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Alternative medium from agricultural by-products used for a starter of vinegar fermentation

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Abstract. The objective of this research was to find a cheap agricultural waste media for the preparation of the effective starter culture of acetic acid bacteria (AAB). The alternative culture medium for AAB was compared to potato medium, preparing from pineapple peel extract (PPE) and banana juice. These media was adjusted the reducing sugar to 25 gL⁻¹ and initial pH of 5.0 used for growing *Acetobacter pasteurianus* FPB2-3. The results found that this strain grew well in PPE medium comparable to potato medium and viable cells achieved the level of at least 9 log CFUmL⁻¹ when cultured for 36 hr. While addition of banana juice, led cell growth decreased. The highest alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) activities in cell free extract were gained by using potato medium while the alternative media showed decrease of activity. However, these pre-culture gave acetic acid production same as the control medium, which cell completely oxidize ethanol to acetic acid. Consistently, analysis by FT-IR indicated insignificantly difference on cell components among these media. The result shows that constitutes of such agricultural by-product can be utilized as an alternative cheap inoculation media for AAB.

1. Introduction

AAB are gram negative, obligate aerobic and play important role on industrial fermentation. *Acetobacter* and *Komagataeibacter* (formally named *Gluconacetobacter*) are predominant AAB strains for industrial vinegar fermentation due to their great capability of ethanol oxidation and acetic acid resistance [1]. In order to improve them on fermentation capability, research on either isolation of the more effective strain, or investigation on some mechanisms underlying on ethanol, acetic acid, and temperature resistance have been carried out [2]. In addition, the appropriated number of inoculum is also important for fermentation. Recently, the cheap inoculation media using agricultural by-product was used to compare with typical commercial media [3-4]. The results demonstrated that a comparable of cell viability could be obtained from such low cost medium [4]. However, there was no comparing the ability of those inoculum on acetic acid fermentation. Requirement of larger volume of inoculum in scale-up fermentation resulted in high cost of production and being difficult providing for local



maker. Pineapple is a major agricultural fruit product in Nong Khai. The production trends to be increased and may cause overproduction. Moreover, pineapple peel, a waste product after processing is also accumulated and consequently become environmental problem if do not utilized further. These by-products still contain sugars, macronutrient and some trace elements available for microbial growth [5]. In this study, we then preliminary investigated the effect of the media prepared from the extraction of pineapple peel on cell growth and enzyme activity. In addition, capable of acetic acid production by those alternative media was also discussed.

2. Materials and Methods

2.1 Bacterial strain and growth conditions

AAB using in this study was *A. pasteurianus* FPB2-3 isolated from fermented plant beverage. The stock cell culture of this strain was streaked on Potato medium [6] and incubated at 30 °C for 2 days and kept at 4 °C.

2.2 Preparation of pineapple peel extract and banana juice

Pineapple peels and over-ripen banana obtained from local fresh market at Nong Khai were dried by sun light for 2 days. Then, it was boiled separately with distilled water (1:3 by weight) for 10 min. After cooling, the filtrate was obtained by passing through cotton cloth and centrifuged at 6,000 rpm for 10 min. The each supernatant was kept at -20 °C, until used. The supernatant was adjusted concentration of reducing sugar to equal to 25 gL⁻¹. The mixture of alternative media was supplement with different concentration of banana juice (BJ) at 0 (PPE), 25, 75 and 100 % (vv⁻¹) into supernatant of PPE. These media was then transferred into 100 mL Erlenmeyer flask and then sterilized by autoclave at 110 °C for 10 min.

2.3 Determination of alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase activity (ALDH)

The cells of *A. pasteurianus* FPB2-3 was collected by centrifugation at 6,000 rpm for 10 min. The cell pellet was washed twice with McB buffer pH 5.0 and resuspended with the same buffer as previous described. The cell suspension was subjected to ultrasonic (VCX500 Sonic & Materials Inc. USA) to lysis cells. The cell free extract (CF) was obtained by centrifugation at 6,000 rpm for 10 min and used for enzymatic measurement. ADH and ALDH activities were determined using Ferricyanide-dupanol method [7]. The protein concentration in CF was measured by Lowry method [8] using bovine serum albumin (BSA) as protein standard.

2.4 Acetic acid fermentation

5% (vv⁻¹) of cell after washing by normal saline was transferred into medium which was prepared from PPE (25 gL⁻¹ of reducing sugar) and supplement ethanol to final concentration of 6% (vv⁻¹). While acetic acid fermentation from rice wine (Sato), the initial ethanol was adjusted at approximately 70 gL⁻¹. The fermentation was carried out under shaking condition at 200 rpm and 30°C.

2.5 Analytical methods

The reducing sugar was measured by using the 3, 5-dinitrosalicylic (DNS) acid method and the total sugar was measured by phenol sulfuric with the standard curve from D-glucose. Total acidity was determined by titration with 0.8 N NaOH using phenolphthalein as pH indicator. The cell growth was enumerated by viable colonies using standard dilution plate count on potato agar. pH was measured using pH meter. Some metal ions were analyzed by atomic absorption flame emission spectrophotometer (AAnalyst400 PerkinElmer Inc. MA, USA).

2.6 Attenuated total reflection Fourier transformed infrared spectroscopy (ATR-FT IR) analysis of dried cell.

The cells obtained from different culture medium were dried using freeze drying. The freeze dried cells were measured at wave numbers ranging from 4000 to 400 cm^{-1} with FT-IR spectrometer Bruker TENSOR27 (Bruker Optics, Germany) equipped with an ATR unit was used for the IR measurements.

3. Results and Discussion

3.1 Approximate physiochemical properties of pineapple peel and banana juice extract

After extraction as described above, the supernatants were determined several physical/chemical parameters as shown in the table 1. The result revealed that the extract contains reducing sugar that would be sufficient for growth of AAB. The monosaccharide was detected in sample including glucose, fructose and sucrose as di-saccharide (data not show). The extract was acidic pH thus required neutralization. Calcium, iron, and zinc act as cofactors for essential enzymatic reactions in the cell were noticeably available which was consistent to previous report [4]. However, these concentration also depends on the weight ratio of the raw material used for extraction.

Table 1. Chemical composition of pineapple peel extract and banana juice

Contents	Pineapple juice	Banana juice
Total sugar (gL^{-1})	244.30 (± 10.53)	393.30 (± 14.86)
pH	3.9 (± 0.03)	4.6 (± 0.02)
Reducing sugar (gL^{-1})	32.66 (± 3.16)	86.98 (± 5.87)
Calcium (mgL^{-1})	48.24 (± 0.01)	15.27 (± 0.06)
Potassium (mgL^{-1})	523.05 (± 0.05)	355.2 (± 0.03)
Iron (mgL^{-1})	2.156 (± 0.02)	0.216 (± 0.013)
Zinc (mgL^{-1})	1.106 (± 0.007)	1.759 (± 0.011)

3.2 Growth profile of AAB cultivating in the alternative cheap media

In order to investigate growth ability of AAB on alternative cheap media, the combination of pineapple peel extract (PPE) and banana juice (BJ) were conducted by adjusting equal concentration of reducing sugar as 25 gL^{-1} . Growth was expressed as viable cell number as shown in figure 1A. As can be seen, growth was sharply increased within 48 hr and steady, except growth of cell in BJ (only banana juice) gave the lowest growth. Even Potato medium gave the highest growth, PPE also showed comparable growth by giving cell viability almost 9 log CFU mL^{-1} within 60 hr. In contrast, use of banana juice suppressed growth obviously. This result indicated that PPE itself is sufficient while addition of banana juice may cause increasing of growth inhibitor. We also found that more banana juice added forms more precipitate similar to previous report and may lead to prevent the oxygen transfer for bacterial uptake as mentioned previously [4].

Reducing sugar was consumed consistent to growth as almost constant after 60 hr (figure 1B). Glucose in potato medium was almost completely exhausted while reducing sugar in other media was still remained. The other cheap media contained fructose and sucrose that obtained from their nutritional facts and externally supplemented, showing the similar trend in reducing sugar pattern. In fact, another carbon source, glycerol is presence in the potato medium can be further utilized through pentose phosphate pathway and can be oxidized by membrane-bound dehydrogenase to form dihydroxyacetone or glyceric acid [9]. Consuming of sugar resulting in increased the population during incubation time, oxidizing product was consequently produced and led the pH values decrease. In the most of treatments, pH values reduced significantly in the first 24 hr and kept constant (figure 1C)

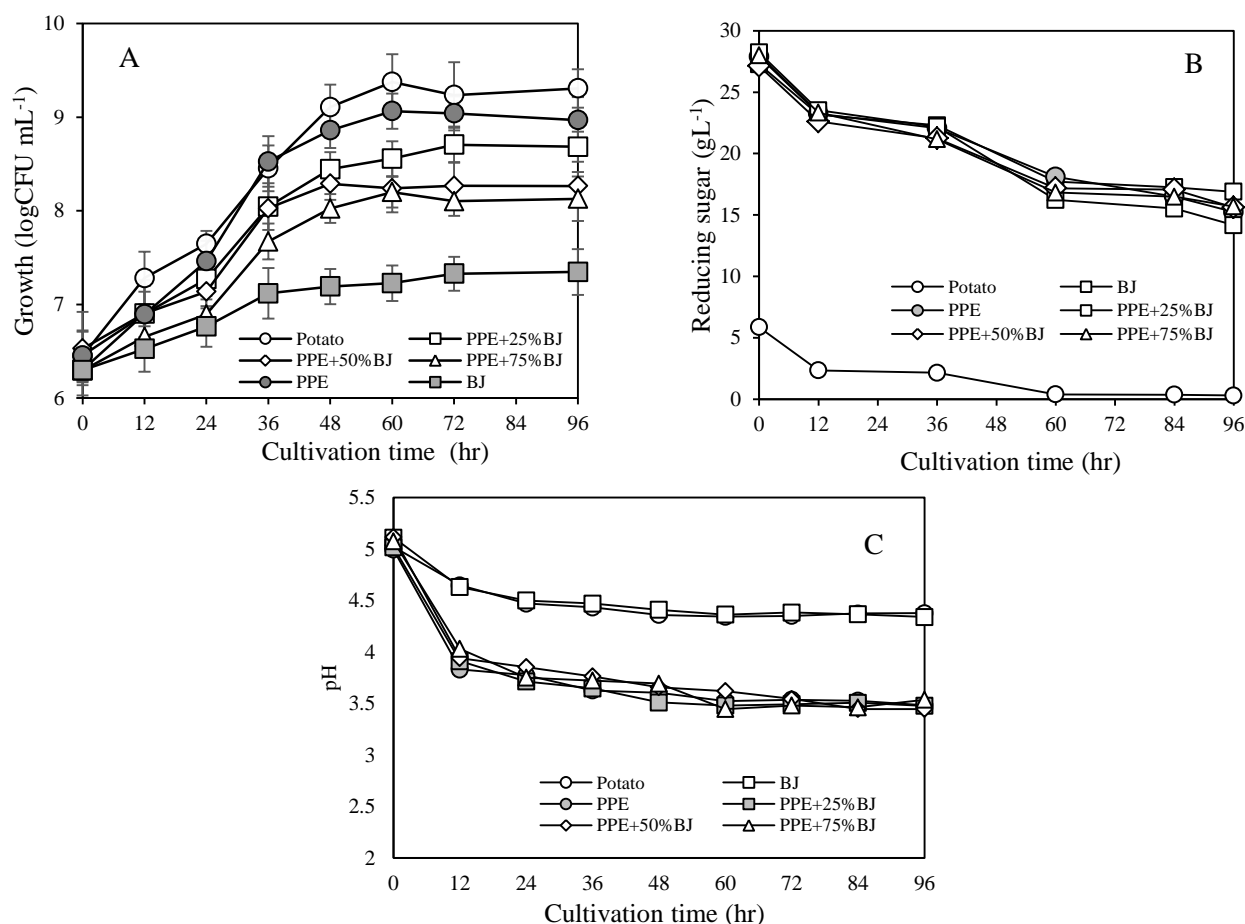


Figure 1. Growth profile of *A. pasteurianus* FPB2-3 on alternative cheap media compared to potato.

Due to lower concentration of glucose in the potato medium, pH decrease was then lower than other. The fact that AAB are able to oxidize carbon sources incompletely to ketoacids as products by membrane-bound dehydrogenases in the periplasm rather than being metabolized to the pentose phosphate pathway and the Entner-Doudoroff pathway [10]. In generally, the growth of AAB on glucose, the major part of this sugar is oxidized in the periplasm. A membrane-bound pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (mGDH) catalyzes the initial reaction linked to the generation of a proton motive force [11]. This direct oxidation led to the rapid accumulation of gluconate, and can be subsequently oxidized by membrane-bound and respiratory-chain-coupled quinoprotein or flavoprotein dehydrogenases to form 5-ketogluconate (5-KGA), 2-ketogluconate (2-KGA), and 2,5-diketogluconate (2,5-DKGA) [2].

3.3 Comparison on membrane-bound ADH and ALDH from cell grown on different culture media

Being effective medium for preparing inoculum of AAB, cell biomass and their capable of fermentation would be considered. Therefore, two medium giving comparable cell number to potato medium, was then further subjected for measurement activity of two membrane-bound dehydrogenase responsible for acetic acid production. As shown in figure 2 ADH and ALDH activities were lower than cell grew on potato almost 2 folds.

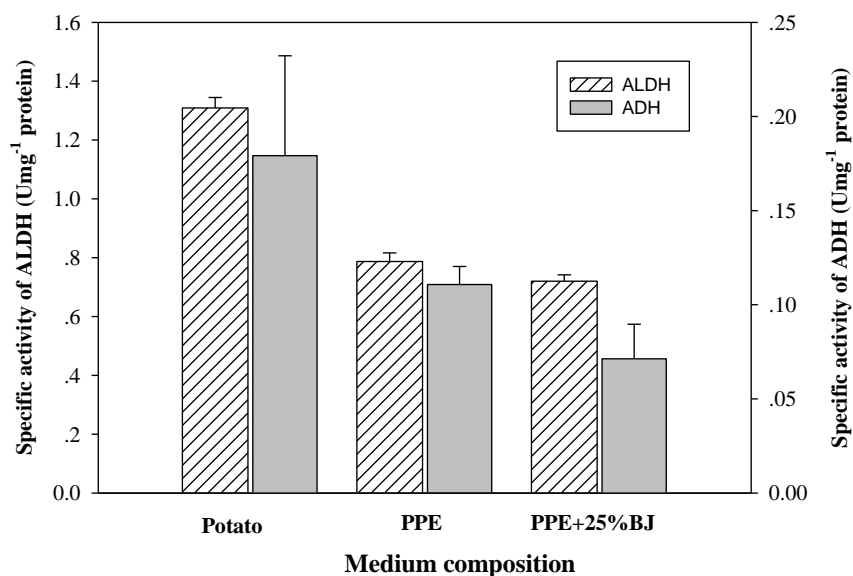


Figure 2. Membrane-bound ADH and ALDH activities in cells grown on different culture medium.

We also observed that adding of ethanol can be activated the ADH activity in the cell membrane (data not shown). The lower enzyme activity may due to inhibitor presence in those media. Saifi-Abolhassan et al., (2007) reported inhibition of purified ADH from *Acetobacter* by several metal ion [12]. It has been reported that at lower pH also cause of the dissociation of the active ADH become inactive [13]. In addition, ALDH had been reported to have optimal pH at acidic condition and can be inhibited by several metal ions but not EDTA at the same concentration [13].

3.4 Acetic acid production by cell inoculum from alternative cheap medium

In order to investigate their ability on acetic acid production, cell inoculum was transferred into PPE supplement with 6 % (v/v) of ethanol as shown in figure 3A. Acetic acid accumulation was slightly observed in 2 days and drastically increased to the maximum production with 6-7 days. This may be due to either high concentration of initial ethanol or insufficient inoculum. Inoculum from PPE medium was able to oxidize ethanol almost completely same as from potato medium. Figure 3B showed acetic acid production from rice wine which was adjusted ethanol concentration at 7% (v/v) before fermentation. Similarly, the accumulation of acetic acid by inoculum from PPE was comparable to using potato medium as inoculum media. The highest acetic acid was obtained after cultivation for 5 days at concentration of 7 % (w/v).

In order investigate that whether the culture medium affect cell component, freeze-dried cells obtained from culturing in the PPE and BJ medium was used to determine their characteristics by FT-IR compared to cell grown on potato medium as showed in figure 4. The obvious spectrum region corresponding to a specific functional group were recorded for instance, at 3000–2800 cm⁻¹ was commonly assumed to be dominated by fatty acid related compounds; the region of 1700–1500 cm⁻¹ indicated the carbonyl residual of proteins (Amide I and II); free amino acids, and polysaccharides was in the region of 1450–1400 cm⁻¹ and the region of 1250–1200 cm⁻¹ by RNA/DNA and phospholipids [14]. According to the spectrum pattern, these were similar to each other, except a sharp peak at 3000–2800 cm⁻¹ were observed in the cell grown on PPE medium. This may explained why the cells from the alternative media still keep the effective properties on acetic acid production.

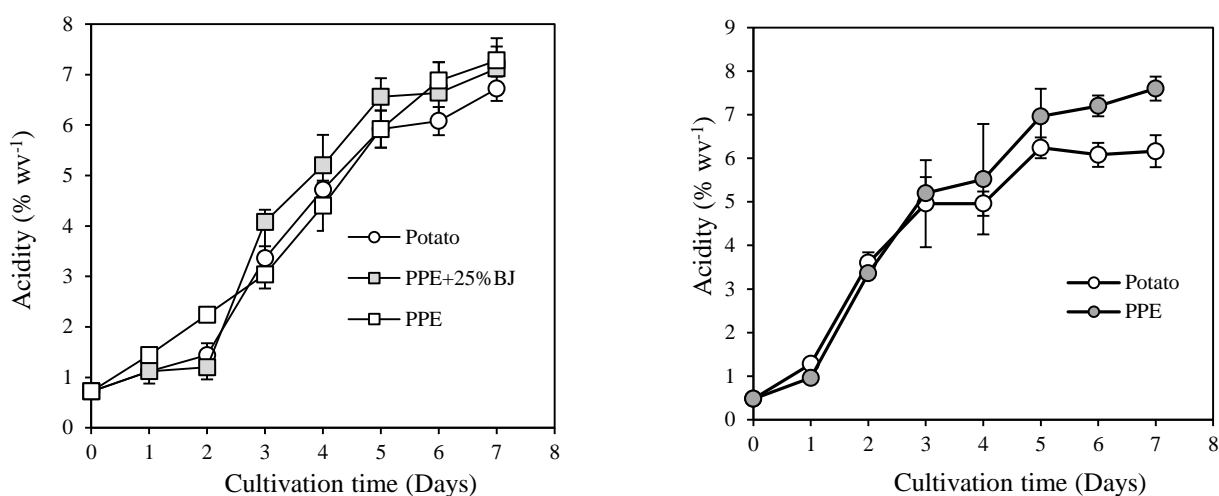


Figure 3. Acetic acid production from PPE and supplement with 6 % ethanol (vv⁻¹) (left panel) and produce from rice wine (right panel).

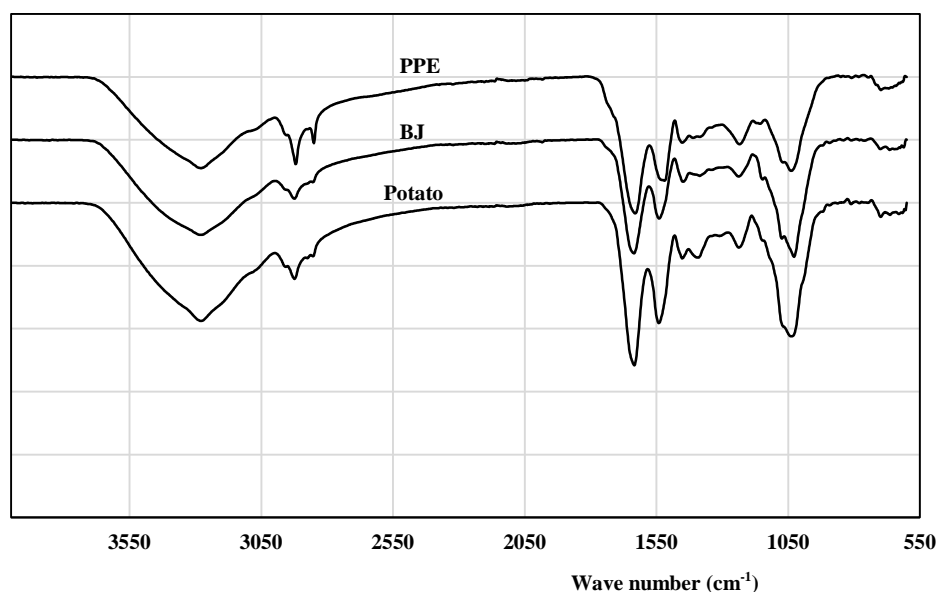


Figure 4. FT-IR analysis of cell obtained from different medium.

4. Conclusion

This study demonstrated that pineapple peel, agricultural waste product can be used as cheap inoculation media for acetic acid bacteria. The inoculum obtained from this medium still showed a great capability of fermentation comparable to the commercial one. This result would be advantage on cost reduction and simple procedure for small manufacturer in rural area. However, to optimize the composition of PPE medium for improving growth of AAB should be further carried out.

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