(Format No. 3)

SUMMARY OF DOCTORAL THESIS

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Title: Studies on Acetic acid Fermentation and Acetic acid Resistance Abilities in Thermotolerant *Acetobacter pasteurianus* Strains
（耐熱性酢酸菌 *Acetobacter pasteurianus* の酢酸発酵能および酢酸耐性能に関する研究）

Acetic acid bacteria (AAB) are Gram-negative, strictly aerobic bacteria that are widely used for the commercial production of vinegar, gluconate and sorbose. AAB, especially *Acetobacter* and *Gluconacetobacter*, have been used for acetic acid fermentation because of their powerful ability to oxidize ethanol and to tolerate to high acetic acid concentration accumulated in the medium. They convert ethanol to acetic acid by two sequential reactions of membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), which are localized on the outer surface of the cytoplasmic membrane. Modern industrial vinegar production is usually carried out by continuous submerged fermentation which gives high fermentation rate as well as high acetic acid yield. However, this process requires precise control of fermentation temperature for the efficient vinegar production. The optimum temperature is about 30ºC and slight increase in temperature leads to a remarkable defect in both fermentation rate and yield of acetic acid. A large scale cooling system is necessary to maintain the cultivation temperature since the oxidation process generates a great deal of heat. In order to reduce the cooling cost and to escape from the growth defect, use of a thermotolerant strain with high resistance to ethanol and acetic acid is of interest. Ethanol oxidation and acetic acid resistance abilities are a crucial factor for AAB to stably perform the fermentation. ADH involved in ethanol oxidation of these thermotolerant strains is especially important for the high temperature fermentation. In *Acetobacter* species, *Acetobacter tropicalis* SKU1100 and *Acetobacter aceti* IFO3284, have been shown to produce capsular polysaccharides as a kind of pellicle or biofilm (Moonmangmee et al. 2002a, 2002b), and have two different types of cells, R (rough colony) and S (smooth colony) strains, where R strain but not S strain produces the pellicle (Deeraksa et al. 2005; Azuma et al. 2009). Thus, the pellicle formation may also be one of the defense mechanism for acetic acid resistance. Therefore, in this work, thermotolerant AAB were isolated, and characterized in their ethanol and acetic acid resistance at high temperature. From the strains, ADH was purified and characterized. And also the relationship among acetic acid fermentation, acetic acid resistance and pellicle polysaccharide formation of *A. pasteurianus* were investigated.

In the first chapter, I have isolated several thermotolerant *Acetobacter* species from fruits and flowers in Thailand. These isolates were characterized for their growth on potato agar plates at different temperatures and their tolerance to acetic acid or ethanol on YPGD medium containing 1-4% acetic acid or 1-15% ethanol. It was found that MSU10 strain, identified as *Acetobacter pasteurianus*, could grow well on agar plates at 41ºC, tolerate to 1.5% acetic acid or 4% ethanol at 39ºC, similarly seen with *Acetobacter pasteurianus* SKU1108 previously isolated. While, mesophilic IFO3284 and IFO3191 strains could not grow at such a high temperature (although IFO3284 could grow at 39ºC

with or without 1% acetic acid). The MSU10 strain showed a higher acetic acid productivity in a medium containing 6% ethanol at 37ºC than SKU1108, while SKU1108 strain could accumulate more acetic acid in a medium supplemented with 4-5% ethanol at the same temperature. The fermentation ability at 37ºC of these thermotolerant strains was superior to that of mesophilic *Acetobacter pasteurianus* IFO3191 strain having weak growth and very delayed acetic acid production at 37ºC even at 4% ethanol. ADHs were purified from MSU10, SKU1108 and IFO3191 strains, and their properties were compared related to the thermotolerance. The optimal temperature of the enzyme activity from MSU10 and SKU1108 strains were found to be 35ºC, but 30ºC in IFO3191. All the enzymes among these strains were stable up to 50ºC for 30 min and retained the activity >50%, but at higher temperature. ADHs of MSU10 and SKU1108 showed a little higher stability than IFO3191 enzyme. Besides, ADHs from MSU10 and SKU1108 exhibit higher resistance to ethanol and acetic acid than IFO3191 enzyme at elevated temperature. The results indicate that ADHs of thermotolerant MSU10 and SKU1108 strains have thermotolerant properties. The ADH genes were cloned and the amino acid sequences of ADH subunit I, subunit II and subunit III were compared. The difference in the amino acid residues could be seen, seemingly related to the thermotolerance, among MSU10, SKU1108 and IFO 3191 ADHs.

In the second chapter, *Acetobacter pasteurianus* strains, IFO3283, SKU1108, and MSU10, were grown in acetic acid fermentation condition, and their growth behavior was examined together with the ability of acetic acid resistance and pellicle formation. In the fermentation process, the cells became aggregated and covered by some amorphous materials at the late-log and stationary phases, but dispersed again at the second growth phase (over-oxidation). In addition, the sugar content of cells was changed and increased from early ethanol oxidation phase (EO-phase) to acetic acid resistance phase (AR-phase), and finally decreased again at over-oxidation phase (AO-phase). The sugar content of the cells has been shown to be related to the polysaccharide attached to the cells which could be deteced only in R strain (rough colony). Thus, the morphological change of the cells was accompanied with the change of sugar contents of the cells, which may be related to pellicle polysaccharide formation. The culture seemed to be rich in the cells having pellicle polysaccharides at late EO-phase to AR-phase, which may be related to the acetic acid resistance of the cells. To know the relationship between the pellicle formation and the acetic acid resistance, a pellicle-forming R strain and non-forming S strain (smooth colony) were isolated, and compared in their fermentation ability in the YPGD medium containing 4% ethanol at 37ºC. In all three *A. pasturianus* strains, R strains showed clearly high ability for acetic acid fermentation, where the cells produced high acetic acid production nearly 3.5% with the typical diauxic growth of EO-, AR-, and AO-phases. Whereas, S strains could not complete the fermentation so as to produce only ~1.5% acetic acid, and thus have no AO-phase. Thus, R strain, having the pellicle polysaccharide, seems to have higher acetic acid resistance ability than S strain not producing the pellicle. These data suggest that the polysaccharide surrounding the cells may be related to acetic acid diffusion into the cells. Thus, since the pellicle polysaccharides were expected to disturb acetic acid diffusion into the cells, acetic acid uptake or diffusion into the cells was examined with both R and S strains. It was found that the R strain had lower acetic acid accumulation than S strain in the acetic acid diffusion experiment. The results suggest that the pellicle formation is directly related to acetic acid resistance ability, and thus important for the acetic acid fermentation in these *A. pasteurianus* strains.