

Optimization of culture conditions for *Acetobacter aceti* TISTR 102 in coconut water with supplementary banana juice

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Abstract

The culture of *Acetobacter aceti* TISTR 102 in coconut water with banana juice was studied to optimize culture conditions for the highest cells with low cost production. The effects of supplementary banana juice, ammonium sulfate, yeast extract and shaking speed were investigated. Coconut water was a potential culture medium for *A. aceti* TISTR 102 with the viable cells comparable to those in synthetic GY (glucose and yeast extract) medium. Banana juice volume from 25% to 100% (v/v) in coconut water showed the significant effect on the bacterial growth ($p \leq 0.05$). The highest cell viability attained in coconut water supplemented with 50% (v/v) banana juice: 8.82-9.07 log CFU/mL in the stationary phase within 18-24 hrs. The ammonium sulfate at concentrations of 0, 0.1, 0.3 and 0.5% (w/v) did not enhance the cell viability ($p > 0.05$) with the specific growth rates ranged from 0.196 hr⁻¹ to 0.204 hr⁻¹. Similarly, the bacterial cells grew ineffectively in the presence of yeast extract concentrations of 0, 0.2, 0.6 and 1.0% ($p > 0.05$); the specific growth rates ranged from 0.145 hr⁻¹ to 0.150 hr⁻¹. In contrast, the shaking speed significantly affected to the cell viability ($p \leq 0.05$): the higher shaking speed was applied, the higher cell viability was obtained. The culture in stationary phase attained 8.56-8.67 log CFU/mL at 150 rpm; while 8.46-8.57 log CFU/mL was recored at 120 rpm.

Keywords

Acetobacter aceti
Coconut water
Banana juice

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Introduction

Acetic acid bacteria are gram-negative or gram-variable microbes, with ellipsoid or cylindrical shape and 0.4-1.0 μm in width and 0.8-4.5 μm in length (Guillamón and Mas, 2009). They grow aerobically in either sugar rich or alcohol rich environments to oxidize substrates into organic acids as final products. The optimal growth conditions were ranges from 5.0 to 6.0 for pH and 30°C for temperature (Hommel and Ahnert, 2000). For vinegar fermentation, *Acetobacter* genus, especially *Acetobacter aceti* with a high yield of acetic acid, is predominant for production of vinegar (Solieri and Giudici, 2009). A high cell biomass is requisite for starter preparation, resulted in taking culture conditions into consideration. Several synthetic media used to culture acetic acid bacteria such as YGP (yeast extract-glucose-peptone), YGM (yeast extract-glucose-mannitol), etc. (Ndoye *et al.*, 2009).

Acetobacter aceti TISTR 102 was a good strain to be the starter culture for vinegar production from palm sap (Wongsudaluck, 2012). The expensive nutrient sources such as HS medium (D-glucose,

20g/L; peptone, 20g/L and yeast extract 5g/L), GYE medium (GEY medium [D-glucose, 20 g/l; yeast extract, 5 g/l; 95% ethyl alcohol, 5% (v/v) or GY medium (D-glucose, 100 g/l; yeast extract, 10 g/l) were typical media used to culture this strain. However, *Acetobacter aceti* TISTR 102 cultivated in coconut water had comparable cell viability; a remarkable viable cell was obtained in mixture of coconut water and banana juice (obtained by enzymatic hydrolysis) at ratio 1:1 (v/v) (Supakod and Wongwicharn, 2012). The novel medium from coconut and banana juice with low cost is potentially applied into microbial mass production, especially for starter culture preparation.

The objective of this research was to optimize the growth conditions for *Acetobacter aceti* TISTR 102 in a simple and low cost medium from coconut water with supplementary banana juice. The effects of banana juice, ammonium sulfate, yeast extract and shaking speed were investigated. The results hopefully would replace the conventional synthetic media to reduce cost and effectively utilize the agricultural waste products.

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Materials and Methods

Microorganism

Acetobacter aceti TISTR 102 strain was bought from the Thailand Institute of Scientific and Technological Research and was inoculated in GYC agar (D-glucose, 100 g/L; yeast extract, 10 g/L; agar, 20 g/L and calcium carbonate, 10 g/L). It was sub-cultured every month and stored at 4°C.

Starter culture preparation

A full loop of *A. aceti* TISTR 102 from GYC agar plate was inoculated into GY broth (D-glucose, 100 g/L; yeast extract, 10 g/L). The culture was incubated on rotary shaker at 30°C and 120 rpm within 18-24 hrs to measure optical density at 600 nm absorbance (OD_{600}). The OD_{600} was adjusted to 0.5 ± 0.05 before inoculating into experimental culture media.

Substrates preparation and physiochemical properties determination

Coconut water was collected from the coconut milk store in Pattani province (Thailand) and then was filtered through a thin cotton cloth for determination some physiochemical properties. Namwa bananas (*Musa sapientum* L.), which were still slightly green and bought in the local market in Pattani province (Thailand), were naturally ripe at ambient temperature. The bananas with yellow colour and slightly brown spot were washed in water, peeled and chopped into small pieces before adding hot water at ratio 1 water: 1 banana pulp. The banana mash was heated in water bath at 95°C for 1 hr and was filtered through a thin cloth to obtain the banana juice for physiochemical analyses.

The pH values and total soluble solid were recorded by a digital pH meter (Metler Toledo, SevenEasy S-20K) and a hand-refractometer (Atago N1), respectively. Total sugar and reducing sugar were determined by phenol sulfuric acid method (Dubois *et al.*, 1956) and DNS method (Miller, 1959), respectively. The Kjeldahl method (AOAC, 2000) was applied to estimate total nitrogen. Phosphorous, sulfur, potassium, sodium, calcium, magnesium, zinc, copper and iron were estimated with atomic absorption spectrometry (AOAC, 2000).

Effects of banana juice volume, ammonium sulfate, yeast extract and shaking speed on bacterial growth

Completely randomized design was serially applied to optimize the banana juice volume, ammonium sulfate, yeast extract and shaking speed. Coconut water standardized to approximate 10% (w/v) total sugar by commercial sucrose was

supplemented with banana juice in different ratios: 0, 25, 50, 75 and 100% (v/v). All experimental media were adjusted to pH 5.0 ± 0.05 by either HCl 0.1N or NaOH 0.1N and subsequently autoclaved at 121°C for 15 minutes. The GY medium was used as the reference medium. In the coconut water with the optimal banana juice volume, the ammonium sulfate was added with concentrations of 0, 0.1, 0.3 and 0.5% (w/v). The yeast extract concentrations of 0, 0.2, 0.6 and 1.0% (w/v) were supplemented into coconut water and banana juice culture medium. Two shaking speeds including 120 rpm and 150 rpm were applied.

In all experiments, starter culture at 5% (v/v) was inoculated into 300 mL working volume and incubated in rotatory shaker for 72 hours at 30°C. Sampling the culture was carried out at every 6 hours in the first 48 hours and every 12 hours for consequent time. Samples were analyzed to determine pH, reducing sugar content and cell viability. The cell viability was considered as the crucial criterion to assess the effectiveness of experimental medium.

Analyses

The reducing sugar was detected using the 3, 5-dinitrosalicylic (DNS) acid method, with the standard curve from D-glucose solution (Miller, 1959). In term of cell viability, the cell growth was enumerated by viable colonies using standard dilution plate count on GYC agar and expressed in log CFU/mL pH values was recorded with pH meter (Metler Toledo, SevenEasy S-20K).

Statistical analysis

The collected data were analyzed based on ANOVA and presented as mean values with standard deviations. Significant differences within the treatments were analyzed by Duncan's multiple range test (DMRT) at a 5% probability level ($p \leq 0.05$). All analyses were run in triplicate.

Results and Discussion

Approximate physiochemical properties of coconut and banana juice

The approximate physiochemical properties of coconut and banana juice are presented in Table 1. In coconut water, the total soluble solid, pH, total sugar and nitrogen were 4.23 ± 0.25 , 5.42 ± 0.03 , 2.61 ± 0.13 (g/100mL) and 1.20 ± 0.10 (mg/mL), respectively that consistent with several previous studies. For examples, total soluble solid was 4.0 ± 0.2 °Brix and nitrogen content was approximately 10 mg/L (Unagul *et al.*, 2007). The pH was 5.36 ± 0.00 and total sugar

Table 1. Approximately physiochemical properties of coconut water and banana juice

Properties & Compositions	Coconut water	Banana juice
Total soluble solid (°Brix)	4.23±0.25	14.1±0.10
pH	5.42±0.03	4.44±0.02
Total sugar (% or g/100 mL)	2.61±0.13	10.70±0.13
Reducing sugar (% or g/100mL)	1.85±0.08	8.86±0.13
Nitrogen (mg/100 mL)	1.20±0.10	2.70±0.30
Minerals (mg/L)		
Calcium	82.30±4.17	16.20±0.35
Magnesium	55.80±1.52	138.90±1.84
Potassium	1017.40±23.74	1604.40±21.28
Sodium	97.00±0.50	20.10±0.14
Phosphorus	90.00±0.00	330.00±0.00
Iron	0.07±0.001	1.52±0.08
Zinc	0.22±0.04	1.02±0.06
Copper	0.03±0.004	0.11±0.01

was 2.03±0.01 (Supakod and Wongwicharn, 2012). In the research, the amount of reducing sugar was 1.85±0.08 g/100mL, occupied more than 50% (w/w) of total available sugar in coconut water. In term of microbiology, carbon source such as sugar was essential to form carbon-containing-substances, for example protein, fats, carbohydrates or lipids. The macronutrients, including calcium, magnesium, potassium, sodium and phosphorus revealed to the cellular composition and inner enzymatic reactions were available with noticeable amounts: 82.30±4.17, 55.80±1.52, 1017.40±23.74, 97.00±0.5 and 90.00±0.00 (mg/L), respectively. Tan et al (2013) studied on physiochemical changes of coconut water in Malaysia within different mature stages and they reported that most of minerals contained in overly-mature coconut were remarkably available. For examples, calcium, magnesium, potassium and sodium were 23.98±0.054, 31.65±0.038, 35.11±0.133 and 36.51±0.020 (mg/100mL), respectively. The trace elements such as zinc, copper and iron were not mentioned in previous studies, but they were available in coconut water with small amounts 0.22±0.04, 0.03±0.004 and 0.07±0.001 (mg/L).

For banana juice, a turbid and slightly purple juice was extracted from the pulp, which consequently became darker with cloudy precipitate formation after sterilization in the autoclave. Generally, most of nutrients determined in extracted banana juice were significantly available as compared to coconut water. Indeed, the major nutrients such as magnesium, potassium and phosphorus were presented with considerably high amounts: 138.90±1.84; 1604.40±21.28 and 330±0.00 (mg/L). But, calcium and sodium were present with 16.20±0.35 and 20.10±0.14 (mg/L) that less than amounts contained

in coconut water. The trace elements, including zinc, copper and iron act as cofactors for essential enzymatic reactions in the cell were noticeably available with 1.02±0.06; 0.11±0.01 and 1.52±0.08 (mg/L), respectively. The pH value was recorded with 4.44±0.02, while pH 4.25±0.01 was found in enzyme-extracted banana juice from namwa banana (e.g. *Musa sapientum* L.) (Supakod and Wongwicharn, 2012). The total soluble solid was 14.1±0.10 °Brix; the total sugar and reducing sugar were remarkably 10.70±0.13 and 8.86±0.13 (g/100mL), respectively. The nitrogen known as required substance for the synthesis of proteins, amino acids, DNA and RNA was available with 2.70±0.3 mg/100 mL and was twice as much as the nitrogen in coconut water.

Effects of different experimental culture substrates

The comparison of culturing ability to culture *Acetobacter aceti* TISTR 102 in different media was investigated. Results are shown in Figure 1. *A. aceti* TISTR 102 grew well in most of the experimental media and attained at least 10⁷ CFU/ml from 6 hours onwards. Obviously, the early stationary phase was observed at 18-24 hours within all treatments. The growth pattern of *A. aceti* TISTR 102 in the coconut water medium was comparable, even markedly better than the one in synthetic GY medium at the same 10% (w/v) total sugar content. The explanation conceivably was due to the abundance of nutrients and sufficient amount of sugar could entirely support the requirement for biomass production (Supakod and Wongwicharn, 2011). Approximately 2.61±0.13 g/100mL sugar content in coconut water insufficiently supports carbon source for microorganisms, results in sugar enhancement for their consumption. Thus, a good growth of *A. aceti* TISTR 102 in coconut water

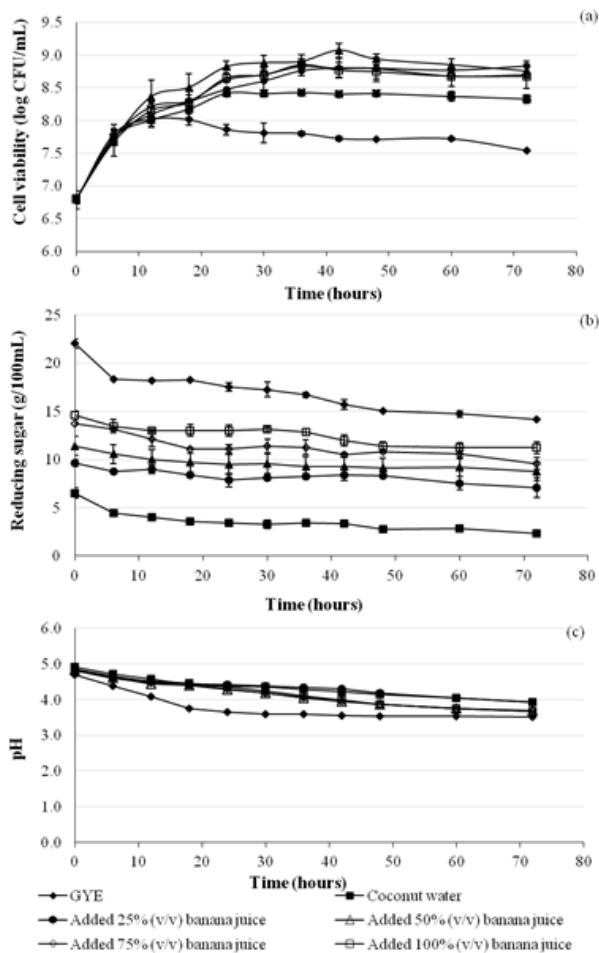


Figure 1. The growth of *A. acetii* TISTR 102 (a), reducing sugar content (b) and pH change (c) in different culture media

with additional sucrose was clearly observed.

In Figure 1a, during the first 18 hours, the viable cells in all experiment media were insignificantly different. However, the media with presence of banana juice had statistically more cell viability than the coconut water as sole medium, especially during stationary phase. The increase in additional volume of banana juice from 25% to 100% (v/v) resulted in the enhancement of cell viability, up to more than 8.48 log CFU/mL. It might be explained by the presence of norepinephrine that was known as neurochemical substance. Norepinephrine was found in banana pulp (*Musa x paradisiaca*) with 325 ng/mL extract that predominantly had enhancement effects on the growth of several gram-negative bacterial species (Lyte, 1997). Norepinephrine belongs to catecholamines with the catechol-ring structure (i.e. siderophores or iron carriers) that enables to scavenge iron from the environment and to make the minerals in bacteria (Kinney *et al.*, 2000). Norepinephrine interaction was a biochemical metabolism that not all bacteria could perform and respond with positive effect (Belay, 2003), although gram-negative bacteria

could respond better (Belay and Sonnenfeld, 2002). Thus, the enhancement effect of norepinephrine on *A. acetii* TISTR 102 growth has been doubt, resulted in nutritional factors in banana juice such as trace elements, minerals, etc also might involve in the bacterial growth. The maximum growth pattern attained at 50% (v/v) added banana juice with approximately 8.82–9.07 log CFU/mL at stationary phase. The externally added banana juice up to 75% and 100% (v/v) showed the statistically insignificant growth pattern as compared to the pattern at 50% (v/v) added banana juice. Vega *et al.* (1988) reported that banana juice at 21% was the optimal concentration for culturing the lipid-accumulating yeast *Apiotrichum curvatum* ATCC 20509. Besides, they also pointed out the higher concentrations of banana juice even caused the adverse effect on the growth without explanation or hypothesis. In this experiment, in the treatment 50% (v/v) added banana juice, the juice was diluted with 6 folds from the original pulp that approximately similar concentration as previous research. In addition, the more banana juice added might form more precipitate, leading to prevent the oxygen transfer for bacterial uptake. The addition of enzyme-extracted banana juice into coconut water with ratio 1:1 (v/v) could enhance the cell viability, nearly 1.2 folds (Supakod and Wongwicharn, 2012). However, in this research, the same fold increase could be attained with banana juice volume reduced to half (i.e. 1 coconut water : 0.5 banana juice) and 0.221 hr⁻¹ specific growth rate.

Different culture media had differently initial reducing sugar content, depending on the substrate and the volume of added banana juice (Figure 1b). The synthetic GY medium containing glucose as the sole carbon source had the highest reducing sugar and sharply declined during the incubation time. The other media contained sucrose that obtained from their nutritional facts and externally supplemented, beside glucose. They had the similar trend in reducing sugar pattern which slightly decreased during the incubation time.

Bacteria consumed sugar and increased the population during incubation time, acetic acid was consequently produced as the biochemical product to make the pH values decrease. In most of treatments, pH values reduced significantly in the first 24 hrs and changed insignificantly after that time (Figure 1c). pH of the synthetic GY medium with glucose as the main carbon source dramatically decreased and significantly different from the others, that previously confirmed by the consumption sugar pattern. Because of acidic properties of banana juice with pH 4.44±0.02 (Table 1), the more volume of

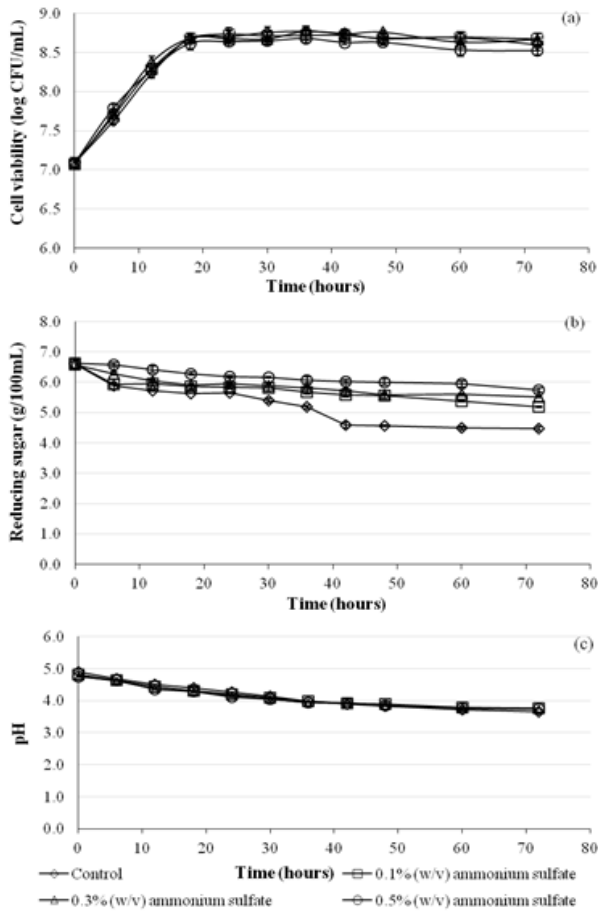


Figure 2. The growth of *A. acetii* TISTR 102 (a), reducing sugar content (b) and pH change (c) of coconut water with 50% (v/v) added banana juice medium at different concentrations of ammonium sulfate (w/v)

banana juice was added, the lower pH values were obtained and significantly different one another.

Effects of ammonium sulfate

The effects of ammonium sulfate with different concentrations of 0, 0.1, 0.3 and 0.5% (w/v) as nitrogen source on the growth of *A. acetii* TISTR 102, reducing sugar content and pH change are presented in Figure 2. Ammonia is known as an important source of nutrients for bacteria because it contains nitrogen, which is used to make proteins and nucleic acids. Figure 7a pointed out that *A. acetii* TISTR 102 attained the early stationary phase after 18 hours, did not grow remarkably in the presence of ammonium sulfate. There was an insignificant difference in cell viability at stationary phase within 0, 0.1 and 0.3% (w/v) concentrations: 8.68-8.77, 8.67-8.72 and 8.68-8.77 log CFU/mL. Correspondingly, the specific growth rates were 0.204, 0.202 and 0.200 hr⁻¹. Ammonium sulfate added up to 0.5% (w/v) showed relatively lower growth pattern with 8.62-8.68 log CFU/mL of viable cells and exponential growth rate

0.191 hr⁻¹. The physicochemical properties of coconut water and banana juice (Table 5) could explain for the results. Obviously, nitrogen content in coconut water was approximately 1.20±0.1 g/100 mL that was as same as the nitrogen content (i.e from 1% (w/v) yeast extract) in synthetic GY medium. The nitrogen content was in excess within the presence of banana juice (nitrogen 2.70±0.1 g/100 mL) and further supplemented ammonium sulfate. So far, there has not been exactly nitrogen content sufficient for this bacteria, but the nitrogen from ammonia salt varied among bacterial species and depended on culture medium. The ammonium sulfate concentrations of 0.2, 0.4, 0.6 and 0.8% (w/v) could enhance the growth of *A. acetii* TISTR 102 in palm sap juice medium; but no significant difference in viable cells among the experimental treatments (Wongsudaluk, 2012). The lower growth pattern obtained at 0.5% (w/v) ammonium sulfate concentration might be due to osmolarity of the medium (Müller *et al.*, 2006). In the other hand, Figure 2b shows the reduction of reducing sugar content during the incubation time. Generally, the reducing sugar contents in all of the treatments dramatically decreased during the first 18 hours that corresponding to exponential phase of bacteria. During the later time, bacteria at stationary phase consumed sugar less than last phase did, resulted in the reducing sugar contents decreased slightly. Among treatments, there was an insignificant difference in reducing sugar contents at the first 24 hrs; but at the 36th hr onwards, reducing sugar was continuously consumed more in control treatment as compared to those of counterparts. The reduction of reducing sugar at 0.5%(w/v) ammonium sulfate treatment was less than the others, which supportingly confirmed the results of growth patterns. The pH values decreased during incubation time are presented in Figure 2c. The initial pH values were statistically different as adding ammonium sulfate concentrations. However, all of the pH patterns tended to change in similar trend: significant decrease in the first 24 hours and slight change in consequent time.

Effects of yeast extract

Yeast extract has been shown that apart from organic nitrogen source, it is an essential source of vitamin, trace elements, etc. The results of cell viability of *A. acetii* TISTR 102, reducing sugar content and pH change with various yeast extract concentrations of 0, 0.2, 0.6 and 1.0% (w/v) are presented in Figure 3. Obviously, in Figure 3a, yeast extract does not show enhancement effect on the bacterial growth through concentrations of 0, 0.2, 0.6 and 1.0% (w/v). Indeed, viable cells without yeast extract attained 8.37-8.50

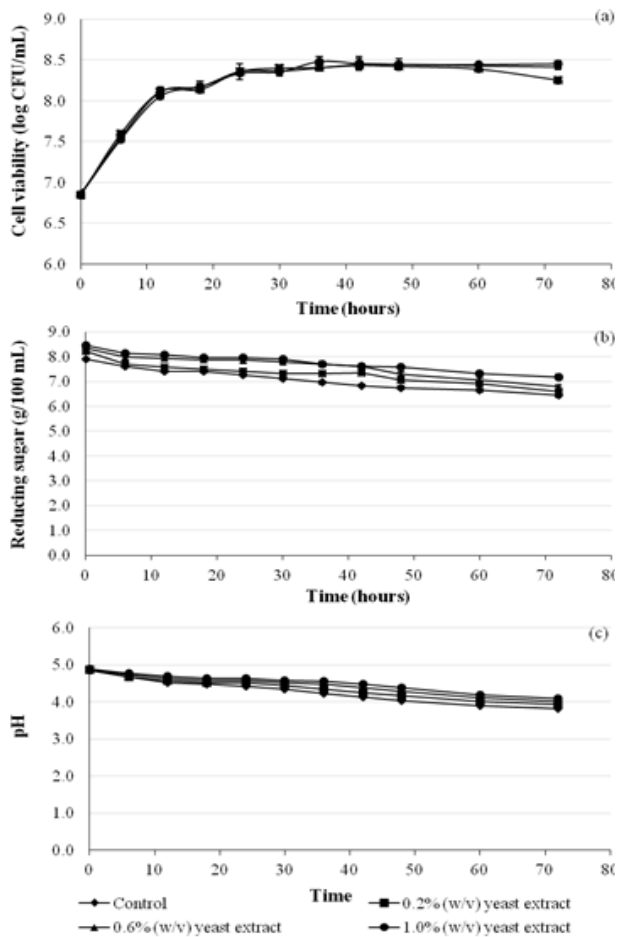


Figure 3. The growth of *A. aceti* TISTR 102 (a), reducing sugar content (b) and pH change (c) of coconut water with 50% (v/v) added banana juice medium at different concentrations of yeast extract (w/v)

log CFU/mL in stationary phase with the specific growth rate 0.144 hr^{-1} . Cell viability 8.36-8.43; 8.28-8.54 and 8.38-8.48 log CFU/mL were obtained at 0.2, 0.6 and 1.0% (w/v) yeast extract, respectively. Correspondingly, 0.150, 0.141 and 0.142 h^{-1} were the recorded specific growth rates. In several studies, yeast extract efficiently stimulated cell growth, even increased the production yield. For examples, yeast extract concentrations of 0.2, 0.4, 0.6 and 0.8% (w/v) significantly enhanced the cell viability of *A. aceti* TISTR 102 in palm juice medium and 0.4% (w/v) yeast extract was the optimal concentration to produce the highest cell viability (Wongsudaluk, 2012). Otherwise, yeast extract just showed its positive effect on *Apiotrichum curvatum* ATCC 20509 biomass within the banana juice concentrations less than 19%; otherwise the enhancement effect was not observed at the higher 19% concentrations (Vega et al., 1988). This study was in agreement with the experimental results to strongly confirm the sufficiently chemical requirements of banana juice for microbial growth enhancement. Thus, in case, yeast extract might be ignored, which is a beneficial result

involves to economic issues. In Figure 3b, in spite of the difference in initial reducing sugar contents depending on the yeast extract concentration, the reducing sugar contents in all of the treatments tended to have similar trend. Generally, the reducing sugar content was remarkably reduced in the first 24 hours and insignificantly consumed in the later time that corresponded to for exponential phase and stationary phase, respectively. These results possibly confirmed the result of growth pattern above that did not have the significant difference in cell viability among these treatment. The changes of pH are performed in Figure 3c with the significant decrease was observed in the first 24 hours. The higher concentration of yeast extract was supplemented, the pH values decreased more slightly.

Effects of shaking speed

Acetobacter aceti TISTR 102 culture in coconut water and 50% (v/v) added banana juice without any external supplementation was incubated at two different shaking speeds 120 and 150 rpm for 72 hours at 30°C . The results were represented in Figure 4. Figure 4a pointed out that the shaking speed had a noticeable effect on the viable cells: the higher speed was applied, the greater cell viability was obtained (Figure 4a). A range from 8.46-8.57 log CFU/mL and 8.56-8.67 log CFU/mL attained in the stationary phase at 120 and 150 rpm, respectively. Correspondingly, the specific growth rates were 0.167 h^{-1} and 0.177 h^{-1} . The effect of shaking speed was reported in several previous researches. For examples, *Acetobacter aceti* TISTR 102 cultured in palm sap medium attained highest cell viability up to 7.8×10^{14} at 180 rpm, while 4.1×10^{10} and 2.3×10^{13} were recorded at 100 and 130 rpm, respectively after 4 days (Wongsudaluk, 2012). *Acetobacter* TISTR 975 (*A. xylinum*) cultured in coconut water at 50 rpm showed the smallest number of cells as compared to those obtained from other speeds 100 and 150 rpm (Tantratia et al., 2005).

Acetobacter aceti TISTR 102 belongs to the aerobic microorganisms whose rate of growth and metabolism depend upon the amount of dissolved oxygen available. But, oxygen demonstrates the dissolving limitation, so a compulsory liquid aeration might be necessary to provide dissolved oxygen. Indeed, shaking breaks the large bubbles formed at the entrance of air by bacterial clumps or biofilm formation, then oxygen transferred from the gas phase to the liquid phase increases (Tesfeya et al., 2000). Additionally, shaking obviously helps the medium to be almost equally distributed and prevents the bacteria sediment in the bottle of the flask. Besides, the presence of turbid suspension from insoluble solid

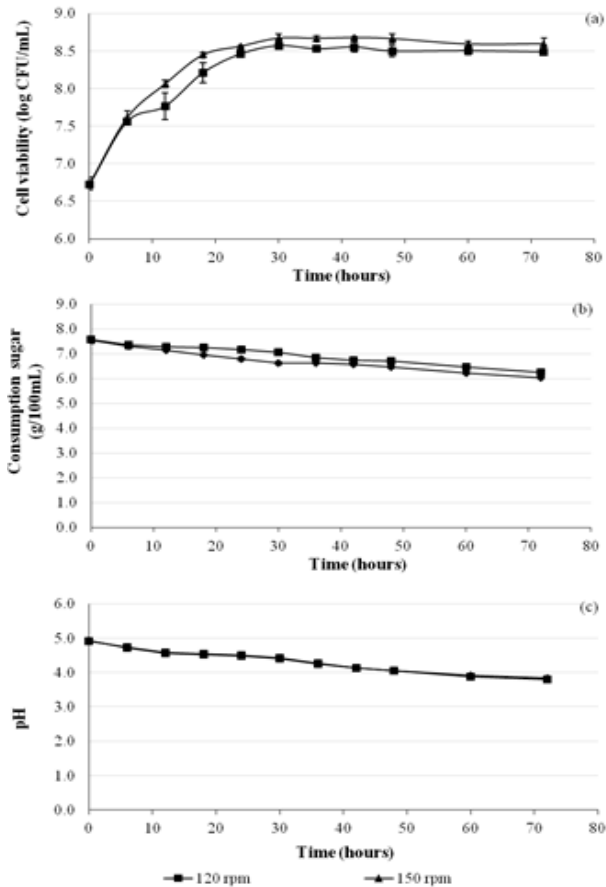


Figure 4. The growth of *A. aceti* TISTR 102 (a), reducing sugar content (b) and pH change (c) of coconut water with 50% (v/v) added banana juice medium at different shaking speeds

substances in banana pulp inhibited the microbial growth was a significant issue. Thus, the shaking, in this case, possibly prevents culture medium from precipitation for increase in oxygen uptake. The decrease in reducing sugar was observed during the incubation time; and the higher consumption rate attained at the higher shaking speed (Figure 4b). Otherwise, the shaking speed did not influence on pH values because of the insignificant difference in pH values between two treatments (Figure 4c).

Conclusion

Coconut water was an economic and effectively comparable medium to culture *A. aceti* TISTR 102. The presence of banana juice in coconut water could enhance the growth of *A. aceti* TISTR102 and the highest cell viability at stationary phase was 8.82–9.02 log CFU/mL with 50% (v/v) added banana juice. Besides, ammonium sulfate and yeast extract did not positively influence on the bacterial growth, resulted in cost reduction for culture this bacterium. The higher shaking speed 150 rpm enhanced the cell

viability with significantly viable cells as compared to those obtained at the lower shaking speed 120 rpm.

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