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*Research Article*

# An investigation of simultaneous pineapple vinegar fermentation interaction between acetic acid bacteria and yeast

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## Abstract

Simultaneous pineapple vinegar fermentation interaction between two microorganisms was investigated, wherein the interaction between acetic acid bacteria (AAB) *Acetobacter aceti* WK and a flocculate yeast *Saccharomyces cerevisiae* M30 was conducted. The inoculated yeast was allowed to ferment for 1 - 4 days prior to inoculating the AAB into the fermenting mash. Non-aerated conditions for alcohol fermentation were used to enhance the ability of *S. cerevisiae* M30, whereas aeration was supplied to assist vinegar fermentation by *A. aceti* WK. During aeration, alcohol production by *S. cerevisiae* M30 was drastically decreased and observed, while *A. aceti* WK cells increased for two more log cycles within 10 days and produced 1.6 – 2.1% acetic acid. By this simultaneous fermentation, the commensalisms interaction was primarily observed by supplying of alcohol to AAB by yeast, while the AAB provided nothing to yeast. The accumulating acetic acid produced by *A. aceti* WK caused an antagonistic effect on growth of *S. cerevisiae* M30.

**Keywords:** *Acetobacter aceti, Saccharomyces cerevisiae* M30, simultaneous pineapple vinegar fermentation, biological interaction, acetic acid bacteria, yeast, commensalisms, antagonistic effect, Thailand.

## Introduction

Acetic acid bacteria (AAB) are ubiquitous organisms which are well adapted to sugar and a rich environment [1]. AAB are also well known for their ability to spoil wines. Principally, these bacteria cause acetification of the wine by oxidizing ethanol to acetic acid [2]. Naturally, the production of vinegar depends on a mixed fermentation involving both yeast and AAB [3]. The organisms concerned in vinegar production usually grow at the top of the

substrate and form a jelly like mass. This mass is known as "mother of vinegar". The mother is composed of both *Acetobacter* and yeast working together. The yeast and AAB simultaneously exist in a form known as commensalism [4]. Much research work has indicated that growth of AAB in fermenting mash during alcoholic fermentation may contribute to the problem of stalled or incomplete alcoholic fermentation [5, 6, 7]. On the other hand, some research mentions that AAB accompanied with lactic acid bacteria and yeast took place during accumulation of alcohol in wine [8]. Using mother of vinegar, the vinegar is produced in simultaneous fermentation. However, vinegar is normally produced by a two - stage fermentation process, the first one being the conversion of fermentable sugars to ethanol by yeasts, usually *Saccharomyces* species, and the second the oxidation of ethanol by bacteria, normally *Acetobacter* species [9, 10, 11]. Even though some evidence suggests that the growth of AAB species can influence the growth activities of *S. cerevisiae* during alcoholic fermentation [12, 13], some yeast strains such as the genus *Zygosaccharomyces* are assumed to be a commensalistic interaction with AAB in traditional balsamic vinegar [11].

As mentioned, the AAB causes a negative effect on yeast due to the fermentation condition and acetic acid produced. However, there is no evidence of any studies on the biological interaction between AAB and yeast in simultaneous vinegar fermentation. To better understand the effect of AAB on growth of yeast, the suitable environment for both microorganisms was investigated. As a result of previous research on vinegar production from various kinds of tropical fruit, pineapple was selected as being an interesting source. Therefore, simultaneous pineapple vinegar was chosen as an example for this study. The interaction model of simultaneous pineapple vinegar fermentation using yeast -*S. cerevisiae* M30 and AAB -*A. aceti* WK is then investigated.

## Materials and Methods

### *Yeast strain and pineapple wine fermentation*

The *Saccharomyces cerevisiae* M30 strain is usually simply called “M30” and is a flocculate yeast kindly provided by the Yeast Biotechnology Laboratory, Department of Microbiology, Kasetsart University. M30 was used for pineapple wine fermentation. The medium used for pineapple wine fermentation was composed of the following ingredients: (per 100 litres of water) pineapple 1 kg, yeast extract 10g, sucrose 15 kg, $(NH_4)_2SO_4$ 50 g, $MgSO_4.7H_2O$ 20 g, pH 5.5. The fermenting mash was put in a 100L cell recycle reactor without aeration. After inoculation of 5% M30 which was cultivated in YM medium (composed of 10g yeast extract, 10g malt extract and 1 litre of distilled water) at 30°C for 1 day, the fermentation was controlled at 30°C for 150 hrs. The yeast cells, alcohol content, invert sugar and acidity (expressed in terms of lactic acid) were determined during fermentation.

### *Bacterial strain and vinegar fermentation*

The *Acetobacter aceti* WK, called “WK”, is an acetic acid bacteria (AAB) which has been adapted for vinegar production by using pineapple wine as substrate. The complex medium used for vinegar production had the compositions as mentioned in our previous investigation [14]: (per litre of water) glucose 5.0 g, yeast extract 2.5 g, peptone 1.0 g. After autoclaving, the 4.5% total concentration consisting of 1% acetic acid of vinegar and 3.5% ethanol were added into the medium and inoculated with 5% AAB-WK, which was cultivated in the same complex medium at 30°C for 2-3 days. Vinegar was fermented in 2L Erlenmeyer flask with ambient air supply. The viable cells of WK, alcohol content and acidity (expressed in terms of acetic acid) during 14 days of fermentation were determined.

***Simultaneous pineapple vinegar fermentation (SPVF)***

The 5% M30 cultivated in the medium mentioned previously was primarily inoculated into the sterile fermenting medium composed of pineapple juice (containing 18% sugar) and the following nutrients: (per litre of water) yeast extract 10g, $(NH_4)_2HPO_4$ 20g, $(NH_4)_2SO_4$ 50 g, $MgSO_4.7H_2O$ 20 g, pH 5.5. Subsequently, the 5% AAB-WK inoculum was inoculated into the fermenting medium in fermentation vessel. As shown in Table 1, there were 5 models of SPVF according to the different inoculation time of AAB-WK consisting of 0, 1, 2, 3 and 4 days, respectively. The yeast cells, viable cells of AAB-WK, alcohol content and acidity (expressed in terms of acetic acid) were determined during 20 days of fermentation at 30°C.

**Table 1**. **Experimental model for simultaneous pineapple vinegar fermentation (SPVF) by using yeast-*S. cerevisiae* M30 and AAB-*A. aceti* WK.**

| Experimental Model | Inoculation day | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| SPVF-1 | M30&WK | | | | |
| SPVF-2 | M30 | WK | | | |
| SPVF-3 | M30 | | WK | | |
| SPVF-4 | M30 | | | WK | |
| SPVF-5 | M30 | | | | WK |

***Analytical procedure***

The viable yeast-M30 cells and AAB-WK cells were enumerated by using spread plate technique on YM medium and GYE medium (composed of glucose 100g, yeast extract 10g, agar 15g and distilled water 1 litre), respectively. The acidity by titration and invert sugar by Lane and Enon method were analyzed according to AOAC [15]. Alcohol content was monitored by Ebulliometer in comparison with alcohol hydrometer.

***Experimental design and statistical analysis***

All experiments were designed by using complete randomized design (CRD). All experiments were carried out in triplicate. The results expressed by mean are shown in each figure.

**Results and Discussion**

The flocculating yeast *S. cerevisiae* M30 is usually used for alcohol fermentation. For suitable suspending of this sediment yeast in fermenting mash, the cell recycle reactor is designed. Normally, the cell recycle is a simple process, stable operation [16] and has been used to produce onion alcohol by using repeated batch process [17].

The fermentation profile of pineapple wine by M30 was investigated in 100L cell recycle reactor without aeration. As shown in Figure 1, the exponential growth of yeast was observed during the early 24 – 48 hrs of fermentation time due to good suspension of flocculating yeast in the fermenting broth. Simultaneously, alcohol was rapidly produced with the productivity of 0.177 - 0.275 g/L/hr in this fermentation period. Finally, this pineapple wine containing 9.6% alcohol and 0.5% acidity was obtained when the alcoholic fermentation was completed at 150 hrs.

From the fermentation profile, it could be implied that fermentation with no aeration during the first 48 hrs was suitable for alcohol fermentation by M30. Then the aeration should be applied soon after to encourage the AAB in producing pineapple vinegar in the simultaneous fermentation system.

The pineapple wine obtained was used for vinegar production by AAB-WK under ambient air supply. 1% acetic acid was added in the fermenting medium as a carbon source for AAB-WK. As shown in Figure 2, it can be observed that the AAB-WK cells could grow exponentially in the pineapple wine within 4 days of fermentation time at 30°C while the alcohol was rapidly

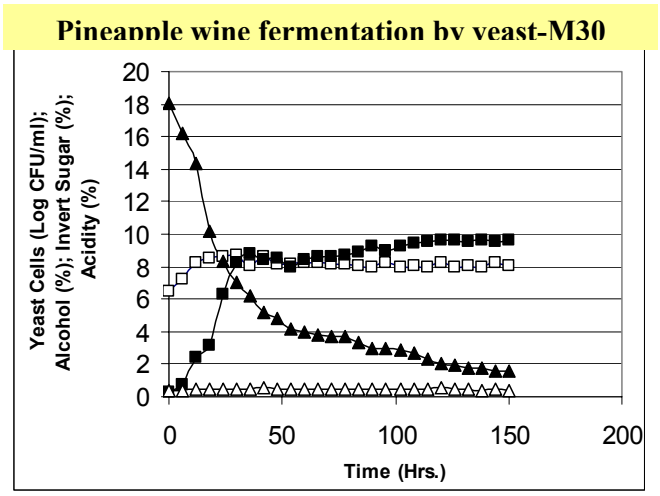


**Figure 1**. **Time course profiles of pineapple wine production by *S. cerevisiae* M30 in 100L cell recycle reactor at 30°C for 150 hrs.**
The symbols represent the following: yeast cells, empty square; alcohol, filled square; invert sugar, filled triangle; acidity, empty triangle.

utilized. Results showed that AAB-WK could promptly adapt itself in the fermenting medium. The adaptation of AAB in new fermenting medium is necessary for vinegar production. Additionally, this pineapple vinegar fermentation profile by AAB-WK implied that AAB-WK cells could well adapt themselves in pineapple wine and can be used for further study of simultaneous pineapple vinegar fermentation. However, the acetic acid was produced at least 0.8 – 0.9% during 14 days. This low productivity of acetic acid (0.0026 – 0.0028 g/L/hr) resulted from the small amount of alcohol left after 4 days because most of alcohol was used for adaptation of *A. aceti* WK. This result was different from Silva *et al.* [18] that no adaptation period was observed during cashew wine vinegar production.

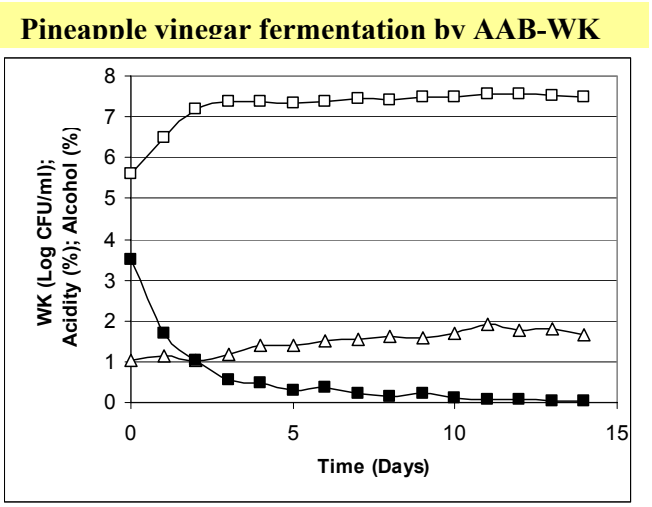


**Figure 2. Time course profiles of pineapple vinegar fermentation by *A. aceti* WK in 2L fermentation vessel with ambient air supply for 14 days at 30°C.**
The symbols represent the following: *A. aceti* WK cells, empty square; acidity, empty triangle; alcohol, filled square.

The simultaneous pineapple vinegar fermentation (SPVF) by yeast-M30 and AAB-WK was conducted. The interaction between both organisms during fermentation was the aim of this study. Five experimental models of SPVF were investigated. In the first model, both organisms were simultaneously inoculated into the fermenting medium called "SPVF-1 model", as shown in Table 1. However, there were different conditions of alcohol and acetic acid fermentation. As shown in Figure 1, the set up for the first 2 days of fermentation was no aeration for alcohol fermentation by M30. Aeration was then started to promote the acetic acid production by AAB-WK.

As shown in Figure 3, M30 could produce alcohol for the first 2 days under a no aeration fermentation system. However, increased growth of yeast cells was observed as resulting from the aeration effect. The metabolism of the yeast was changed from alcohol fermentation to respiration which encouraged more budding within the next 5 days. The rapid decrease in alcohol was observed after 2 days because of the oxidation of alcohol to acetic acid by AAB-WK. The low growth rate of AAB-WK in this SPVF was observed and it caused low acetic acid production. From this result, it was noticed that the commensalism between M30 and AAB-WK occurred during the first 7 days of SPVF due to the supply of alcohol from yeast to AAB for its oxidation. After 7 days of fermentation, the rapid decrease in yeast was clearly found. This result was due to the population of AAB up to 6 Log CFU/ml in fermenting medium which is sufficient to cause the death of *S. cerevisiae* as reported by Grossmann and Becker [19]. It demonstrates that AAB causes an antagonistic effect to the yeast. However, the mechanism of antagonism was not elucidated. The growth of AAB-WK with M30 induced the incomplete fermentation with low alcohol (4.35%). This result is similar to that mentioned by Drysdale and Fleet [5]. However, this low amount of alcohol was oxidized by AAB-WK during SPVF until alcohol was used up after 15 days. Then, the reduction of *A. aceti* WK was clearly observed, but it still remained in the level of 3.7 Log CFU/g at the end of fermentation.

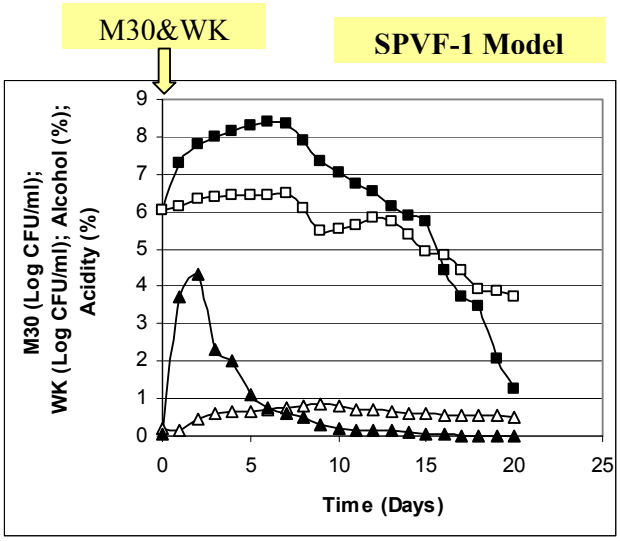


**Figure 3. Time course profiles of SPVF-1 model by simultaneously inoculating *S. cerevisiae* M30 and *A. aceti* WK in 2L fermentation vessel at 30°C. Ambient air was supplied after 2 days of fermentation.**

The symbols represent the following: *A. aceti* WK cells, empty square; *S. cerevisiae* M30, filled square; acidity, empty triangle; alcohol, filled triangle.

The second model of SPVF called "SPVF-2 model" is shown in Figure 4. The AAB-WK was inoculated into the fermenting medium after 1 day of yeast fermentation. More alcohol (4.92%) was found in the fermenting medium after 1 day of yeast fermentation because of the rapid increase of M30 in fermenting medium. Nevertheless, the yeast still produced alcohol when the fermenting medium was inoculated with AAB-WK and started to aerate within the next 1 – 2 days. This was due to the positive effect of oxygen on yeast ability to produce ethanol as reported by Alexandre and Charpentier [20], because it increases fatty acid and sterol biosynthesis in the yeast. The ability of alcoholic fermentation under aerobic conditions has been observed in some Traditional Balsamic Vinegar (TBA) species, especially *Zygosaccharomyces bailii* and in *S. cerevisiae* [21]. The results showed that M30 has an ability to carry out alcoholic fermentation despite aerobic conditions.

During 5 days after inoculation under ambient air supply, the AAB-WK was still well adapted to the fermenting medium. After completely adapting, the AAB-WK could grow at two more log cycles at 6 days after inoculation and maintained the amount of cells (approx. 7.9 – 8.6 Log CFU/g) until the end of 20 days of this SPVF. No reduction of AAB-WK was observed. However, the growth of yeast was rapidly decreased after 12 – 13 days of fermentation by the antagonistic effect of 2% acetic acid produced from the AAB-WK. The reason for this negative effect on growth of *S. cerevisiae* by acetic acid was explained by Zhao *et al*. [13] in that not only the glycolytic enzymes but also the NADH dehydrogenase of yeast *S. cerevisiae* were sensitive to acetic acid. After the death of yeast, the excretion of various growth stimulatory nutrients occurs in the fermenting medium which can stimulate the growth of AAB, as reported by Alexandre *et al*. [22]. Thus, a high amount of AAB-WK cells still remained in the fermenting medium.

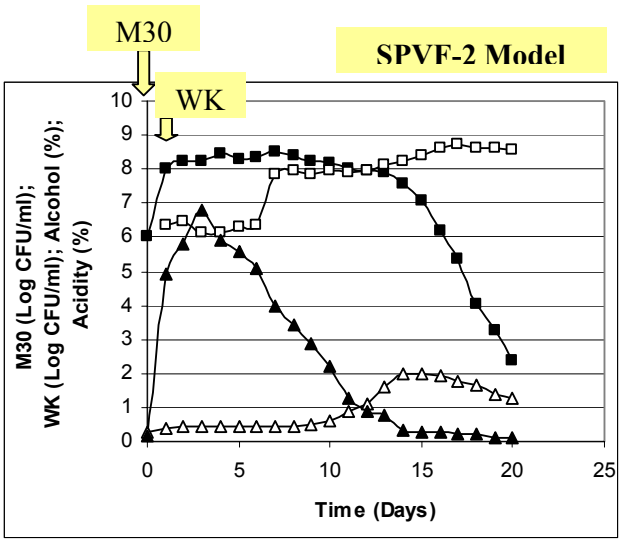


**Figure 4. Time course profiles of SPVF-2 model by inoculation of *A. aceti* WK after 1 day of yeast fermentation by *S. cerevisiae* M30 in 2L fermentation vessel with ambient air supply at 30°C.**

The symbols represent the following: *A. aceti* WK cells, empty square; *S. cerevisiae* M30, filled square; acidity, empty triangle; alcohol, filled triangle.

Figure 5 shows the result of the third model of SPVF called "SPVF-3 model". The AAB-WK was inoculated into the fermenting medium after 2 days of yeast fermentation. The yeast-M30 could produce more alcohol when compared with that obtained from the first model, SPVF-1 model (as shown in Figure 3), but was similar to that obtained from the second, SPVF-2 model. The result of alcoholic fermentation confirmed that M30 has an ability to carry out alcoholic fermentation under aerobic conditions. The rapid production of alcohol was found in the first 2 days and the rapid reduction of alcohol was observed after 5 days of fermentation (or the 3 days of SPVF). The high amount of yeast was maintained until 10 days of fermentation and then exponential reduction of yeast growth occurred after 10 days until no yeast cells were found at 18 days.

The reduction of alcohol in the fermenting medium occurred after 3 days of inoculation with AAB-WK and it was an indicator that the AAB-WK rapidly adapted. Then the AAB-WK cells gradually increased about two more log cycles within the next 4 days and slightly increased until the end of this SPVF (20 days). When the AAB-WK grew to approx. 8.3 log CFU/ml at 11 days of total fermentation time (or 8 days after inoculation), the yeast cells of M30 started to decrease following the increase of acetic acid accumulation produced by AAB-WK. This result implied that the growth of AAB-WK discouraged the growth of yeast-M30.

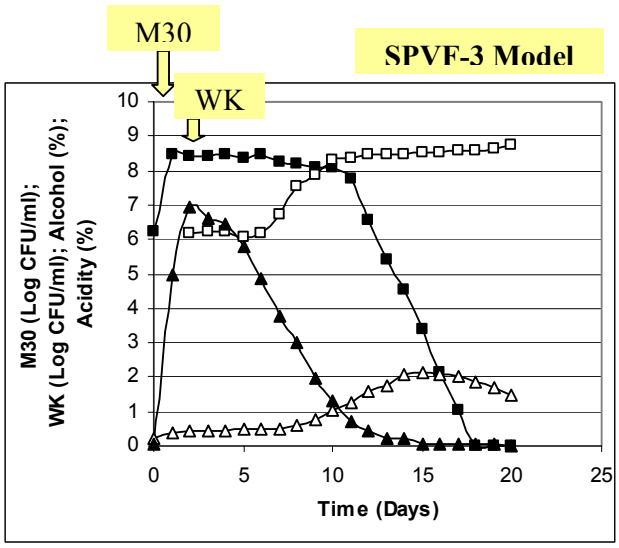


**Figure 5. Time course profiles of SPVF-3 model by inoculation of *A. aceti* WK after 2 days of yeast fermentation by *S. cerevisiae* M30 in 2L fermentation vessel with ambient air supply at 30°C.**

The symbols represent the following: *A. aceti* WK cells, empty square; *S. cerevisiae* M30, filled square; acidity, empty triangle; alcohol, filled triangle.

The "SPVF-4 model" or the fourth model of SPVF is shown in Figure 6. The AAB-WK was inoculated into the fermenting medium after 3 days of yeast fermentation. The profile of M30 growth and alcohol fermentation was similar to that obtained from the second model (SPVF-2 model) and the third model (SPVF-3 model) as shown in Figures 4 and 5, respectively. It implied that the 3 days of yeast fermentation was not necessary in this SPVF. The AAB-WK also showed rapid adaptation after inoculation into the fermenting medium. Two more log cycle increases of AAB-WK were also found. When the AAB-WK cells reached to 8.3 log CFU/ml (within 6 days after inoculation), the yeast cells started to reduce. No yeast cells were found at 18 days, while the large amount of AAB-WK cells were still maintained.

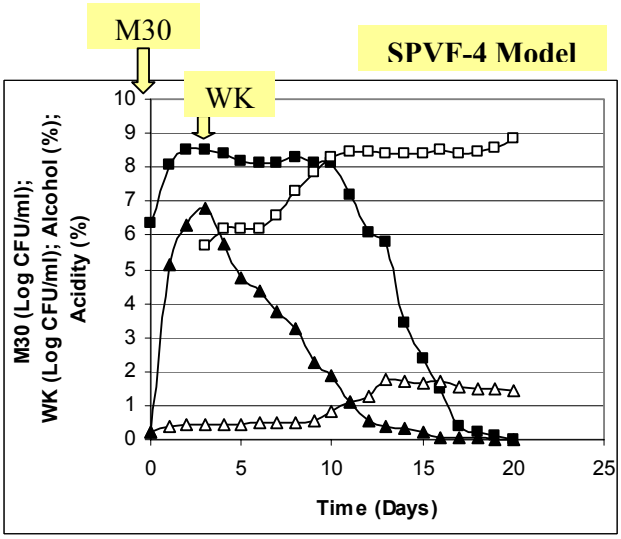

**Figure 6. Time course profiles of SPVF-4 model by inoculation of *A. aceti* WK after 3 days of yeast fermentation by *S. cerevisiae* M30 in 2L fermentation vessel with ambient air supply at 30°C.**

The symbols represent the following: *A. aceti* WK cells, empty square; *S. cerevisiae* M30, filled square; acidity, empty triangle; alcohol, filled triangle.

The fifth model of SPVF called "SPVF-5 model" is shown in Figure 7. The AAB-WK was inoculated into the fermenting medium after 4 days of yeast fermentation. No effect of extending yeast fermentation period on alcohol produced found. The yeast cells were also exponentially reduced when the growth of AAB-WK arrived at 8 log CFU/ml, while the high amount of AAB-WK was maintained until the end of fermentation.

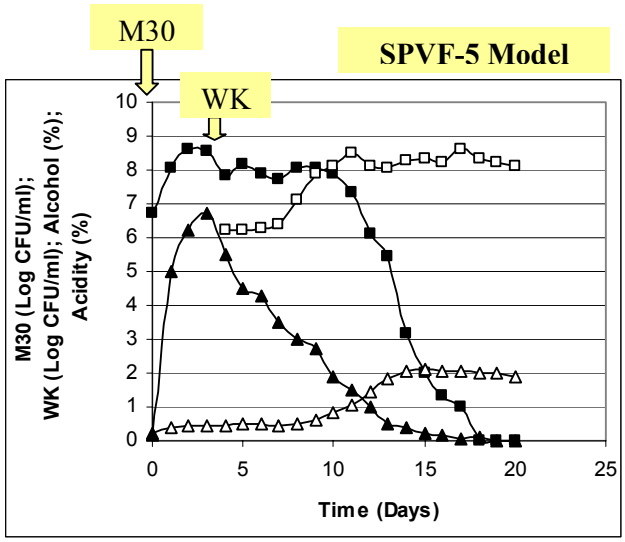


**Figure 7. Time course profiles of SPVF-5 by inoculation of *A. aceti* WK after 4 days of yeast fermentation by *S. cerevisiae* M30 in 2L fermentation vessel with ambient air supply at 30°C.**

The symbols represent the following: *A. aceti* WK cells, empty square; *S. cerevisiae* M30, filled square; acidity, empty triangle; alcohol, filled triangle.
(M30 = *S. cerevisiae* M30; WK = *A. aceti* WK)

## Conclusions

From these results, it could be considered that there are two biological interactions between yeast and AAB in this simultaneous pineapple vinegar fermentation. One is commensalism, which occurs during the early stage of fermentation as yeast-M30 provided alcohol to AAB-WK for further oxidation. This result complied with the investigation by the Food and Agriculture Organization [3]. The period of commensalism relates to the inoculation of both organisms. When the simultaneous inoculation of both organisms was used, the shorter period occurred due to low alcohol provided by yeast-M30. However, 10 days of commensalism period could be noticed when the inoculation of AAB-WK after yeast fermentation for 1 – 4 days. During SPVF, aeration was supplied to promote the growth of AAB-WK and its oxidation activity. However, it was noticed that there was a positive effect of oxygen on yeast- M30's ability to produce more ethanol which in turn was supplied to AAB-WK and caused the longer period of commensalisms.

As mentioned by Grossmann and Becker [19], the population of AAB-WK up to 6 Log CFU/ml in fermenting medium is sufficient to cause the death of yeast-M30. This is similar to these results when simultaneous inoculation of both organisms was used. Additionally, the negative effect of AAB-WK up to 8 Log CFU/ml on yeast-M30 was found in the case of inoculation of AAB-WK after yeast fermentation for 1 – 4 days. This was due to low acetic acid produced during the commensalism period between yeast-M30 and AAB-WK. It indicated that ethanol was utilized as a carbon source for AAB-WK growth but not for oxidation to produce acetic acid. This indicates that there is significant advantage of commensalisms in the first phase of this biological interaction.

Moreover, it could be noticed that the growth of AAB-WK during this interaction period can influence the growth activities of yeast-M30 and causes a lower amount of alcohol produced when compared with conventional yeast alcoholic fermentation - fermentation by yeast-M30 without AAB-WK.

Another interaction between yeast-M30 and AAB-WK is antagonism. AAB-WK causes an antagonistic effect on growth of yeast-M30. This result was in line with the research conducted by many investigators [2, 5, 6, 13]. There are two reasons from two groups of investigators. The first reason was reported by Pampulha and Loureiro-Dias [23]. Weak lipophilic acids and low pH have a synergic effect and reduce intracellular pH below the normal physiological values, inhibiting the yeast growth. Moreover, acetic acid seems to directly affect transport or enzymatic activities, such as enolase, a key enzyme of glycolysis. In addition, Zhao *et al*. [13] explained that not only the glycolytic enzymes but also the NADH dehydrogenase of yeast *S. cerevisiae* were sensitive to acetic acid. However, the mechanism of antagonism was not elucidated and needed more investigation.

As the result of this research, only the antagonistic effect of AAB-WK on yeast-M30 was reported while the impact of yeast-M30 on AAB-WK was not found. In contrast to the results by Drysdale and Fleet [2], high ethanol values (up to 7 – 8%) can inhibit AAB, especially at low pH values. The reason that this yeast-AAB antagonism in the SPVF was not found was because the lower 7% alcohol content was found in the fermenting medium and alcohol was rapidly utilized by AAB-WK in the first phase of interaction. Only small amounts of alcohol remained for the second interaction, therefore, no inhibitory effect on AAB-WK took place.

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