

STUDY ON POSSIBILITY OF THE USE OF *Kluyveromyces marxianus* DMKU3 (TROPICAL YEAST) AND THERMOADAPTED *Acetobacter pasteurianus* TH3 IN SIMULTANEOUS VINEGAR PRODUCTION AT HIGHER TEMPERATURE

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ABSTRACT

Increasing temperature in recent years poses a substantial influence on fermentation industries and thus, huge cooling systems are required to maintain the optimum temperature leading to additional cost of production. In the present study, simultaneous vinegar fermentation with thermotolerant yeast (*Kluyveromyces marxianus* DMKU3) and thermo-adapted acetic acid bacteria (*Acetobacter pasteurianus* TH3) was performed at 37, 40 and 41°C with 10% initial glucose in shaking and static cultures. The highest ethanol production (4.952±0.02 % v/v) by tropical yeast and the highest acetate production (4.44±0.01 % w/v) was observed at 37 °C when both strains were added together at the beginning of fermentation. Moreover, it was found that higher vinegar production was achieved under shaking culture than the static culture. Moreover, the tropical yeast and the thermoadapted TH3 strains were able to use in simultaneous coconut water vinegar production at 37 and 40 °C successfully with an acetic acid production of 2.53±0.01% (w/v) and 2.34±0.03% (w/v) respectively. Thus, thermotolerant yeast and thermo-adapted bacteria could be successfully used in vinegar fermentation under higher temperature conditions.

Keywords: Fermentation, Simultaneous vinegar production, Thermo-adapted acetic acid bacteria, Tropical yeast

INTRODUCTION

Vinegar production and use of vinegar have been made by mankind for thousands of years. The fermentation of vinegar has been known from very ancient time by natural fermentation of ethanol containing solutions without understanding the nature of the process. The first description of vinegar fermentation was made by Pasteur in 1862 (Saeki *et al.* 1997). He recognized that the mother of vinegar was a mass of living organisms that caused acetic acid fermentation. Among the genera of acetic acid bacteria (AAB), *Acetobacter* and *Gluconacetobacter* are the most popular, and have been used for acetic acid fermentation due to their powerful ability to oxidize ethanol and to tolerate high acetic acid concentration accumulated in the medium (Kanchanarach *et al.* 2010b). This vinegar production is done by two sequential reactions catalyzed by

membrane bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH).

Usually, the industrial vinegar fermentation is performed around 30 °C with the use of mesophilic strains which can grow and perform oxidative fermentation under 30 °C. Thus, the increasing temperature in recent years poses a serious challenge to the fermentation industry. Also, during submerged culture, a large amount of heat is generated during fermentation. A temperature increment of 2-3 °C causes a serious failure in both fermentation rate and efficiency. Thus, a large cooling system is required for maintaining the optimum temperature to obtain a good production. Further, the use of mesophilic strains is a barrier for the development of vinegar fermentation in tropical regions where the temperature is regularly higher than 30 °C and the cooling

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water expenses could be a great problem. Thus, the development of strains which can grow and perform oxidative fermentation at temperatures around 40 °C will be beneficial.

Acetobacter pasteurianus SKU1108 is a thermotolerant AAB strain isolated from Thailand, and can grow and produce acetic acid at 37 °C (Saeki *et al.* 1997). *A. pasteurianus* TH3 strain is the thermo-adapted strain of SKU1108 strain, and was obtained by repeated cultivation of the wild type (*A. pasteurianus* SKU1108) at higher temperatures (Matsutani *et al.* 2013). This strain can grow and produce acetic acid even at 40 °C.

Kluyveromyces marxianus DMKU3-1042 is described as thermotolerant yeast which is phylogenetically related to *Saccharomyces cerevisiae*, and is a sister species to the better-known *K. lactis* (Melani and Morrissey 2010). *K. lactis* was recorded as the first yeast after *S. cerevisiae* to be named as GRAS (Generally Regarded as Safe) organism (Bonekamp and Oosterom 1994). This yeast is known as 'the tropical yeast' as it can grow and produce ethanol at temperatures above 40 °C (Sanom *et al.* 2008). Moreover, *K. marxianus* offers additional benefits including a high growth rate, and the ability to utilize a wide variety of industrially relevant substrates such as sugar cane, corn silage juice, molasses and whey powder. Also due to its long history of association with food products, helped this yeast to achieve GRAS and QPS (Qualified Presumption of Safety) in the United States and European Union, respectively (Melani and Morrissey, 2010). Thus, this yeast has been currently promoted as a viable alternative to *S. cerevisiae* as an ethanol producer.

This study was done to examine the ability of usage of thermo adapted *K. marxianus* DMKU3-1042 and *A. pasteurianus* TH3 strains in simultaneous vinegar production at higher temperatures under laboratory conditions.

Moreover, the possibility of the use of the above strains in simultaneous coconut water vinegar production at 37 °C and 40 °C was also performed.

MATERIALS AND METHODS

Used strains and culture media

Thermotolerant (*A. pasteurianus* SKU1108) and thermo adapted (*A. pasteurianus* TH3) strains of AAB and the tropical yeast (*K. marxianus*) used in this study were obtained from Yamaguchi University, Japan. YP medium (1 g of yeast extract and 2 g of polypeptone) plus 2 g of glucose in 100 ml of tap water (YPD medium) was used for pre-culture, and YP medium with 5 g or 10 g of glucose in 100 ml of tap water was used to monitor the growth and ethanol production of the tropical yeast at higher temperatures. Potato medium (1 g of yeast extract, 1 g of polypeptone, 2 g of glycerol, 0.5 g of glucose and 20 ml of potato extract, filled up to 100 ml with tap water) was used as the seed culture medium for pre-culturing AAB. YPG medium (0.2 g of yeast extract, 0.2 g of polypeptone, 0.5 g of glycerol and 0.5 g of glucose in 100 ml of tap water) supplement with 4% or 6% ethanol was used as the main culture medium to monitor the growth and acetic acid production of thermotolerant and thermo-adapted AAB and to monitor the simultaneous vinegar production.

Ethanol fermentation of tropical yeast

The overnight-cultured tropical yeast in YPD medium was inoculated into 100 ml of YP medium with 5% (w/v) and 10% (w/v) glucose in 500 ml flasks, and incubated at 37 °C, 40 °C and 41 °C in both shaking cultures (150 rpm) along with a static culture. Samples were collected at 0 h, 3 h, 6 h, 9 h, 12 h, and every 24 h intervals for two days period, to monitor the growth, ethanol production and glucose consumption at each temperature level.

Acetic acid fermentation of thermotolerant and thermo-adapted acetic acid bacteria

Required AAB strain was pre-incubated in potato medium for 24 h with 200 rpm shaking conditions. The above seed culture (5 ml) was inoculated in 100 ml of YPG medium with 4% (v/v) and 6% (v/v) ethanol, and was incubated at 30 °C, 37 °C, 40 °C and 41 °C for 10 days period in both shaking (200 rpm) and static culture. Samples were collected at 0 h, 3 h, 6 h, 9 h, 12 h, and every 24 h intervals

to measure the growth, acidity and ethanol percentage.

Simultaneous vinegar production by tropical yeast (*K. marxianus* DMKU3-1042) and thermo-adapted acetic acid bacteria (*A. pasteurianus* TH3)

K. marxianus and TH3 strains were pre-incubated for 24 h period in YPD and potato medium respectively at 30 °C in a bio shaker with 200 rpm. Subsequently, 5% of pre-cultured inoculums of *K. marxianus* and TH3 were inoculated into 100 ml of the main culture (YPG + 10% Glucose) as shown in Table 1. Simultaneous vinegar fermentation was monitored at both shaking culture and static culture at 37 °C, 40 °C and 41 °C.

Samples were collected at 0 h, 3 h, 6 h, 10 h, and every 24 h intervals for 10 days to measure the growth, ethanol %, acidity % and alcohol %.

Production of coconut water vinegar

The use of thermotolerant *K. marxianus* with *A. pasteurianus* SKU1108 and TH3 strains in simultaneous coconut water vinegar production was examined at 37 °C and 40 °C. Variation in ethanol, acetic acid and glucose levels were monitored by taking samples at 0 h, 3 h, 6 h, 9 h, 12 h and each 24 h intervals for 10 days.

Other analytical procedures

Growth of *K. marxianus* and TH3 strains was monitored by measuring optical density at 600 nm using the UV visible spectrophotometer. Ethanol in culture medium was measured enzymatically using membrane fraction obtained from *Gluconobacter frateurii* with ferricyanide as an electron acceptor (Ameyama, 1982).

Table 1: Experimental model for Simultaneous Vinegar Fermentation (SVF)

Experimental model	Inoculation days	
	0 day	1 day
SVF 1	<i>K. marxianus</i> and TH3	
SVF 2	<i>K.marxianus</i>	TH3

Glucose was measured enzymatically with quinoprotein glucose dehydrogenase (TOYOBO). The acidity of the culture medium was measured by titration with 0.8N NaOH using phenolphthalein as a pH indicator.

Statistical analysis

Each experiment was repeated three times while taking all the measurements in triplicates. Obtained results were statistically analyzed using the Two-way ANOVA method by GraphPad Prism 5 software.

RESULTS AND DISCUSSION

Ethanol fermentation of tropical yeast

Ethanol production of *K. marxianus* was observed at 37, 40 and 41 °C with 5% (w/v) and 10% (w/v) initial glucose concentrations, and the obtained results are summarized in Figure 1. As shown in Figure 1, *K. marxianus* showed similar profiles of growth either with 5% (w/v) or 10% (w/v) initial glucose levels at all the tested temperature levels. Moreover, *K. marxianus* has given its highest ethanol production 4% (v/v) with 10% (w/v) initial glucose either at 37 °C or 40 °C. Moreover, even at 41 °C, it showed a rapid growth either with 5% and 10% initial glucose level and, gave an ethanol production of 2% (v/v) and 3.3% (v/v) respectively. However, the ethanol production was found to be comparatively less than that of 30 °C and 37 °C. Moreover, when compared to the growth and ethanol production in shaking culture, both the growth and ethanol production of *K. marxianus* at all the tested temperature levels under static culture conditions was found to be very poor (data not shown). As it is reported by Fonseca *et al.* (2007), *K. marxianus* is an aerobic microorganism. As a result, due to lack of oxygen presence under static culture will badly affect the performance of *K. marxianus*.

A study by Matsumoto *et al.* (2018) on the comparison of growth of *S. cerevisiae* and *K. marxianus* at 30 °C and 45 °C revealed that both species could grow well with similar growth profiles at 30 °C. However, in contrast to *S. cerevisiae*, *K. marxianus* showed their growth even at 45 °C even though the growth level is slightly less than that of 30 °C.

Furthermore, when compared to *S. cerevisiae*, they confirmed that *K. marxianus* can withstand a heat shock at 50 °C. Accordingly, the *K. marxianus* DMKU3 used in the current study was also performed a good growth and ethanol production at 41 °C showing its potential to be used in ethanol production at higher temperatures.

Acetic acid fermentation of thermotolerant and thermo-adapted acetic acid bacteria

The acetic acid production of the thermotolerant SKU1108 (Figure 2) and its thermo-adapted TH3 (Figure 3) strains were observed with 4% (v/v) and 6% (v/v) ethanol levels at 30, 37, 40 and 41 °C under both shaking and static culture. According to the results of their growth profiles, SKU1108 strain was found to be grew well at both 30 and 37 °C either with 4% (v/v) or 6 % (v/v) initial ethanol. As it is shown in Figure 2, SKU1108 strain showed an acetic acid

production of about $3.44\% \pm 0.052$ (w/v) and $3.127\% \pm 0.25$ (w/v) with 4% (v/v) initial ethanol at 30 or 37 °C respectively, showing a complete conversion of added ethanol. Moreover, a considerable amount of acetic acid production was observed even with 6% (v/v) initial ethanol level too. Further, both at 30 °C and 37 °C the SKU1108 strain was found to be growing well without showing a measurable lag phase. However, at 40 °C, SKU1108 showed $3.34\% \pm 0.058$ (w/v) and $2.297\% \pm 0.038$ (w/v) of acetic acid production either with 4% (v/v) or 6% (v/v) initial ethanol respectively. Nevertheless, at 40 °C, the SKU1108 strain showed a considerable lag phase showing that the strain requires some time to adjust to the new stressful environment. Furthermore, when compared to the lag phase at 4% (v/v) initial ethanol level, it has taken about 6 days of lag phase at 6% (v/v) ethanol level since both the higher temperature and ethanol level has increased

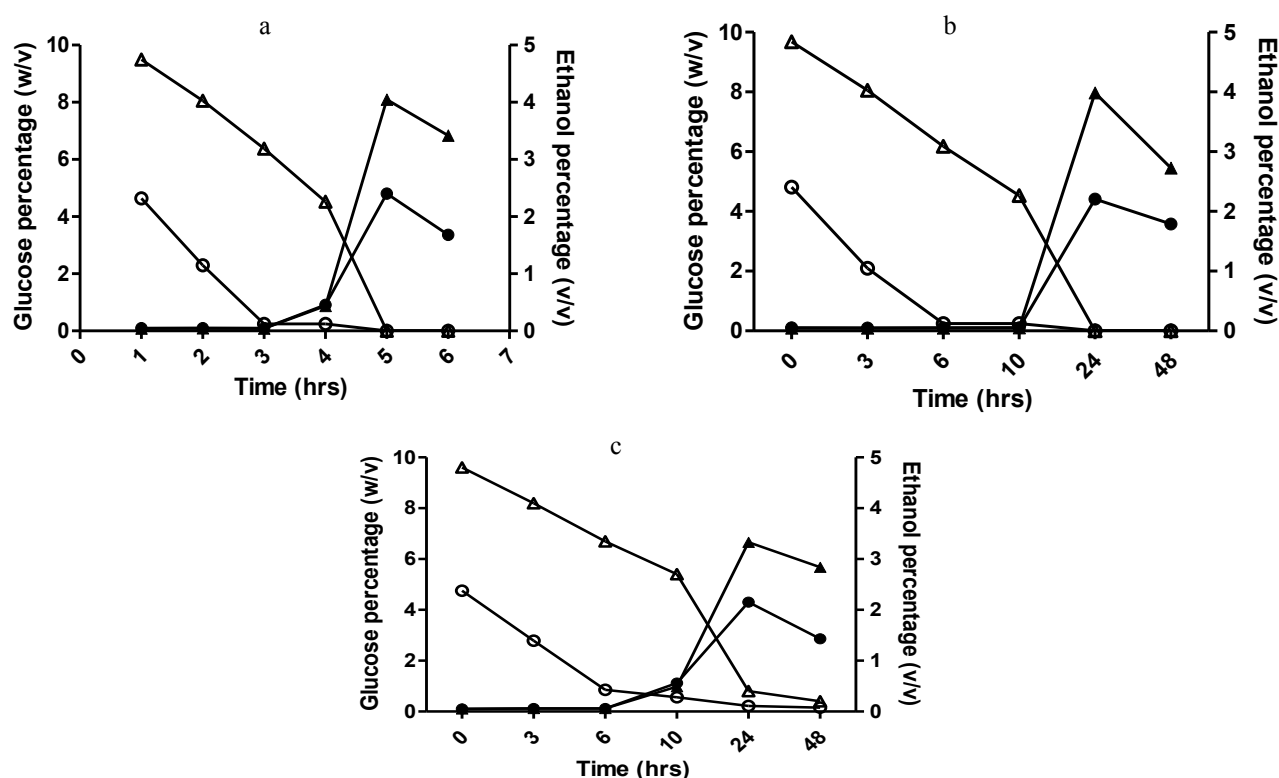


Figure 1: Glucose consumption and ethanol production profiles of *K. marxianus* with 5% and 10% initial glucose levels at (a) 37 °C, (b) 40 °C and (c) 41 °C under shaking culture (150 rpm). White circles and white triangles represent the glucose consumption with 5% (w/v) and 10% (w/v) glucose levels respectively. Black circles and black triangles represent the ethanol production with 5% (w/v) and 10% (w/v) glucose respectively.

their stress level. As a whole, the SKU1108 strain gave its highest acetic acid production of $3.42\% \pm 0.052$ (w/v) at 30°C with 4% initial ethanol, and it was found to be significantly higher (99% probability level) than that of other temperature levels. Moreover, it gave a significantly higher (95% probability level) acetic acid level of $3.34\% \pm 0.072$ (w/v) even at 40°C . These results suggest that the SKU1108 strain can be successfully used in acetic acid production at higher temperature levels. Moreover, the SKU1108 strain did not show any growth or acetic acid production at 41°C (data not shown).

A study done by Kanchanarach *et al.* (2010a) showed a similar profile of acetic acid production by *A. pasteurianus* SKU1108 at 30 and 37°C . They further compared the acetic acid production of the thermotolerant SKU1108 strain with mesophilic *A. pasteurianus* IFO3191 and found that

IFO3191 is showing a very poor growth performance and acetate production at 37°C even with 4% (v/v) initial ethanol level. Moreover, they have examined the activity of the membrane-bound alcohol dehydrogenase (ADH) enzymes of SKU1108 and the results were compared with those of the mesophilic IFO3191 strain. According to the obtained results, ADH activity of SKU1108 strain was found to be high when the cells were grown at 37°C indicating that the high acetate production of SKU1108 strain at higher temperatures is due to the increase in ADH activity when the cells were grown at higher temperatures.

Subsequently, the acetic acid production of the thermo-adapted TH3 strain was examined at 37 , 40 and 41°C , and the obtained results are summarized in Figure 3. According to Matsutani *et al.* (2013), the thermo-adapted TH3 strain was taken by repeated cultivation of the thermotolerant SKU1108 strain at

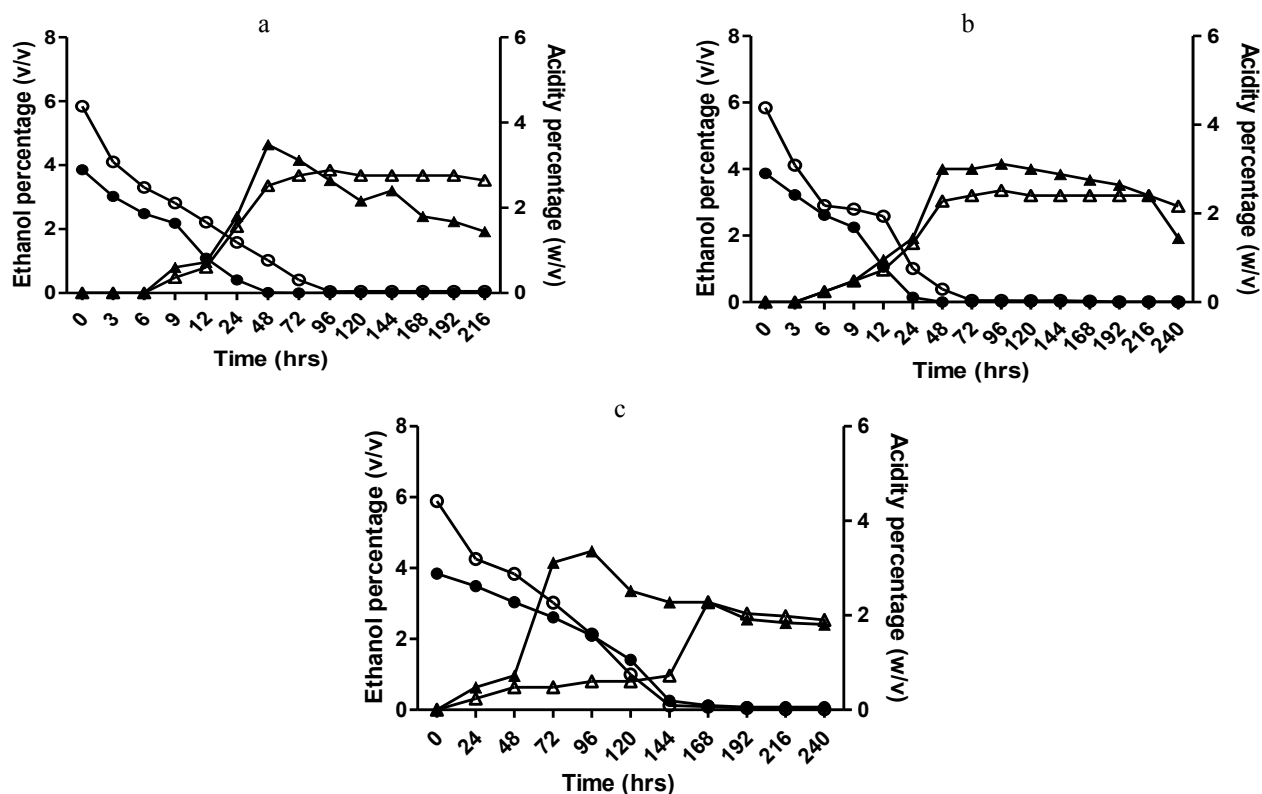


Figure 2: Acetate production of SKU1108 strain at (a) 30°C , (b) 37°C and (c) 40°C levels with 4% and 6% ethanol in shaking culture (200 rpm).

Black and white circles represent percentage ethanol at 4% and 6% ethanol, respectively. Black and white triangles represent percentage acidity with 4% and 6% ethanol, respectively.

higher temperatures. As a result, the thermo-adapted TH3 strain could grow and perform oxidative fermentation at higher temperatures effectively. According to the obtained results, the TH3 strain showed its highest acetic acid production at 37 °C where the initially added ethanol was almost completely converted into acetic acid during the fermentation process. In addition, the maximum level of the acetic acid production by TH3 strain (5.277%±0.086 w/v) was observed at 37 °C and it was found to be significantly higher (at 0.001 probability level) than that of SKU1108 strain at 30°C. Moreover, even at 40 °C, when compared to SKU1108 strain, the TH3 strain gave a considerable amount of acetic acid production either with 4% (v/v) or 6% (v/v) initial ethanol level. However, in contrast, to the thermotolerant SKU1108, the thermo-adapted TH3 strain shows a 3% (w/v) acetic acid production at 41 °C with 4% (v/v) initial

ethanol showing its ability to grow and perform oxidation at higher temperatures.

Interestingly, unlike the thermotolerant SKU1108 strain, the thermo-adapted TH3 strain does not show any acetate overoxidation, which is beneficial in the fermentation industry. This is mainly due to the production of the higher amount of acetic acid which suppress the acetate overoxidation ability of AAB. According to Saeki *et al.* (1997), in acetic acid fermentation, acetate overoxidation was observed when the final acetic acid content in the culture medium is less than 3.7% (w/v). In the current study, the acetic acid production of the thermotolerant SKU1108 strain was found to be less than 3.7% (w/v), and thus a strong acetate overoxidation was observed at all three tested temperature levels. In contrast, the acid production of TH3 strain at 37 and 40 °C were found to be greater than 3.7% (w/v), and

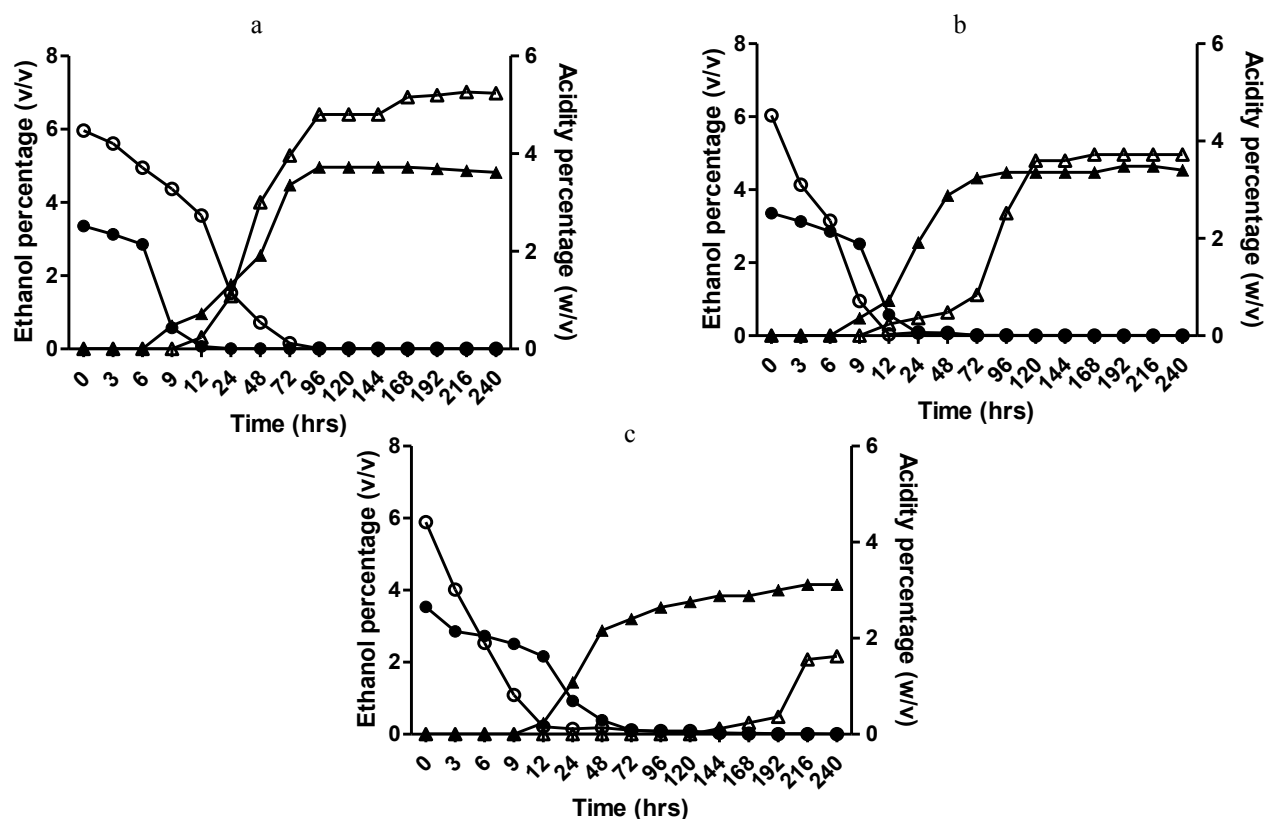


Figure 3: Acetate production of TH3 strain at (a) 37 °C, (b) 40 °C and (c) 41°C levels with 4% and 6% initial ethanol in shaking culture (200 rpm).

Black and white circles represent percentage ethanol at 4% and 6% ethanol, respectively. Black and white triangles represent percentage acidity with 4% and 6% ethanol, respectively.

consequently no acetate oxidation was observed. Moreover, as it stated by Perumpuli *et al.* (2014) even though the produced acetic acid level is less than 3.7% (w/v), acetate over oxidation was not observed by thermotolerant AAB strains when they are grown in a culture medium with 6% (v/v) initial ethanol level. By considering all the obtained results, *A. pasteurianus* TH3 strain can be identified as a potential candidate in vinegar production at higher temperatures.

Simultaneous vinegar production by tropical yeast (*K. marxianus* DMKU3-

1042) and thermo adapted acetic acid bacteria (*A. pasteurianus* TH3)

Normally, vinegar production is a mixed fermentation of yeast and AAB with two-stage fermentation where during the first stage, the fermentable sugar will be converted to ethanol by yeast, and secondly the produced ethanol will be converted in to acetic acid through AAB. During this process both the yeast and AAB form a pellicle on the top of the vinegar mass knowing as the 'mother vinegar'. Thus, the possibility of the use of tropical yeast and the thermo-adapted TH3 strain in simultaneous vinegar production was examined at 37 and 40 °C using 10% (w/

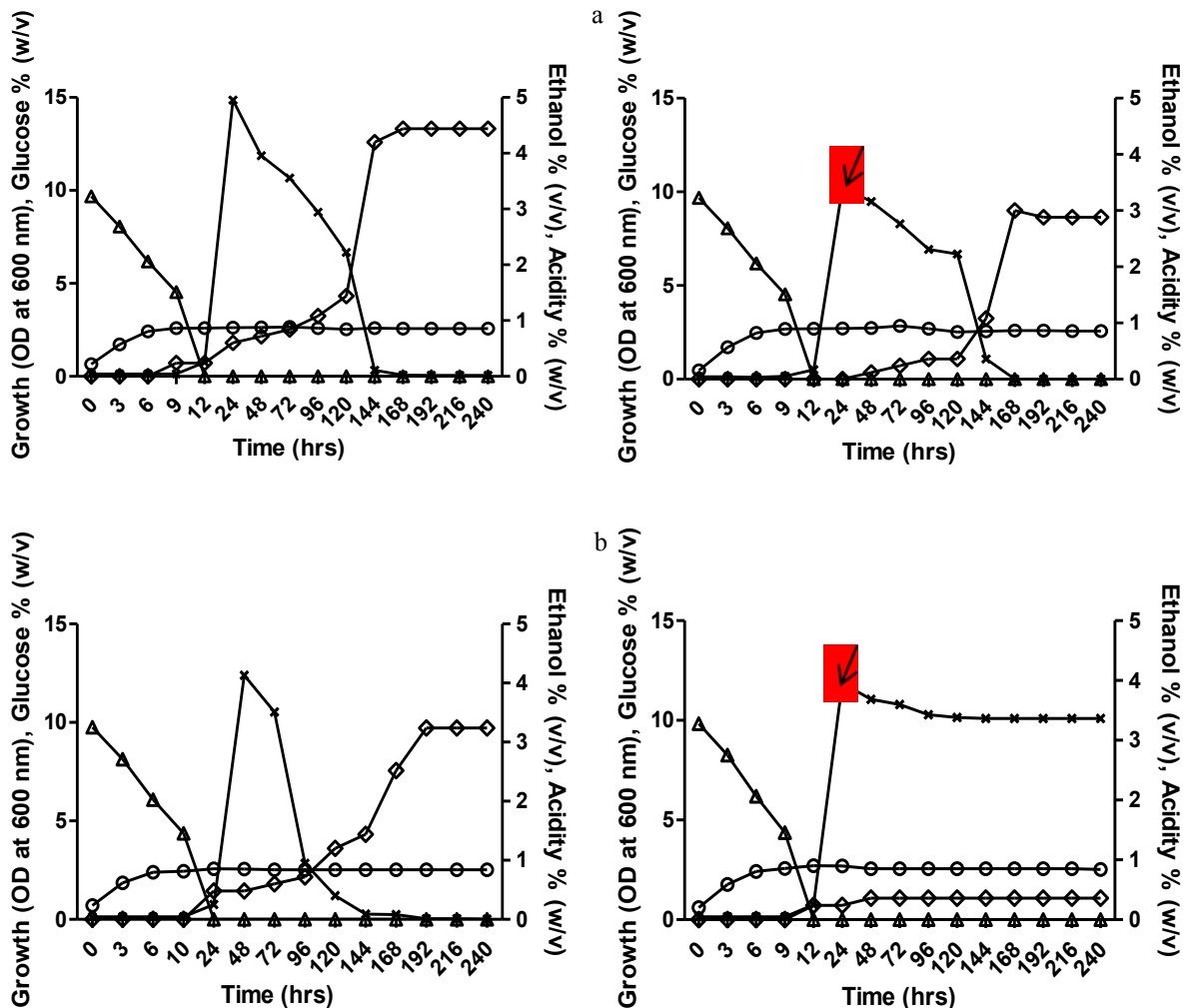


Figure 4: Simultaneous vinegar production (left panel - adding both yeast and AAB together, right panel - sequential addition of yeast and AAB) of *K. marxianus* and TH3 strain at (a) 37 °C, (b) 40 °C under shaking culture.

Circles represent the growth, crosses represent the ethanol percentage (v/v), diamonds represent the acetic acid percentage (w/v) and triangles represent the glucose percentage (w/v). Arrows indicate the time for inoculation of TH3 strain.

v) initial glucose level (Figure 4). Moreover, the ability to add both the yeast and AAB together from the beginning of the fermentation or sequential addition of the yeast and the AAB where the AAB was added at the peak alcohol production level was also examined.

As shown in Figure 4, both at 37 and 40 °C, better production of acetic acid was observed when the yeast and AAB were added together from the beginning of the ethanol fermentation. At both 37 and 40 °C, simultaneous addition of yeast and acetic acid bacteria from the beginning gave significantly higher acetic acid production at 99% probability with a final acetic acid production of $4.44\% \pm 0$ (w/v) and $3.24\% \pm 0$ (w/v) respectively. In sequential addition of the two strains, the TH3 strain was added to the culture medium after one day of ethanol production, where the peak ethanol production was observed and the level of glucose in the culture medium has reached a minimum level due to complete conversion of glucose into ethanol by yeast. However, for the growth and proper oxidative fermentation of AAB, a carbon source other than ethanol is essential to be present in the culture medium. Thus, due to the absence of glucose for initial growth and development, AAB will lead to poor acetate production during sequential addition of yeast and AAB in simultaneous vinegar production. Thus, adding both yeast and AAB together at the beginning of the fermentation will give a better production of acetic acid in vinegar production.

Moreover, a study was done by Drysdale and Fleet (1989) on effect of *Acetobacter* and *Gluconobacter* species on the growth of wine yeast during mixed culture of grape wine production reveals that the viable count of yeast cells was slightly impaired by the AAB. Besides, they found that the amount of sugars (glucose and fructose), ethanol, acetaldehyde, glycerol and other organic acids in the fermented juice also significantly influence the performance of wine yeast.

According to the results given in Figure 4, the highest amount of ethanol production

(4.952 ± 0.02 % v/v) by tropical yeast was observed at 37 °C when both the yeast and AAB were added together, and accordingly the highest amount of acetic acid production (4.44 ± 0.0 % w/v) was also observed. In contrast, both ethanol and acetic acid production at 40 °C were found to be comparatively low which may be due to the stress caused by high temperature. However, a considerable amount of alcohol (4.13 ± 0.04 % v/v) and acetic acid (3.24 ± 0.01 % w/v) was found to be produced even at 40 °C when both yeast and AAB were added together from the beginning. These results suggest that the tropical yeast and the thermo-adapted strain can be used in simultaneous vinegar production even at 40 °C, and further improvements are needed to increase the acetic acid production while reducing acetate overoxidation.

Krusong and Vichitraka (2010) have done a study to understand the interaction between AAB and wine yeast during simultaneous fermentation of pineapple vinegar. Through their study they have found that there is commensalism between the wine yeast and the AAB during the first seven days of fermentation where the wine yeast supplies the required ethanol for AAB for oxidation. There after a rapid decrease in the yeast population has been observed as a result of the increasing population of AAB that demonstrates an antagonistic effect to wine yeast by AAB. According to Zhao *et al.* (2008) the activity of wine yeast is affected by AAB due to the negative effect of acetic acid on both glycolytic enzymes and NADH dehydrogenase in yeast cells that responsible for ethanol fermentation. Furthermore, as it was reported by Drysdale and Fleet (1989) ethanol values that are higher than 7% (v/v) can inhibit the growth and activity of AAB at low pH values. However, in the current study, such antagonism was not observed since the alcohol level in the medium was less than 7% (v/v) and the produced alcohol was rapidly consumed by AAB for the production of acetic acid.

When comparing the acetic acid production of simultaneous vinegar fermentation with

shaking culture, there is poor acetic acid production in the static culture at all three temperature levels irrespective of the time of inoculation (data not shown). One reason for this poor acid production is, low ethanol production by the tropical yeast in static culture due to less amount of available oxygen in the media. According to Fonseca *et al.* (2007) *K. marxianus* requires oxygen for proper growth and ethanol production. Thus, due to the poor aeration condition, the amount of ethanol produced in static culture has become low either at 37 °C or 40 °C. Moreover, AAB is also obligate aerobic bacteria that require oxygen for acetic acid production, and consequently, the acetic acid produced during static culture was found to be low. However, the use of tropical yeast and the thermo-adapted TH3 strains on coconut water vinegar production was also examined at 37 °C and 40 °C under static conditions (Figure 5).

As it is shown in figure 5, through simultaneous coconut vinegar fermentation, the tropical yeast and the thermo-adapted TH3 strain together were able to produce an acetic acid production of $2.53 \pm 0.01\%$ (w/v) and $2.34 \pm 0.03\%$ (w/v) either at 37 °C or 40 °C respectively. The poor acetic acid production by thermo-adapted TH3 strain was

mainly due to the poor performance of tropical yeast under static conditions where glucose was not completely converted into ethanol during alcohol fermentation. As it was mentioned previously, the poor production of ethanol by tropical yeast was mainly due to poor aeration conditions available in static culture condition. However, with the progression of the fermentation, a pellicle was observed on the surface of the vinegar media showing that both the yeast and AAB have come to the surface to get the required aeration condition for their growth and performance. A similar type of research done by Tanamool *et al.* (2020) stated that they were able to perform simultaneous vinegar production from pineapple peel using thermotolerant *A. pasteurianus* FPB2-3 successfully.

CONCLUSION

According to the obtained results, *K. marxianus*, the tropical yeast can grow and produce ethanol effectively even at 41°C, and thus, it can be successfully used in simultaneous vinegar production at higher temperatures successfully. The thermo-adapted TH3 strain could produce considerable amount of acetic acid production even at 41 °C. However, the maximum tolerable initial ethanol level at 41°C was 4%

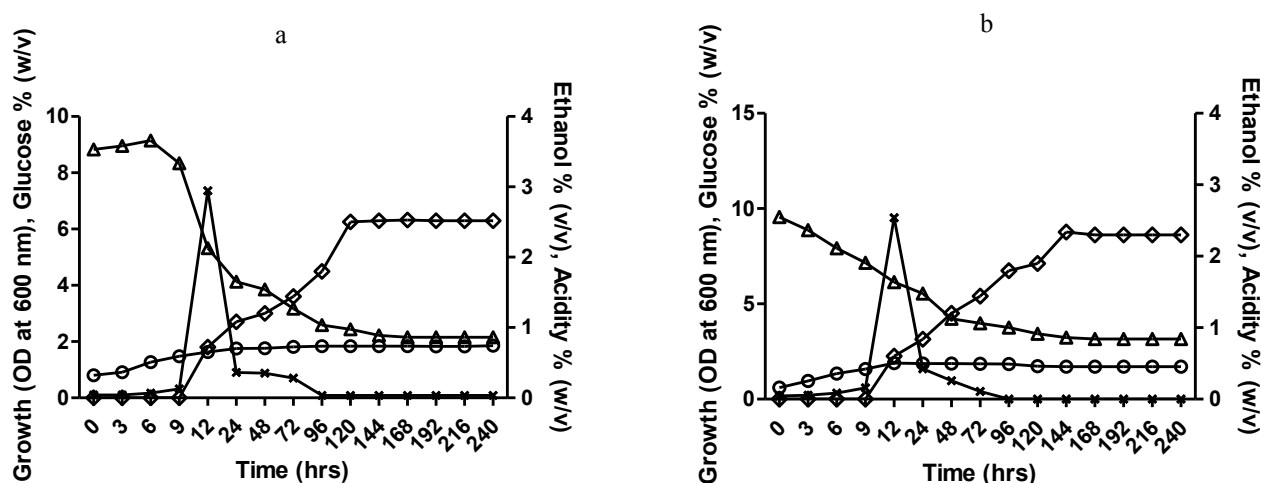


Figure 5: Simultaneous coconut vinegar production of *K. marxianus* and TH3 strain at (a) 37 °C, and (b) 40 °C under static conditions.

Circles represent the growth, crosses represent the ethanol percentage (v/v), diamonds represent the acetic acid percentage (w/v) and triangles represent the glucose percentage (w/v).

(v/v). The results of simultaneous vinegar production revealed that the tropical yeast (*K. marxianus* DMKU3) and the thermo-adapted *A. pasteurianus* TH3 can be successfully used in simultaneous vinegar production up to 40 °C successfully. However, since the commercial level fermentation is mainly done in large fermentation vats, improving the aeration condition is essential for the proper production of vinegar, and thus, further studies are needed.

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Author Contribution

HT and BNP conceptualized and designed the study. BNP performed the experiments. HT and BNP analyzed and interpret the data. BNP contributed in drafting the manuscript and HT critically revised the manuscript.

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