IDENTIFICATION OF HIGH ACETIC ACID-YIELDING BACTERIA FROM NIPA SAP

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ABSTRACT

Eighteen gram-negative bacilli and cocci were selected from 40 isolates in naturally fermenting nipa sap from Paombong, Bulacan. When grown and screened in pasteurized nipa sap, 10 high acetic acid-yielding isolates were further selected. Based on their morphological and physiological characteristics, they were identified as *Acetobacter aceti* **subsp.** *aceti, A. paradoxus* **subsp.** *paradoxus,* **six isolates of** *A. ascendens* **subsp.** *ascendens, A. lovaniensis* **subsp.** *lovaniensis* **and** *A. rancens* **subsp.** *pasteurianus.*

Among the identified bacteria, A. *aceti* **subsp.** *aceti* **produced the highest amount of acetic acid in pasteurized nipa sap. This bacterium responded positively to most of the physiological tests that were used. The utilization of glucose, ethanol and other nutrients by bacteria for acetic acid production was found to be dependent on their physiological characteristics.**

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INTRODUCTION

The most common types of microorganisms for acetic acid production are bacteria that belong to the genus *Acetobacter.* A high acetic acid-producing bacterium from *mabolo,* a sugary fruit, was identified as *Acetobacter rancens* Beijerinck var. *turbidans* Frateur (Maceda and Palo, 1967). In the production of white vinegar, a special strain of *Acetobacter* sp. was used (Ross, 1961).

Rao and Stokes (1953a) reported that for optimum growth of *Acetobacter* bacteria, the medium must contain the required growth factors and appropriate sources of carbon. These growth factors include pantothenic acid, nicotinic acid, *p*-aminobenzoic acid and thiamine. Suitable sources of carbon include glucose and glycerol but not other simple sugars and ethanol. When these conditions are satisfied, acetic acid bacteria particularly *A. suboxydans* and *A. melanogenum* can utilize ammonium nitrogen for growth. On the other hand, some acetic acid bacteria including *A. rancens* require apparently new and

unidentified factors which could be found in yeast autolysate. However, Rao and Stokes (1953b) found that small amounts of glucose, fructose, mannitol and glycerol could successfully replace yeast autolysate. It was also noted that growth of bacteria cannot be supported by their metabolic processes such as oxidation of ethanol to acetic acid, unless organic substances like acetate or glucose were provided in the medium (Pasteur, 1968). It follows then that when these sugars and related substances are incorporated in the medium, growth of these bacteria will be established hence, they can oxidize ethanol to acetic acid.

Among the locally available fermented food products, nipa sap was found to be a good source of acetic acid bacteria. Baguinon (1982) found that 26.5 percent of the microbial population in nipa sap were acetic acid bacteria. A total titratable acidity of 0.37 percent in freshly gathered nipa sap was reported by Cantalejo and Ilaga (1982) and Alcasid (1982). These results clearly indicate that acetic acid producing bacteria are naturally present in nipa sap.

This study aimed to isolate, screen and identify high acetic acid-yielding bacteria from naturally fermenting nipa sap.

MATERIALS AND METHODS

Isolation of Acetic Acid Bacteria

Three samples of freshly gathered nipa sap (coded as A, B and C) from Bulacan were purchased. They were allowed to ferment at room temperature for 12 days.

Acetic acid bacteria were then isolated from the fermented nipa sap using the method described by Villacorta (1976). Beijerinck solution [$(NH_4)_2$ HPO_4, 0.59 g; KCl, 0.1g; ethanol, 30.0 ml; and tap water, 1.0 l)] was used as an enrichment medium for the acetic acid bacteria. The pH of the solution was adjusted to 6.5 with 0.1N NaOH. Fifty-ml portions of the solution were dispensed in 250-ml Erlenmeyer flasks and plugged with cotton. The medium was sterilized at 15 psi for 15 minutes and then added with ethyl alcohol at the rate of 1.5 ml per flask.

One-ml portions of the sap were aseptically pipetted into each of the flasks containing Beijerinck solution. The flasks were then incubated for one week at ambient temperature under agitated condition. Growth was examined microscopically and the enriched culture was streaked on Beijerinck agar, a medium containing 1 ml of Beijerinck solution, 15 g of agar and 15 ml of 10% Andrade's indicator prepared by mixing 0.5 g acid fuchsin, 16.0 ml NaOH and 100 ml distilled water.

Acid producing bacteria which exhibited pink color on Beijerinck agar with Andrade's indicator were taken out from the plates and transferred to Beijerinck agar slants with Andrade's indicator.

Purification of Acetic Acid Bacteria

The cultures were transferred into triplicate tubes of *Acetobacter* agar slants (10 g yeast extract; 10 g $CaCO_3$; 3 g glucose; 15 g agar, and 1 liter distilled water). They were purified in the Beijerinck agar with Andrade's indicator incorporated with actidione (20 mg/1 of prepared medium). Pour plate method was used to further purify the isolates. A total of 40 isolates was obtained from the samples. Twenty-two isolates were picked out from sample A (coded as A1 to A22), six from sample B (coded as B1 to B6) and 12 from sample C (coded as C1 to C12). The isolates were gram stained and examined microscopically. Gram-negative rods (bacilli) with rounded ends were retained for further study. These isolates were maintained in modified malt yeast agar (5 g yeast extract; 2 g malt extract; 10 g glucose; 1 g $CaCO_3$; 20 g agar; 20 ml alcohol and 1 liter distilled water). YMG medium (5 g yeast extract; 5 g malt extract; 20 g glucose; 55 ml ethyl alcohol; 1 liter distilled water) with a pH of 6.8 was used as starter for screening.

Screening of the Isolates Using Pasteurized Nipa Sap

Freshly gathered nipa sap was analyzed for its glucose and sucrose (Pridham, 1956), ash, total solids and acid content. Pasteurization of sap was done at 80°C for 20 minutes to inactivate the microorganisms and stop fermentation and other processes. After cooling, 100-ml portions of the pasteurized nipa sap were dispensed in 500-ml Erlenmeyer flasks which were then plugged with cotton. Eighteen gram negative bacilli with rounded ends were inoculated in the sap in three replications and were allowed to ferment for 14 days or until the amount of acetic acid produced decreased. The amount of acetic acid was determined every other day by titration using standardized 0.1 N NaOH. The percent acetic acid was computed using the following formula:

$$\% \text{ acetic acid} = \frac{Nb \times Vb \times \frac{MW}{100}}{Vs} \times 100$$

where Nb = normality of NaOH
Vb = volume (ml) of NaOH
Vs = volume (ml) of sample
MW = molecular weight of acetic acid

Identification of Acetic Acid Bacteria

The pure acetic acid bacteria were identified based on their morphological and physiological characteristics. Morphological characteristics were determined based on their gram reaction, size, shape, color, pellicle formation and arrangement of cells (Buchanan and Gibbons, 1974). Physiological characteristics were determined using the method of Carr and Passmore (1979). This was done on the basis of brown pigment formation, growth on ethanol, formation of 5-ketogluconate and 2-ketogluconate or gluconate, ketogenesis in glycerol

and acid, catalase and cellulose production.

RESULTS AND DISCUSSION

Forty colonies of bacteria were isolated from freshly fermented nipa sap from Paombong, Bulacan. Table 1 shows the microflora of nipa sap with their corresponding gram reaction and shape.

Seven isolates (A1, A4, A7, A8, A16, A18 and C12) were found to be gram-positive cocci, two were gram-positive, long and short bacilli (A2 and A9), 13 were gram-positive long bacilli (A5, A6, A10, A11, A13, A14, A15, A19, A22, B6, C3, C5 and C8) and 18 were gram-negative short bacilli with rounded ends (A3, A12, A13, A20, A21, B1, B2, B3, B4, B5, C1, C2, C4, C6, C7, C9, C10 and C11). The gram-negative short bacilli with rounded ends were maintained in *Acetobacter* agar slants for screening.

Acetic Acid Production of Gram-Negative Isolates in Pasteurized Nipa Sap

The 18 purified gram-negative bacilli with rounded ends were inoculated in pasteurized palm sap. The amounts of acid produced were determined every other day and are presented in Table 2. The isolates generally produced maximum amounts of acid on the sixth day except for C1, C2, C4, C6, C9 and C10 which produced highest amounts of acid after 8 days of fermentation. Highest acetic acid amount of 3.57% was produced by A12 followed by B1, with 3.43%. After 10 days, the amount of acetic acid generally decreased up to 14 days of fermentation.

The ten highest acetic acid-yielding isolates, namely A12, A17, A21, B1, B2, B3, B4, B5, C7 and C11 were picked out and their acetic acid yields were compared (Fig. 1). As

Table 1. Characteristics of nipa sap microflora.

Isolates	Gram Reaction	Shape
A1, A4, A7, A8, A16, A18, C12	+	Cocci
A2, A9	+	Long, short bacilli
A5, A6, 10, A11, A13, A14, A15, A19, A22, B6, C3, C5, C8	+	Long bacilli
A3, A12, A17, A20, A21, B1, B2, B3, B4, B5, C1, C2, C4, C6, C7, C9, C10, C11	—	Short bacilli with rounded ends

early as 2 days of fermentation, an abrupt increase in the acid production was observed in A12, B1, B2, B3, B4, B5 and C11 but not in A17, A21 and C7. However after 4 days of fermentation, A17, A21 and C7

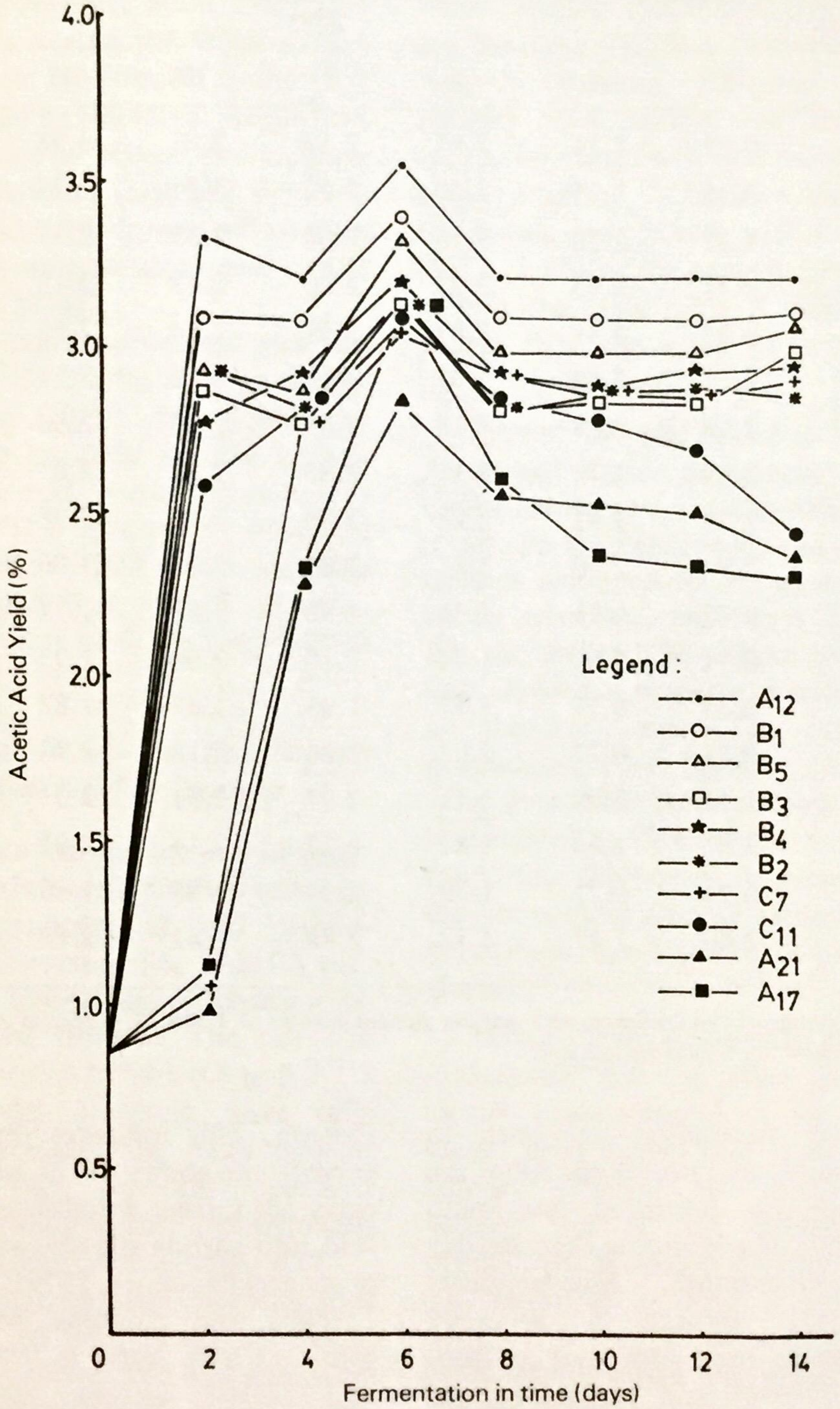

Figure 1. Amounts of acetic acid (%) produced by the ten highest acid-yielding bacterial isolates in pasteurized palm sap.

Table 2. Amount of acetic acid produced by the isolates in pasteurized nipa sap.[1]

Isolate	Amount of Acetic Acid (%)						
	Days						
	2	4	6	8	10	12	14
A3	0.71	1.14	2.63	2.19	2.01	1.98	1.84
A12	3.35	3.21	3.57	3.22	3.21	3.23	3.25
A17	1.27	2.36	3.15	2.64	2.39	2.37	2.33
A20	0.71	0.95	2.53	2.40	2.43	2.15	2.07
A21	0.95	2.31	2.87	2.59	2.54	2.52	2.39
B1	3.11	3.12	3.43	3.13	3.12	3.11	3.15
B2	2.94	2.85	3.18	2.85	2.90	2.90	2.95
B3	2.89	2.79	3.13	2.85	2.87	2.87	3.09
B4	2.79	2.91	3.23	2.96	2.90	2.98	3.06
B5	2.94	2.90	3.35	3.05	3.01	3.06	3.14
C1	0.71	0.71	1.30	1.90	1.82	1.74	1.33
C2	0.71	1.08	1.71	2.10	2.00	1.93	1.42
C4	0.71	0.71	1.77	1.93	1.87	1.82	1.37
C6	0.71	0.72	2.03	2.26	2.18	2.13	1.69
C7	1.14	2.79	3.11	2.97	2.91	2.91	2.90
C9	0.71	1.28	1.87	2.22	2.14	2.07	1.51
C10	0.71	0.71	1.79	2.11	1.95	1.70	1.47
C11	2.60	2.87	3.12	2.85	2.81	2.73	2.47

[1] Average of two replicates with starting alcohol content of 4.12%. Underlined values are the highest acetic acid percentages.

showed substantial increases in amount of acid produced. All of the 10 isolates produced maximum amounts of acid during the sixth day of fermentation. Measurements thereafter showed a generally decreasing trend. Moreover, a steeper decline in acid percentage was noted in A17, A21 and C11 than in the other isolates on the twelfth day thereon. This indicates the differences in the ability of the isolates to carry out further oxidation of acetic acid into carbon dioxide and water (Carr and Passmore, 1979).

Nipa sap contains 6% total solids, 1.03% ash, 0.71% acetic acid, 11.10% sucrose, 2.96% gluclose and 4.12% alcohol. It can essentially supply acetic acid bac-

teria with relatively low amount of sugar in the form of determined glucose which can be oxidized to alcohol. However, upon hydrolysis of sucrose during the fermentation process, an additional amount of glucose can be liberated. Regardless of raw material source, the medium must contain at least 8% sugar so that the finished vinegar will contain the minimum acetic acid level of 4% (Vaughn, 1954).

The alcohol content of nipa sap (4.12%) is sufficient since according to Nickol (1979), 4% alcohol by volume is required for the growth and acetic acid production of Acetobacters. This amount is sufficient to yield the desired acetic acid plus an amount that would allow a residual value of 0.2% unconverted alcohol.

Identification of the Ten Highest Acetic Acid-Yielding Bacteria

The ten highest acetic acid-yielding bacteria were identified based on their morphological and physiological characteristics. Table 3 presents the morphological characteristics of the isolates. The cell size ranged from 0.5-1.0 x 0.5 to 0.5-1.5 x 0.5. All of them have cells arranged singly, in pairs and in chains. They generally appeared white in young culture and yellowish white in older culture except A17.

The physiological characteristics of these 10 isolates are shown in Table 4. Isolate A12 was identified as *Acetobacter aceti* subsp. *aceti.* The formation of gluconate and consequently, acid production by this organism was shown by a cleared area around the bacterial growth due to dissolution of fine white calcium carbonate ($CaCO_3$) powder. Its positive response in Hoyer's medium indicated that ethanol was utilized as carbon source for its growth and for acetic acid production. Its positive reaction to catalase and glycerol tests showed that it produced the enzyme catalase and at the same time a dehydrogenase that converted glycerol to dihydroxyacetone.

Isolate A17 was found to be *A. paradoxus* subsp. *paradoxus.* This isolate exhibited a negative response to all the tests that were used. Six isolates belonged to *A. ascendens* subsp. *ascendens,* namely: A12, B1, B2, B3, B4 and B5. These isolates also showed a negative reaction to all the tests except in catalase production. Their inability to produce gluconate from glucose was investigated by De Ley (1961) and he found that the typical *A. ascendens* did not contain glucose oxidase nor hexokinase, hence it cannot oxidize glucose.

Isolate C7 *(A. lovaniensis* subsp. *lovaniensis)* had the same physiological characteristics as *A. aceti* subsp. *aceti* except that it did not convert glycerol to dihydroxyacetone due to its inability to produce the necessary enzyme. This was shown by its negative reaction to glycerol test. *A rancens* subsp. *pasteurianus* which was isolate C11 did not grow abundantly unlike the other isolates. This kind of growth among *A. rancens* species was also

Table 3. Morphological characteristics of the gram-negative bacilli isolated from nipa sap.

Isolate Number	Size (μ)	Color	Arrangement of Cells	Pellicle Formation
A12	0.5 — 1.2 x 0.5	White to yellowish	Singly, in pairs, in chains	+
A17	0.5 — 1.2 x 0.5	White	Singly, in pairs, in chains	—
A21	0.5 — 1.2 x 0.5	White to yellowish	Singly, in pairs, in chains	—
B1	0.5 — 1.2 x 0.5	White to yellowish	Singly, in pairs, in chains	—
B2	0.5 — 1.2 x 0.5	White to yellowish	Singly, in pairs, in chains	—
B3	0.5 — 1.5 x 0.5	White to yellowish	Singly, in pairs, in chains	—
B4	0.5 — 1.5 x 0.5	White to yellowish	Singly, in pairs, in chains	—
B5	0.5 — 1.0 x 0.5	White to yellowish	Singly, in pairs, in chains	—
C7	0.5 — 1.0 x 0.5	White to yellowish	Singly, in pairs, in chains	—
C11	0.5 — 1.0 x 0.5	White to yellowish	Singly, in pairs, in chains	+

reported by De Ley (1961) and he found that these bacteria which oxidized glucose only to gluconate grew poorly compared to other acetic acid bacteria. It produced acid from glucose and ethanol as shown by its growth on glucose and Hoyer's medium, though in a very small amount.

The physiological characteristics of bacteria measure their ability to produce acetic acid from alcohol. *A. aceti* subsp. *aceti* being the highest acid-yielding isolate, reacted positively to most of the physiological tests that were used.

The ability of acetic acid bacteria to utilize ethanol as carbon and energy source seemed to be an essential physiological activity (De Ley, 1961). The presence of ethanol in the medium thus enabled *A. ascendens* subsp. *ascendens* to produce a small amount of acid although this was partly inhibited by the presence of glucose. This agrees with the findings of Henneberg (1898 as cited by Asai, 1968) that the

Table 4. Physiological characteristics of acetic acid bacteria isolated from nipa sap.[1]

Isolate Number	Formation of Ketogluconate, 2-Ketogluconate or Gluconate	Production of Brown Pigment	Growth in Hoyer's Medium	Catalase Production	Ketogenesis on Glycerol	Production of Cellulose	Organism
A12	+	—	+	+	+	—	*Acetobacter aceti* subsp. *aceti*
A17	—	—	—	—	—	—	*A. paradoxus* subsp. *paradoxus*
A21	—	—	—	—	—	—	*A. ascendens* subsp. *ascendens*
B1	—	—	—	+	—	—	*A. ascendens* subsp. *ascendens*
B2	—	—	—	+	—	—	*A. ascendens* subsp. *ascendens*
B3	—	—	—	+	—	—	*A. ascendens* subsp. *ascendens*
B4	—	—	—	+	—	—	*A. ascendens* subsp. *ascendens*
B5	—	—	—	+	—	—	*A. ascendens* subsp. *ascendens*
C7	+	—	+	+	—	—	*A. lovaniensis* subsp. *lovaniensis*
C11	+	—	(—)[2]	+	—	—	*A. rancens* subsp. *pasteurianus*

[1] Identification based on De Ley and Frateur (1974)

[2] Usually negative

presence of glucose in the medium inhibits growth of *A. ascendens* while other strains can oxidize glucose to acid. It seemed therefore, that two types of *A. ascendens* could have different properties. This was probably the reason for the difference in the amount of acetic acid produced by the six isolates that were identified as *A. ascendens* subsp. *ascendens*. It is also possible that these isolates belong to different strains.

The dramatic increase in acid production by the bacteria could be due to "glucose effect" (Boyer, 1970) which means that glucose in the medium was utilized preferentially before the enzyme for the catabolism of the second substrate could be formed.

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