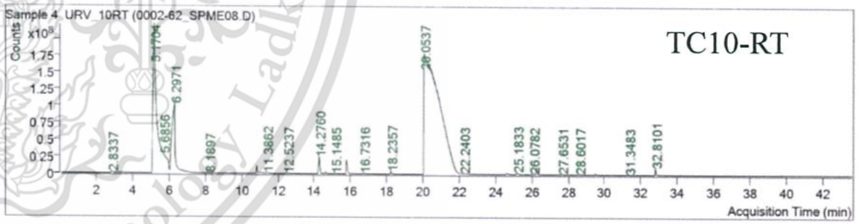






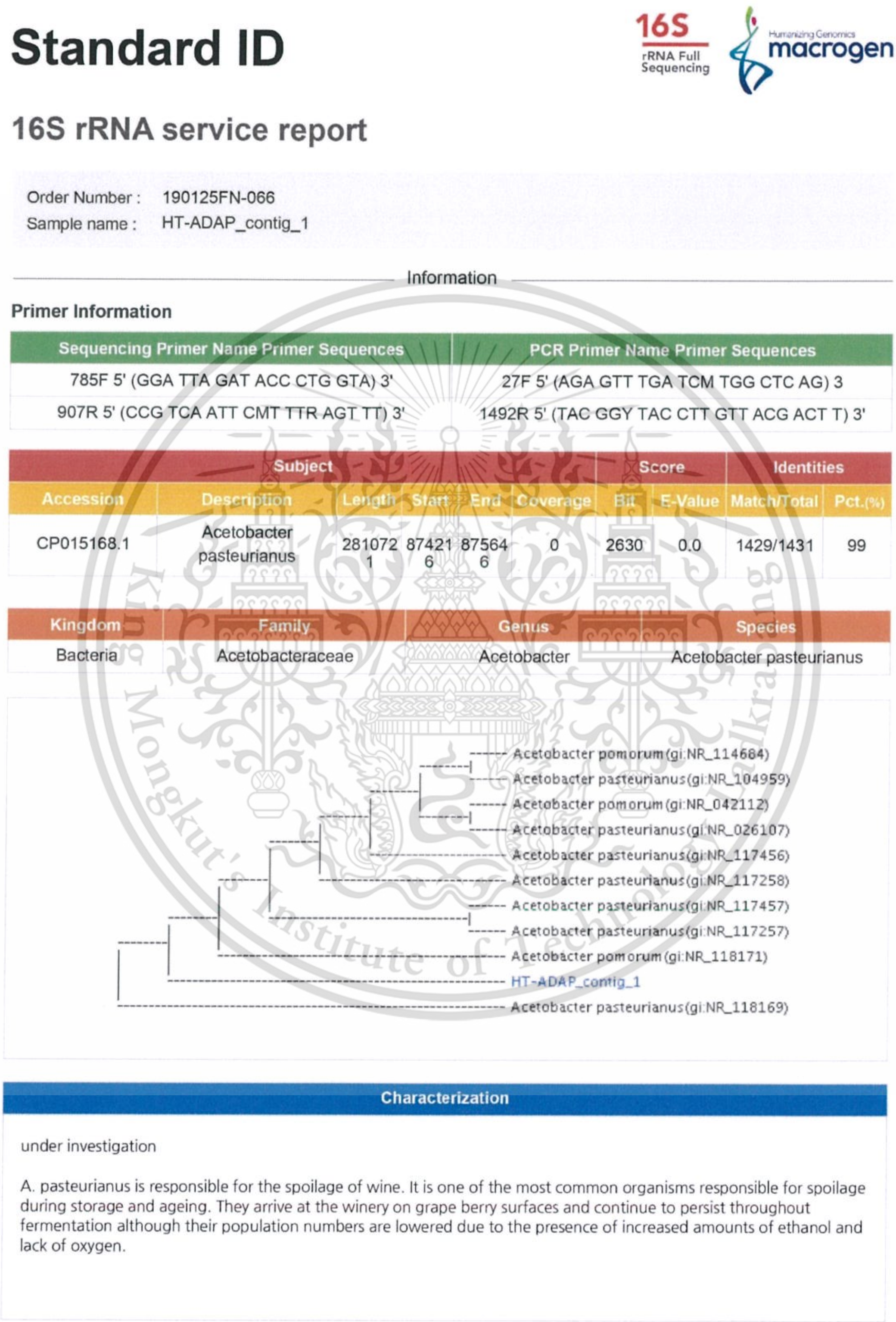






Appendix A5: Phylogenetic tree by 16S rRNA

1. HT-adapted strain at 40°C



Standard ID

16S rRNA service report

Order Number : 190125FN-066
Sample name : HT-ADAP_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
CP015168.1	Acetobacter pasteurianus	2810721	874216	875646	0	2630	0.0	1429/1431	99

Kingdom	Family	Genus	Species
Bacteria	Acetobacteraceae	Acetobacter	Acetobacter pasteurianus

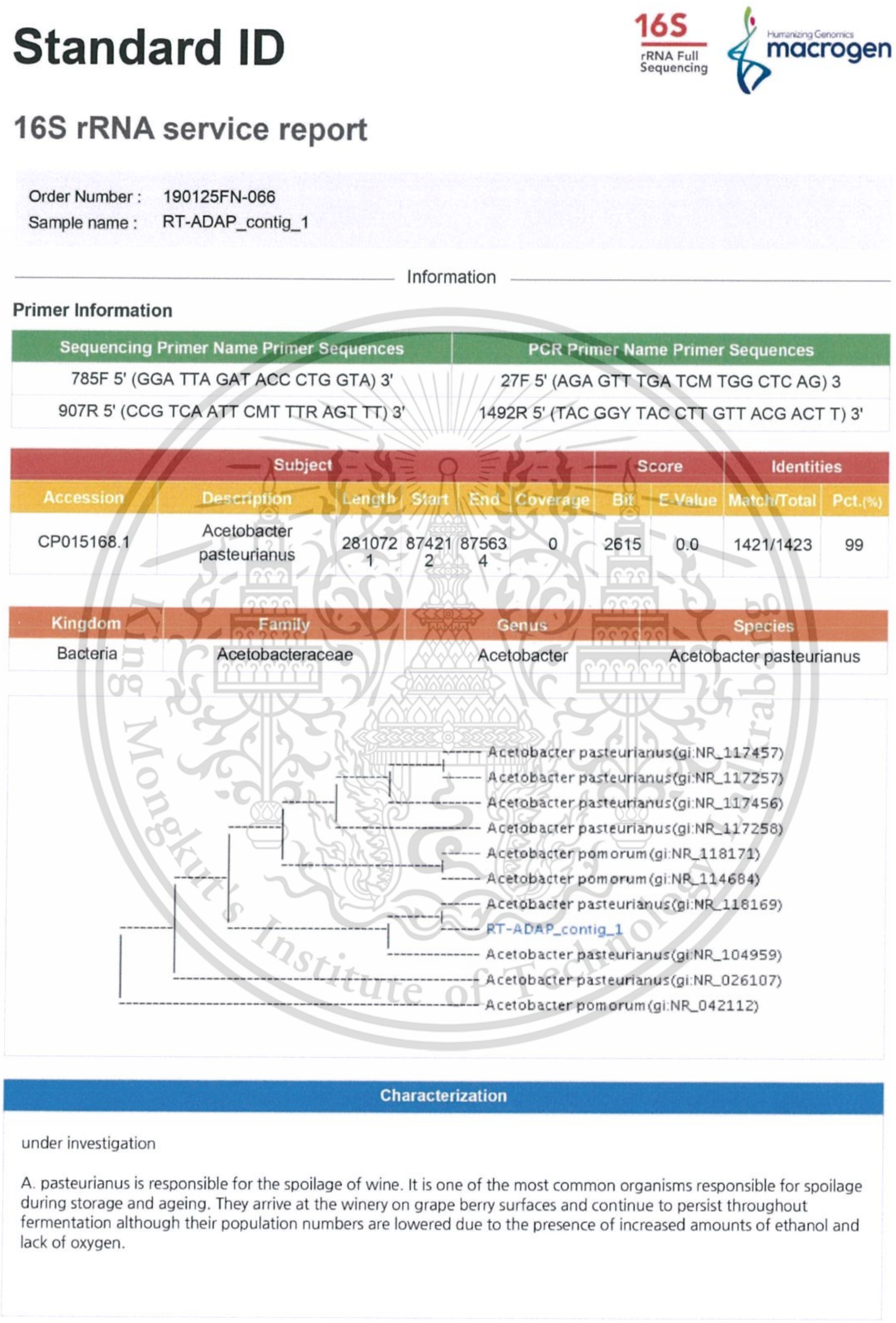
Characterization

under investigation

A. pasteurianus is responsible for the spoilage of wine. It is one of the most common organisms responsible for spoilage during storage and ageing. They arrive at the winery on grape berry surfaces and continue to persist throughout fermentation although their population numbers are lowered due to the presence of increased amounts of ethanol and lack of oxygen.

Appendix A5: Phylogenetic tree by 16S rRNA

2. RT-adapted strain at 30°C



Standard ID

16S rRNA service report

Order Number : 190125FN-066
Sample name : RT-ADAP_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
CP015168.1	Acetobacter pasteurianus	2810721	874212	875634	0	2615	0.0	1421/1423	99

Kingdom	Family	Genus	Species
Bacteria	Acetobacteraceae	Acetobacter	Acetobacter pasteurianus

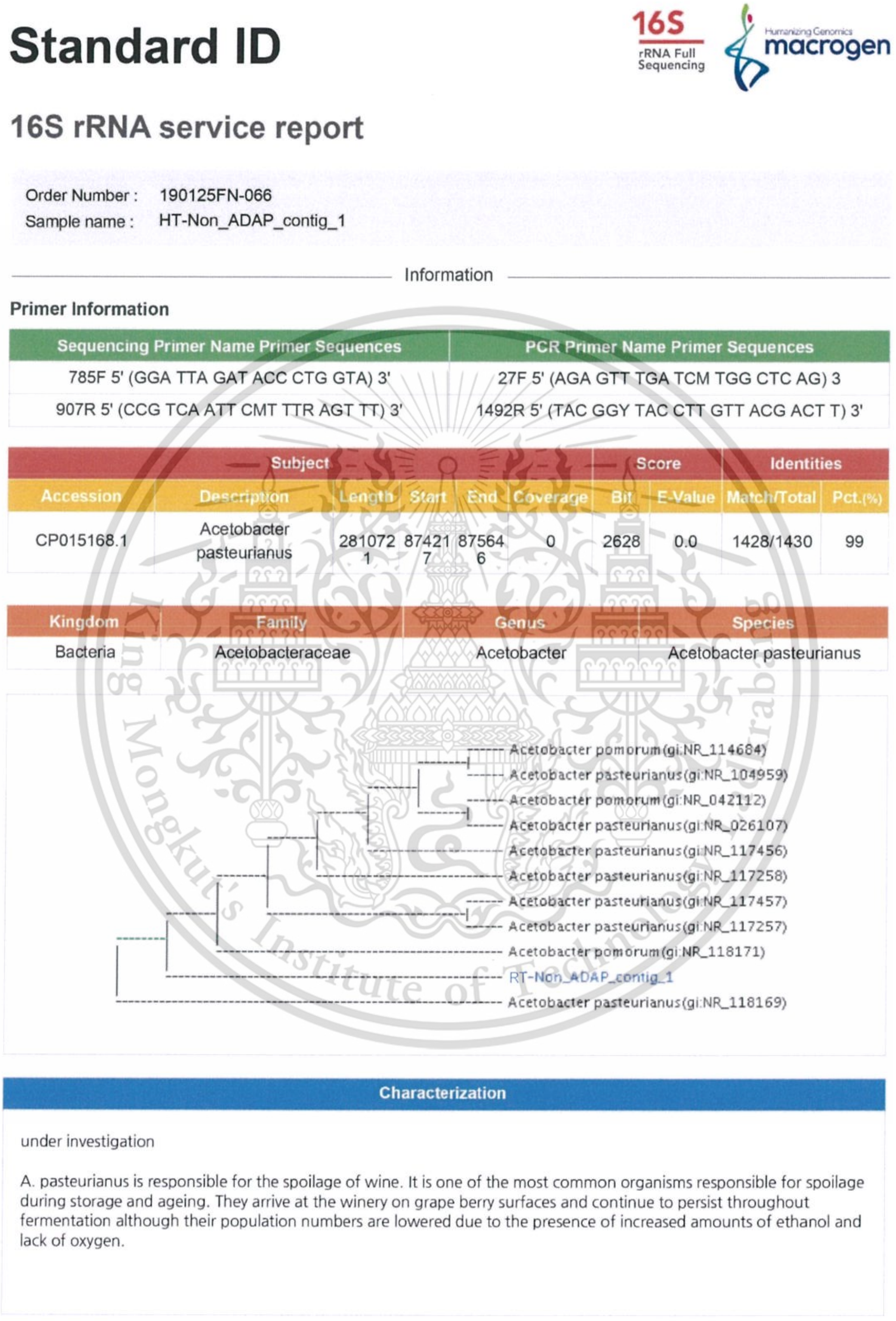
Characterization

under investigation

A. pasteurianus is responsible for the spoilage of wine. It is one of the most common organisms responsible for spoilage during storage and ageing. They arrive at the winery on grape berry surfaces and continue to persist throughout fermentation although their population numbers are lowered due to the presence of increased amounts of ethanol and lack of oxygen.

Appendix A5: Phylogenetic tree by 16S rRNA

3. RTN-wild type strain at 35°C



Standard ID

16S rRNA service report

Order Number : 190125FN-066
Sample name : HT-Non_ADAP_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
CP015168.1	Acetobacter pasteurianus	2810721	874217	875646	0	2628	0.0	1428/1430	99

Kingdom	Family	Genus	Species
Bacteria	Acetobacteraceae	Acetobacter	Acetobacter pasteurianus

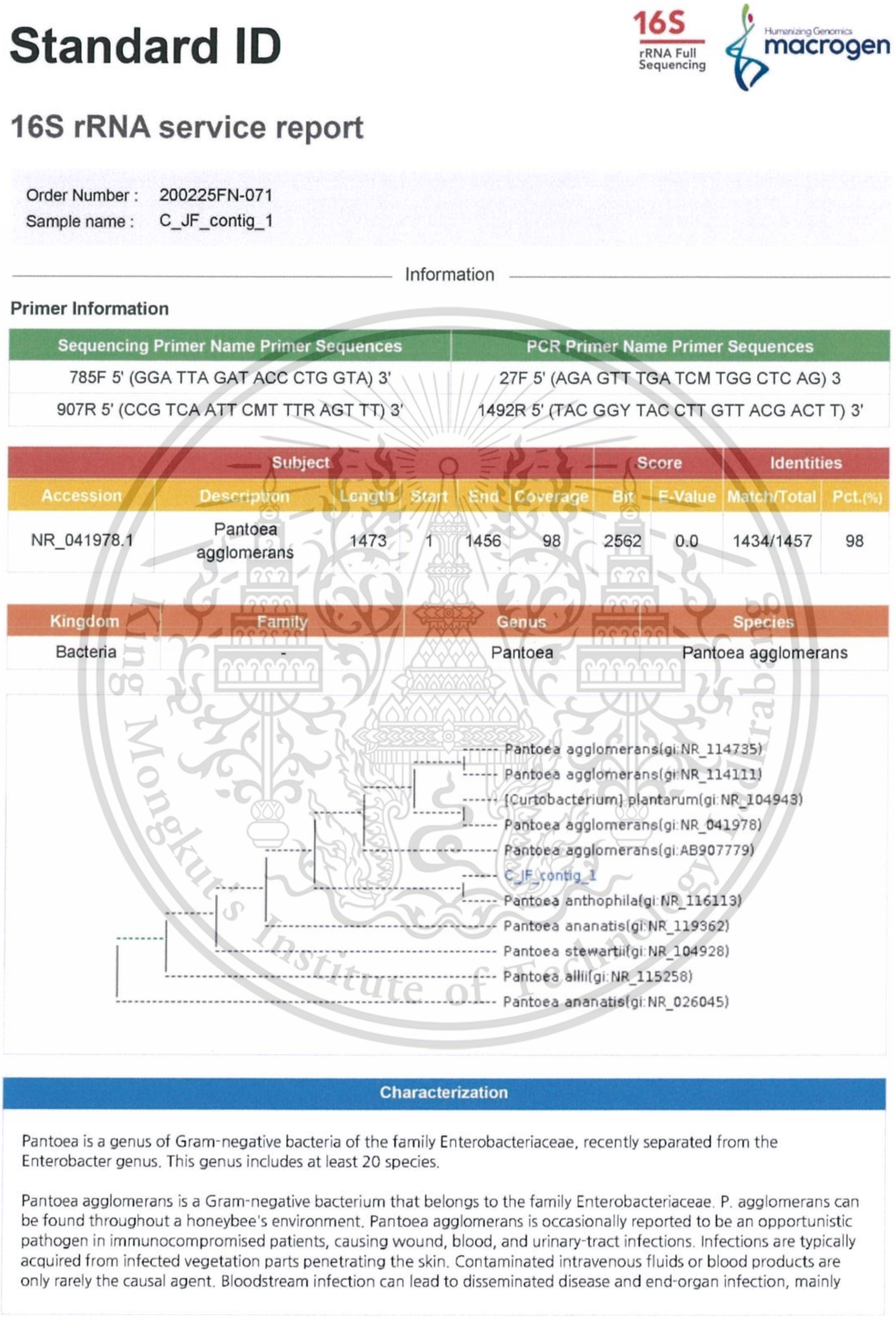
Characterization

under investigation

A. pasteurianus is responsible for the spoilage of wine. It is one of the most common organisms responsible for spoilage during storage and ageing. They arrive at the winery on grape berry surfaces and continue to persist throughout fermentation although their population numbers are lowered due to the presence of increased amounts of ethanol and lack of oxygen.

Appendix A5: Phylogenetic tree by 16S rRNA

4. Phylogenetic tree contaminate in Fresh-cut Jackfruit



Standard ID

16S rRNA Full Sequencing

Humanizing Genomics macrogen

16S rRNA service report

Order Number : 200225FN-071
Sample name : C_JF_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
NR_041978.1	Pantoea agglomerans	1473	1	1456	98	2562	0.0	1434/1457	98

Kingdom	Family	Genus	Species
Bacteria	-	Pantoea	Pantoea agglomerans

Characterization

Pantoea is a genus of Gram-negative bacteria of the family Enterobacteriaceae, recently separated from the Enterobacter genus. This genus includes at least 20 species.

Pantoea agglomerans is a Gram-negative bacterium that belongs to the family Enterobacteriaceae. P. agglomerans can be found throughout a honeybee's environment. Pantoea agglomerans is occasionally reported to be an opportunistic pathogen in immunocompromised patients, causing wound, blood, and urinary-tract infections. Infections are typically acquired from infected vegetation parts penetrating the skin. Contaminated intravenous fluids or blood products are only rarely the causal agent. Bloodstream infection can lead to disseminated disease and end-organ infection, mainly

Appendix A6: The calculation of acetic acid (AP) and acetification rate (ETA) content

The AP and ETA were calculated on the average AP and ETA of each cycles (including operation phase 9 semi-continuous cycles with out start-up phase).

AP (g/L) = (Final acetic acid product (g/L) – Initial acetic acid (g/L))

$$\text{ETA (g/L/d)} = \left[\frac{(Final\ acetic\ acid\ product\ (g/L) - Initial\ acetic\ acid\ (g/L))}{Acetification\ period\ (d)}\right]$$

Appendix B: Sensory Evaluation

Product: Fresh-cut Jackfruits

แบบบันทึกผลการทดสอบการให้คะแนนความชอบของขนุนตัดแต่งพร้อมบริโภค

ชื่อผู้ทดสอบ (Name) ....วันที่

(Date)................................ เวลา (Time)....................

<u>คำแนะนำ</u> ทดสอบตัวอย่างขนุนแล้วให้คะแนนความชอบแต่ละคุณลักษณะของตัดแต่งพร้อมบริโภคตามคำอธิบายคะแนนต่อไปนี้และกรุณาบ้วนปากระหว่างตัวอย่าง

(You are given coded samples. Please evaluate them to right and give a score for each attribute below.)

1 = ไม่ชอบมาก (Very much dislike)	2 = ไม่ชอบ) Moderately dislike)
3 = บอกไม่ได้ว่าชอบหรือไม่ชอบ (Neither like or dislike)	4 = ชอบ) Moderately dislike)
5 = ชอบมาก (Very much like)	

Sample code	คุณลักษณะของตัวอย่าง **(Attributes)**						การยอมรับ ตัวอย่าง
	ลักษณะปรากฎ (Appearance)	สี (Color)	กลิ่น (Aroma)	รสชาติ (Taste)	เนื้อสัมผัส (Flavor)	ความชอบรวม (Overall)	
A							☐ ยอมรับ (Accept) ☐ ไม่ยอมรับ (No Accept) เพราะ..................................
B							☐ ยอมรับ (Accept) ☐ ไม่ยอมรับ (No Accept) เพราะ..................................

"Please neutralize your mouth with water before and after testing"

AUTHOR BIOGRAPHY

Ruttipron Pothimon was born in Sakon Nakhon, Thailand, and grew up there as well. She graduated with her Bachelor of Science degree (majoring in Food Technology - with Honors) in 2013 from Kasetsart University, Thailand, and she was graduated with Master of Science degree (majoring in Food Safety Management) in 2016 from King Mongkut's Institute of Technology Ladkrabang (KMITL), Thailand. During her study master degree, she was a science teacher in Panchasap Minburi School for three years. She obtained her Doctoral of Science degree (majoring in Food Science) in 2020 from King Mongkut's Institute of Technology Ladkrabang (KMITL), Thailand, and was fully supported by Research and Researchers for Industries-RRI Ph.D. Scholarship awarded by the Thailand Research Fund under the Office of the Prime Minister, Royal Thai Government (Code PHD60I0035) for the financial support of the study. During she study in KMITL, Thailand, she working as training assistant in Laboratory of Fermentation Technology at Faculty of Food Industry KMITL, Thailand. She joined an exchange student program in University of Modena and Reggio Emilia (UNIMORE), Italy for six months (2019) under Asst. Prof. Dr. Maria Gullo, with the project of molecular gene mutation in acetic acid bacteria cells. Also, she has gained a lot of knowledge, lab-skills, experiences, and networks. She studied the physicochemical, instrumentation, chemistry and technology of food flavor, genetic engineering, shelf-life, vinegar processing in food industry and identification of volatile compound by using gas chromatography of wine and vinegar, under the supervision of Prof. Dr. Warawut Krusong at the Faculty of Food Industry, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand.

"Don't stop when you are tired but stop when you are done: Do it until you succeed!"

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List of publications

Pothimon, R., Gullo, M., La China, S., Thompson, A. K., and Krusong, W. (2020). Conducting High Acetic Acid and Temperature Acetification Processes by *Acetobacter pasteurianus* UMCC 2951. *Journal of Process Biochemistry, 98,* 41-50. https://doi:10.1016/j.procbio.2020.07.022.

Pothimon, R., Podjanee, U., Krusong, W., Thompson, A. K., and Massa, S. (2020). Inhibition of Pantoea agglomerans contamination of fresh-cut jackfruit by exposure to weak organic acid vapors. *LWT - Food Science and Technology,* xx, xxx. https://doi.org/10.1016/j.lwt.2020.110586.

Pothimon, R., Deetae, P., Krusong, W., and Thompson, A. K. (2020). Vinegar processing methods on volatile organic components and their effects on controlling *Aspergillus flavus* in dried chili peppers. *Food Control*, [submitted manuscript: under reviewer].

Krusong, W., **Pothimon, R**., La China, S., Thompson, A. K. (2021). Consecutive bacterial cellulose production by luffa sponge enmeshed with cellulose microfibrils of *Acetobacter xylinum* under continuous aeration. *3 Biotech*, *11,* 6. https://doi.org/10.1007/s13205-020-02569-8

Krusong, W., **Pothimon, R**., and Vichitraka, A. (2019). Inhibitory impact of vapor-phase ethanol on conidia germination and mycelial growth of *Aspergillus fumigatus* on bread. *Food Control, 95,* 165-169. https://doi.org/10.1016/j.foodcont.2018.08.010.