RESEARCH ARTICLE

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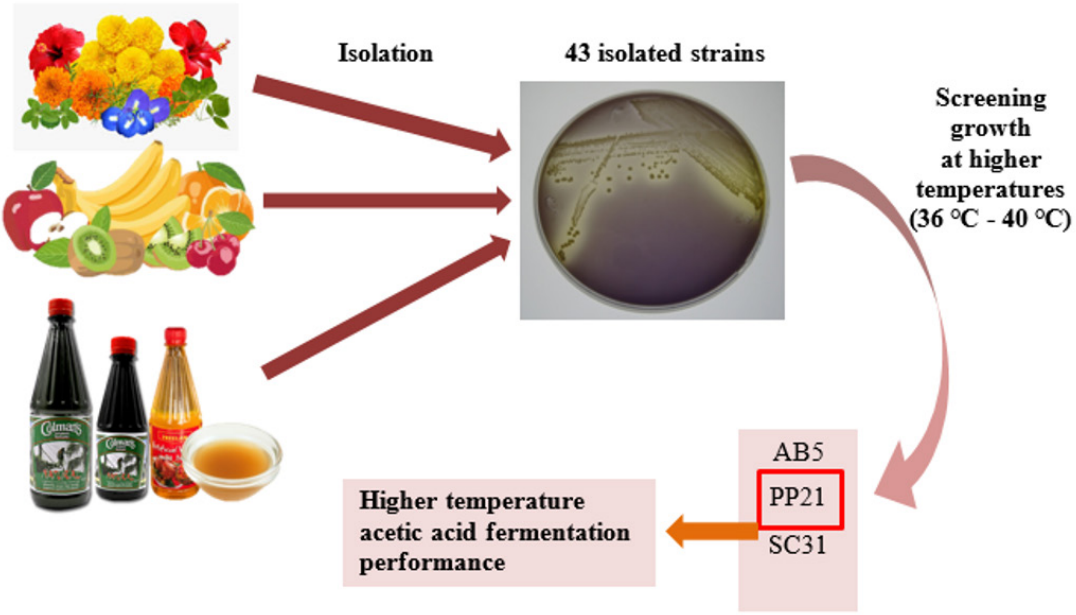
**M.A.A. Buddhika, P.A.B.N. Perumpuli* and M.N.Kaumal**




## Highlights

- Forty three different acetic acid bacteria were isolated from Sri Lanka.
- Three strains that grow even at 40 °C were screened.
- *Acetobacter pasteurianus* PP21 is a potential strain in vinegar production at higher temperatures.

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**RESEARCH ARTICLE**

# Identification and characterization of acetic acid bacteria species isolated from various sources in Sri Lanka

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**Abstract:** Forty-three acetic acid bacteria (AAB) strains were isolated from different types of fruits, flowers and fermented products collected from different regions of Sri Lanka. They were identified using morphological, physiological and biochemical methods. Based on their acetate oxidation pattern, 37 strains of isolated AAB were identified as either *Acetobacte*r or *Gluconacetobacter* while the rest of the strains were recognized as *Gluconobacter*. Along with the results of species level identification, isolated strains were identified as *A. pomorum*, *A. aceti*, *A. pasteurianus*, *Gluconacetobacter* spp. and *Gluconobacter frateurii*. In order to examine the growth characters of the isolates, they were grown on potato agar plates at 30 °C, 37 °C, and 40 °C, and all the isolated strains revealed good growth at both 30 °C and 37 °C while *A. aceti* MS33, *A. pomorum* SC31, *G. frateurii* MN12 and MN18 showed good growth even at 40 °C. Further, it was found that most of the isolated *Acetobacter*-type strains can tolerate up to 10% ethanol even at 37 °C, and in contrast, the acetate tolerance of all the isolated strains was found to be very poor. Then, acetic acid production ability of AB5, PP21 and SC31 was examined statistically either with 4% of 6% (v/v) ethanol at 30 °C, 36 °C and 37 °C, and all strains showed over 3.5% (v/v) acetate production either with 4% or 6% initial ethanol up to 36 °C. Further, *A. pasteurianus* PP21 gave an acetic acid production of 2.2% with 4% initial ethanol even at 37 °C. Thus, the obtained results revealed that all three tested strains are prospective applicants in the production of vinegar at higher temperatures.

***Keywords*:** Acetic acid bacteria; *Acetobacter*; *Gluconacetobacter*; *Gluconobacter*; fermented products.

## INTRODUCTION

Acetic acid bacteria (AAB) are gram-negative α-*Proteobacteria* that require oxygen as the terminal electron acceptor, and thus they are considered as obligate aerobes. They are found commonly in products as vinegar, fruits, flowers, rotten fruits or flowers, and so on and are famous for their capability to oxidize sugars and alcohols, to useful organic acids as their final products. This unique character has made them used in the production of fermented food such as vinegar, sorbose, and dihydroxyacetone. The AAB are at present classified into eight genera: *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconoacetobacter*, *Asaia*, *Kozakia*, *Swaminathania*, *Sacchribacter*, *Neoasaia* and *Granulibacter* (Cleenwerck *et al*., 2002; Greenberg *et al*., 2006; Yamada and Yukphan, 2008).

In recent years, most of the researchers showed diligence to find out novel approaches to perform oxidative fermentation at elevated temperature conditions. Generally, industrial vinegar fermentation is mainly performed using mesophilic AAB strains where their best temperature for growth and acetic acid production lies around 30 °C (Saeki *et al*., 1997). However, since vinegar fermentation in submerged culture is an exothermal process, where temperature increases up to about 35 °C or higher, the performance of traditional mesophilic strains would be badly affected. This is mainly due to the microbial enzyme deactivation and cell membrane damage caused by the increasing temperature (Chen *et al*., 2016). Thus, a sudden reduction in the performance of AAB could be seen, resulting in a severe reduction in fermentation rate and efficiency (Saeki *et al*., 1997). This leads to isolate, identify, and characterize thermotolerant AAB that could perform oxidative fermentation at higher temperature levels. Thermotolerant AAB is AAB that belongs to the same genera or same species as mesophilic strains but can grow at a temperature of 5 to 10 °C higher than generic mesophilic strains do, and these strains differ from thermophilic strains that could grow above 60 °C (Saeki *et al*., 1997). Hence, the application of thermotolerant strains in oxidative fermentation reduces the cost of cooling in the fermentation industry.

Isolation, identification, and characterization of AAB from different sources such as fruits, flowers, vinegar, alcoholic beverages, honey bees, sugar cane juices and soil have been frequently done in Thailand and Indonesia (Saeki *et al*., 1997; Yamada *et al*., 1999; Seearunruangchai *et al*., 2004; Tanasupawat *et al*., 2011). In contrast, there are only a few studies reported on the characterization of AAB from tropical regions (Ndoye *et al*., 2006; Ndoye *et al*., 2007). Furthermore, research on isolation, characterization and identification of AAB from Sri Lanka is also very limited except for the one reported on thermotolerant AAB isolated from pellicle of coconut water vinegar (Perumpuli *et al*., 2014). Furthermore, Atputharajah *et al*. (1986)

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has reported a study on the role of microorganisms and different biochemical reactions on the natural fermentation of coconut palm sap. Thus, due to the lack of information on AAB isolated from Sri Lanka, studying on AAB isolated from different sources in Sri Lanka would be interesting, and it would be advantageous for the development of the country`s fermentation industry.

Consequently, the current study was carried out to isolate, identify and characterize AAB species from different sources collected from Sri Lanka, and to evaluate the thermotolerant properties of the isolated strains especially those belong to genus *Acetobacter* and *Gluconobactor*.

## MATERIALS AND METHODS

### Culture media and cultivation

In order to isolate *Acetobacter* type strains, YPG medium with 0.5 g of each yeast extract, polypeptone, $CaCO_3$, 1 g of glycerol, 1.5 g of agar enhanced with 4 mL ethanol and 3 mg of bromocresol purple per 100 mL tap water were used. To isolate *Gluconobacter* type strains, YPS/YPM medium consists of each 0.1 g of yeast extract, polypeptone, 1 g of D-sorbitol or D-mannitol and 1.5 g of agar added with 3 mg of bromocresol purple per 100 mL distilled water was used. YPGD medium comprises yeast extract, polypeptone, glycerol and glucose each weighing 0.5 g per 100 mL tap water, and 1.5 g of agar was used in studying the growth of isolated strains at various temperature levels.

### Isolation of AAB

Fruits (mango, papaya, star fruit, banana, etc.) flowers (*sal* flower and Ixora), molasses, coconut toddy, vinegar, and many other sources collected from different parts of Sri Lanka were used as the sources to isolate AAB. In order to isolate *Acetobacter* type strains, 5 mL or 5 g of each sample cut into small portions (Fruits and flower samples), were added to sterile bottles contain either sterile distilled water with 2 or 4% (v/v) acetic acid, or 4 or 6% (v/v) ethanol. *Gluconobacter* type strains were isolated by placing the samples into bottles with an isolation medium containing either 10 g of D-sorbitol or D-mannitol, 1 g of yeast extract and 1 g of polypeptone per liter (pH was adjusted to 4.0 by addition of 10 mL of 1 M D-gluconic acid). The bottles were covered with aluminum foil to prevent insect contamination, and were statically incubated at 30 °C for seven days. After incubation of 3, 5 and 7 d, to isolate *Acetobacter* type strains and *Gluconobacter* type strains, loop-full of the sample were streaked on YPG agar plates augmented with 4% (v/v) ethanol and YPS/YPM agar plates respectively, and repeated streaking on the same agar plate was done until pure strains of the isolates were obtained. The obtained pure strains were preserved on slants of potato agar (1 g of yeast extract, 1 g of polypeptone, 2 g of glycerol, 0.5 g of glucose, 10 mL of potato extract and 1.5 g of agar made up to 100 mL with tap water), and stock cultures of the isolates were maintained under frozen conditions (-80 °C), after mixing with an equal volume of sterile glycerol.

### Identification of isolated AAB strains

Identification of the isolated strains was done using their morphological, biochemical and physiological properties as mentioned by Asai *et al*., (1964); Yamada *et al*., (1976); Katsura *et al*., (2002); and Brenner *et al*., (2005).

Gram staining of the isolates was done as described by Hucker and Conn (1923). The isolated bacterial cultures were incubated on D-glucose calcium carbonate agar slants containing 30 g of D-glucose, 2 g of yeast extract, 3 g of polypeptone and 2 g of $CaCO_3$ (precipitated), per liter for 10 d at 30 °C to observe pigmentation (Drysdale and Fleet, 1988).

In order to observe the catalase production, the isolated strains were cultured on potato agar plates for 24 h at 30 °C. Then, the bacterial cells were transferred onto a glass slide, and few drops of 3% hydrogen peroxide were added (Brenner *et al*., 2005).

To observe the acetic acid production from isolated colonies, they were inoculated on agar plates containing 30 mL of ethanol, 30 g yeast extract, 20 g $CaCO_3$ and 20 g agar per liter. Suspension of calcium carbonate around the colonies was taken as the indicator of acetic acid production (Perumpuli *et al*., 2014).

The medium comprising 5 g of yeast extract, 10 g of each carbon source in one liter of distilled water with the addition of 0.003% bromocresol purple (pH 6.8) was used to examine the acid production from glucose, lactose, fructose, sucrose, galactose maltose, xylose, mannitol, sorbitol and rhamnose, and the inoculated agar plates were incubated at 30 °C for 7 d to observe acid formation from different sugar sources.

Production of dihydroxyacetone (DHA) from glycerol was observed by culturing the isolated strains in a medium containing 30 g of glycerol, 5 g of yeast extract, and 10 g of polypeptone per liter. After incubation at 30 °C for 3 d under static culture, 200 µL of Fehling's solution was added to each test tube. The formation of DHA was confirmed by the appearance of orange colour in the medium (Yamada *et al*., 1999).

In order to examine the acetate and lactate oxidation ability to $CO_2$ and $H_2O$, the isolated strains were incubated at 30 °C for 24 h on a culture medium comprising 2 g of yeast extract, 3 g of polypeptone and 2 g of sodium acetate or sodium lactate with 0.003% bromothymol blue as pH indicator (Brenner *et al*., 2005).

To examine the acid production of isolated *Gluconobacter* strains from pentitols, they were inoculated in a culture medium containing 3 g of pentitiols (D-arabitol, L-arabitol or ribitol), 3 g of yeast extract and 3 g of polypeptone per liter. Bromothymol blue (0.003%) was used as the pH indicator, and the cultures were incubated by shaking (200 rpm) at 30 °C for 24 - 48 h (Yamada *et al*., 1999).

The carbon source utilization pattern of the isolated *Acetobacter* strains was examined using salt agar medium containing $(NH_4)_2HPO_4$ (0.02 g), $MgSO_4.7H_2O$ (0.01 g),

$Ca_5(PO_4)_3(OH)$ (0.01 g), glacial acetic acid (0.2 mL) and of agar (1.5 g) in 100 mL of tap water. Cultures were incubated at 30 °C for 10 days (Sokollek *et al*., 1998).

**Growth of isolated AAB strains**

Growth of isolated AAB strains was observed on potato agar plates at 30, 37, 39 and 40 °C for five days. According to Sudsakda *et al*. (2007), the potato medium has been considered as one of the enrichment media for AAB, and thus, the potato medium was used to examine the growth performance of the isolated strains. Furthermore, identified *Acetobacter* strains were cultured on YPGD agar plates supplemented with 1 - 10% (v/v) ethanol or 1 - 4% (v/v) acetic acid, and were allowed to grow at 30, 37, 39 and 40 °C for 5 d period to examine their ethanol and acetic acid tolerance respectively.

**Acetate production ability of the isolated *Acetobacter* strains**

To examine the acetic acid production ability of the identified *Acetobacter* strains, they were allowed to grow under static culture conditions for 10 d at 30, 36 and 37 °C in YPGD medium with 4 and 6% (v/v) ethanol. The acetic acid produced was measured by titration with 0.8N NaOH with phenolphthalein as a pH indicator.

**RESULTS AND DISCUSSION**

**Identification of the isolated strains**

Forty-three different bacterial strains were isolated from 43 different samples such as fruits, flowers, fermented products and rotten fruits collected from different regions in Sri Lanka; Matara (Southern Province), Panadura (Western Province), Ampara (Eastern Province), Ambilipitiya (Sabaragamuwa Province), Horowpothana (Northcentral Province), and isolates forming a yellow zone or halos around the colonies were selected as AAB (Diba *et al*., 2015). All the isolates were found to be Gram-negative, rod-shaped, and catalase-positive, and thus preliminarily identified as AAB. Moreover, these results were further confirmed by the capability of all the isolates to produce acetic acid from ethanol which is a primary character of AAB. Then, further characterization of the isolated strains was performed by many biochemical and physicochemical methods and the results of isolates identified as *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* respectively (data not shown).

According to biochemical and physicochemical identifications, 37 isolates out of the 43 isolated strains, were identified as *Acetobacter* and *Gluconacetobacter* type strains since they were able to oxidize ethanol completely into $CO_2$ and $H_2O$ (Brenner *et al*., 2005). Moreover, all the *Acetobacter* and *Gluconacetobacter* strains were able to convert the colour of the YPGD medium supplemented with bromothymol blue (as an indicator) from blue to yellow during incubation, and further incubation reverses this yellow colour into blue which indicates the ethanol over-oxidation ability of the *Acetobacter* and *Gluconacetobacter* strains. Conversely, the rest of the isolates were identified as *Gluconobacter* strains due to their incomplete oxidation of ethanol where reversing of the yellow colour into the blue was not visible during further incubation. These results not only confirm each strain as *Acetobacter*, *Gluconacetobacter* and *Gluconobater* but also differentiate *Acetobacter* and *Gluconacetobacter* from *Gluconobacter*. These results were further confirmed by examining the acetate and lactate oxidation ability of each isolate where only the isolates recognized as *Acetobacter* and *Gluconacetobacter* were able to oxidized acetate and lactate into carbon dioxide and water (Data not shown). The ability of *Acetobacter* and *Gluconacetobacter* strains to oxidize acetic acid which is known as over-oxidation of acetate is mainly happened through the tricarboxylic acid cycle (TCA cycle), and oxidation of other organic acids such as lactic, pyruvic, malic, succinic, citric and fumaric are also similarly happened. On the contrary, due to the deficiency of *alpha*-ketoglutarate dehydrogenase and succinate dehydrogenase enzymes, *Gluconobacter* strains do not have a functional TCA cycle. This makes *Gluconobacter* strains incapable to metabolize organic acids such as acetic and lactic (Matsushita *et al*., 2004).

Furthermore, all the isolates recognized as *Acetobacter* and *Gluconacetobacter* did not form any brown pigment in the YPG medium, and in contrast, some of the *Gluconobacter* isolates were able to form the brown coloured pigment in the same medium which is unique to *Gluconobater* species (Data not shown). Moreover, these results indicate that most of the strains identified as *Acetobacter* are lacking the ability to oxidize glycerol to DHA as it was found in some isolates of *Gluconacetobacter* and *Gluconobacter.* According to Asai *et al*. (1964), *Acetobacter*-type strains belong to *A. xylinus* are capable of oxidizing glycerol to DHA. Species belong to *Gluconobacter* and some species belongs to *Gluconacetobacter* type are capable of oxidizing glycerol to DHA by membrane-bound glycerol/sorbitol dehydrogenase that exhibits a broad substrate specificity (Mamlouk *et al*., 2013). In contrast, the absence of the above enzyme system in many *Acetobacter*-type strains makes them unable to oxidize glycerol to DHA that is useful in the pharmaceutical industry.

Thereafter, the acid production ability of the isolated *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* strains were examined, and the obtained results are summarized in Table 1. As it is shown in Table 1, none of the *Acetobacter* isolates produced acids from D-fructose, D-galactose, glycerol, maltose, sucrose, lactose, D-sorbitol and D-mannitol, and these results are confirmed with the findings by Kadere *et al*. (2008) and *Gluconacetobacter* isolates produced acids from glycerol, ethanol and D-glucose as it is confirmed by Mamlouk *et al*. (2013). But in contrast, the isolates identified as *Gluconobacter* showed their acid production in all the tested carbon sources except for glycerol and n-propanol. Thus, when considering the acid formation from different carbon sources; it is evident that strains belonging to Genus *Acetobacter* are having a less versatile enzymic arrangement as compared with Genus *Gluconacetobacter* and *Gluconobacter*. When considering

**Table 1:** Acid production of isolated *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* type strains from different sugar sources.

| Strain | D-glucose | D-fructose | D-galactose | Ethanol | Glycerol | n-propanol | Maltose | Sucrose | Lactose | D-Sorbitol | D-Mannitol | D-Arabitoll | L-Arabitol | Ribitol |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **SL13E-2** | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **PP1** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **PP2** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **SF3** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **AB4** [A] | + | – | – | + | – | w | – | – | – | – | – | – | – | – |
| **AB5** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **AN6** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **AN7** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **AN8** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **MN13** [A] | + | – | – | + | – | – | – | – | – | – | – | – | – | – |
| **SB15** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **SB16** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **SB17** [A] | + | – | – | + | – | – | – | – | – | – | – | – | – | – |
| **SB19*** | + | – | – | + | – | – | – | – | – | – | – | – | – | – |
| **PP21** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **PP22** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **PP24** [A] | + | – | – | + | – | – | – | – | – | – | – | – | – | – |
| **CH26** [A] | + | – | – | + | – | – | – | – | – | – | – | – | – | – |
| **SO27** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **AV29** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **SC31** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **SC32** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **MS33** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **MS35** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **SF37** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **RA38** [A] | + | – | – | + | + | + | – | + | + | – | – | – | – | – |
| **RA39** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **CT41** [A] | + | – | – | + | + | – | – | – | – | – | – | – | – | – |
| **CT42** [A] | + | – | – | + | – | + | – | – | – | – | – | – | – | – |
| **Gv43** [A] | + | – | – | + | + | + | – | – | – | – | – | – | – | – |
| **AN9** [GA] | + | – | – | + | + | + | – | – | – | – | – | – | – | – |
| **MN11** [GA] | + | – | – | + | + | – | – | – | – | – | – | | | |
| **MN14** [GA] | + | – | – | + | + | – | – | – | – | – | – | – | – | – |
| **PP20** [GA] | + | – | – | + | + | + | – | – | – | – | – | – | – | – |
| **PP23** [GA] | + | – | – | + | + | w | – | – | – | – | – | – | – | – |
| **PP25** [GA] | + | – | – | + | + | + | – | – | – | – | – | – | – | – |
| **AV30** [GA] | + | – | – | + | + | + | – | – | – | – | – | – | – | – |
| **MS34** [GA] | + | – | – | + | + | w | – | – | – | – | – | – | – | – |
| **MN10**[G] | + | – | – | + | + | – | – | – | – | – | – | + | + | + |
| **MN12** [G] | + | – | – | + | + | – | – | – | – | – | – | + | + | + |
| **MN18** [G] | + | – | – | + | + | – | – | – | – | – | – | + | + | + |
| **SO28** [G] | + | + | + | + | + | w | w | + | + | + | + | + | + | + |
| **SF36** [G] | + | + | + | + | – | – | + | + | + | + | + | + | + | + |
| **CB40** [G] | + | + | + | + | – | – | + | + | + | + | + | + | + | + |

Note: +: Positive; -: Negative; w: weakly positive; A: *Acetobacter* type; GA: *Gluconoacetobacter* type; G: *Gluconobacter* type.

sugar oxidation, the oxidative pentose phosphate pathway (PPP) was considered as the most essential route to break sugars and polyols to $CO_2$, and this pathway is prominently found in AAB strains belongs to Genus *Gluconobacter*. Furthermore, in *Gluconobacter* strains, sugars are more favored as carbon sources rather than *Acetobacter* or *Gluconacetobacter* since they can effectively obtain energy by metabolizing sugars via PPP (Mamlouk *et al*., 2013). In contrast, in *Acetobacter* strains where PPP is less prominent, they use hexose monophosphate pathway and Embden-Meyerhof-Parnas pathways to utilize sugars (Toit *et al*., 2002) and Entner-Doudoroff pathway occurs only *Gluconacetobacter* and *Acetobacter* strains which are capable of producing cellulose from sugars (Mamlouk *et al*., 2013).

Along with this, the species level identification of the isolated *Gluconobacter* strains was done using their acid production pattern from pentitols (D-arabitol, L-arabitol and ribitol). According to Yamada *et al*. (1999), this character is useful in discriminating *Gluconobacter* strains among *G. oxydans*, *G. frateurii* and *G. cerinus.* They further report that isolates belong to *G. frateurii* could produce acids from all the tested pentitols while *G. oxydans* could not produce acids from D-arabitol, L-arabitol or ribitol. On the other hand, *G. cerinus* could produce acids from D-arabitol, but not from L-arabitol or ribitol. According to the results shown in (Table 1), all the obtained *Gluconobacter* isolates showed their acid production from D-arabitol, L-arabitol and ribitol, and thus, all the *Gluconobacter* isolates were identified as *G. frateurii* (MN10, MN12, MN18, SO28, SF36 and CB40).

Further, biochemical identification of the isolated *Acetobacter* strains was done using their carbon source utilization pattern in salt agar and yeast extract/peptone agar medium (Table 2). According to Sokollek *et al*. (1998) *A. pomorum* is the only *Acetobacter* species that could grow well on ethanol, methanol and n-propanol with ammonium as the only nitrogen source. Furthermore, as they described, unlike the *A. pasteurianus* strain, the isolates belong to *A. pomorum* and *A. oboediens* could form DHA upon growth on glycerol. By considering all these factors, PPI, PP2, SF3, AB4, AN6, PP22, SB17, CH26, SC31, SC32, MS35 and RA39 strains were identified as *A. pomorum.*

Moreover, since they did not form DHA from glycerol and do not show any growth in salt agar media with ethanol, methanol, and n-propanol and in a certain case, with sucrose, AB5, SB15, PP21, PP24 and CT42 were identified as *A. pasteurianus.* On the other hand, as it was clarified by Sokollek *et al*. (1998), the strains capable of growing on ethanol and n-propanol with ammonium as an only nitrogen source can be classified as *A. pasteurianus.* Furthermore, as it was reported by Ohmori *et al*. (1980) and Lisdiyanti *et al*. (2000), *Acetobacter* strains that produce DHA from glycerol, and are able to produce acids from glucose, ethanol and propanol can be identified as *A. aceti*. Consequently, since they showed production of DHA from glycerol and acid production from glucose, ethanol and propanol, AN7, AN8, SB16, SB19, SO27, AV29, MS33, SF37 strains were classified as *A. aceti*.

According to Sokollek *et al*. (1998) strains fitting to the genus *A. europeus* did not grow well on glycerol. But, according to the results obtained, all the isolates were found to be growing well on glycerol as the only carbon source for their growth. Thus, none of the strains isolated from this study were not belonging to *A. europeus*.

Moreover, as well as AAB from genus *Acetobacter*, those of genus *Gluconacetobacter* are also able to oxidize the generated acetic acid, which is known as acetate over-oxidation *via* the TCA cycle (Matsushita *et al*., 2004). However, in contrast to *Acetobacter*-type strains, AAB belongs to the genus *Gluconacetobacter* could produce acids from glycerol (Mamlouk and Gullo, 2013). Hence, the strains PP20, PP23, PP25, AN9, MN11, MN14, AV30, MS34, RA38, CT41 and GV43 were categorized as *Gluconacetobacter*.

Consequently, the identified isolates from different sources were summarized in Table 3 along with their respective sources of isolation.

**Growth characteristics of the isolated strains**

Thereafter the growth characters of isolated *Acetobacter*, *Gluconobacter*, and *Gluconacetobacter* strains were observed on potato agar plates at 30, 37 and 40 ℃. As it is given in Table 4, most of the *Acetobacter* isolates showed their growth at 37 ℃ in potato agar plates while *A. pomorum* SC31 and *A. aceti* MS33 isolated from sugarcane and molasses respectively, showed a good growth even at 40 ℃. Furthermore, almost all the isolated *Gluconobacter* strains showed a good growth up to 37 ℃ on potato agar plates which is characteristic to *Gluconobacter* spp. But in contrast, *G. frateurii* MN12 and MN18 isolated from mango showed a good growth even at 40 ℃, which is interesting to *Gluconobacter* spp. According to Brenner *et al*. (2005), the optimum temperature for the growth of AAB was found to be in the range of 25 to 35 ℃. But, during the examination of growth characteristics, almost all the *Acetobacter* and *Gluconobacter* strains were found to be able to grow up to 37 ℃. As it was reported by Saeki *et al*. (1997), since they show their best growth at 30 ℃, most of the AAB are known to be mesophilic. However, there are some *Acetobacter* strains that are able to grow up to 40 ℃ (Saeki *et al*., 1997; Ndoye *et al*., 2006; Sengun *et al*., 2011), and *Gluconobacter* strains that could grow up to 37 ℃ (Moonmangmee *et al*., 2000; Toyama *et al*., 2005). These strains are known to be thermotolerant AAB strains that are useful in oxidative fermentation in tropical countries. Thus, according to the obtained results, *A. pomorum* SC31, *A. aceti* MS33, *G. frateurii* MN12 and *G. frateurii* MN18 isolated from sugarcane, molasses, and mango respectively, can be characterized as thermotolerant AAB since they were able to grow even at 40 ℃, and further studies needed to be conducted.

Other than that, the ethanol and acetic acid tolerance of isolated strains were also examined at 37 ℃ to further characterization of their thermotolerant properties. According to the results in Table 4, almost all the isolates except for RA38 and GV43 showed a good growth up to

**Table 2:** Carbon source utilization pattern of isolated *Acetobacter*, and *Gluconacetobacter* strains.

| | D-glucose | D-mannitol | Ethanol | Methanol | Glycerol | n-propanol | D-Fructose | Sucrose | Maltose | Lactose | D-sorbitol | D-Galactose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **SL13E-2** | +++ | ++ | – | – | ++ | – | + | – | – | – | – | – |
| **PP1** [A] | + | + | + | + | ++ | +++ | ++ | – | + | + | – | – |
| **PP2** [A] | + | + | + | + | ++ | +++ | ++ | – | – | – | – | – |
| **SF3** [A] | +++ | + | + | + | +++ | +++ | + | – | – | + | – | – |
| **AB4** [A] | ++ | + | + | + | + | ++ | ++ | – | – | – | – | – |
| **AB5** [A] | +++ | + | – | – | + | ++ | + | – | – | – | – | – |
| **AN6** [A] | ++ | + | + | + | ++ | ++ | ++ | ++ | + | + | – | – |
| **AN7** [A] | ++ | + | + | – | ++ | ++ | ++ | + | + | + | – | – |
| **AN8** [A] | + | – | – | – | + | – | ++ | – | – | + | – | – |
| **MN13** [A] | ++ | – | + | + | + | +++ | + | – | – | – | – | – |
| **SB15** [A] | +++ | + | – | – | ++ | – | +++ | – | – | – | – | – |
| **SB16** [A] | ++ | + | – | – | + | – | +++ | ++ | – | + | – | – |
| **SB17** [A] | ++ | – | + | + | +++ | ++ | – | – | – | – | – | – |
| **SB19** [A] | +++ | + | – | – | + | +++ | + | – | + | + | – | – |
| **PP21** [A] | + | – | – | – | ++ | – | – | – | – | – | – | – |
| **PP22** [A] | ++ | + | + | + | ++ | +++ | ++ | + | + | + | – | – |
| **PP24** [A] | ++ | – | – | – | ++ | ++ | + | – | – | – | – | – |
| **CH26** [A] | +++ | ++ | + | + | +++ | +++ | +++ | ++ | ++ | – | – | – |
| **SO27** [A] | ++ | + | – | – | ++ | ++ | ++ | + | + | – | – | – |
| **AV29** [A] | +++ | ++ | – | – | +++ | ++ | + | + | + | + | – | – |
| **SC31** [A] | +++ | + | ++ | ++ | +++ | +++ | +++ | – | – | – | – | – |
| **SC32** [A] | ++ | + | + | + | +++ | + | ++ | – | + | – | – | – |
| **MS33** [A] | +++ | ++ | – | – | +++ | +++ | +++ | ++ | +++ | + | – | – |
| **MS35** [A] | +++ | ++ | + | + | ++ | ++ | ++ | – | ++ | + | – | – |
| **SF37** [A] | +++ | +++ | ++ | – | +++ | + | – | ++ | ++ | – | – | – |
| **RA38** [A] | +++ | +++ | – | – | +++ | – | – | – | – | – | – | – |
| **RA39** [A] | +++ | + | + | + | +++ | + | – | – | – | – | – | – |
| **CT41** [A] | – | – | – | – | ++ | – | – | – | – | – | – | – |
| **CT42** [A] | – | – | – | – | +++ | – | – | – | – | – | – | – |
| **Gv43** [A] | ++ | + | – | – | ++ | – | – | – | – | – | – | – |
| **AN9** [GA] | + | – | – | – | ++ | – | ++ | + | + | + | – | – |
| **MN11** [GA] | ++ | + | – | – | ++ | – | ++ | + | + | – | – | – |
| **MN14** [GA] | ++ | – | – | – | + | – | ++ | + | + | – | – | – |
| **PP20** [GA] | ++ | – | – | – | ++ | – | ++ | + | – | – | – | – |
| **PP23** [GA] | ++ | + | – | – | ++ | ++ | + | – | – | + | – | – |
| **PP25** [GA] | +++ | + | – | – | ++ | +++ | – | – | – | – | – | – |
| **AV30** [GA] | ++ | – | – | – | ++ | – | ++ | + | – | – | – | – |
| **MS34** [GA] | ++ | + | – | – | ++ | ++ | + | – | – | + | – | – |

Note: +++: Good growth; ++: Moderate growth; +: Poor growth; -: Negative growth; A: *Acetobacter* type; GA: *Gluconoacetobacter* type.

**Table 3:** Species-level identification of isolates.

| Source | Code of Isolate | Isolated Location | Identified AAB Species |
|---|---|---|---|
| **Papaya (*Carica papaya*)** | PP1 | Kamburupitiya | *A. pomorum* |
| | PP2 | Kamburupitiya | *A. pomorum* |
| | PP20 | Ambilipitiya | *Gluconacetobacter* sp. |
| | PP21 | Ambilipitiya | *A. pasteurianus* |
| | PP22 | Ambilipitiya | *A. pomorum* |
| | PP23 | Ambilipitiya | *Gluconacetobacter* sp. |
| | PP24 | Ambilipitiya | *A. pasteurianus* |
| | PP25 | Ambilipitiya | *Gluconacetobacter* sp. |
| **Star fruit (*Averrhoa carambola*)** | SF3 | Kamburupitiya | *A. pomorum* |
| | SF36 | Akuressa | *G. frateurii* |
| | SF37 | Akuressa | *A. aceti* |
| **Ambarella (*Spondias dulcis*)** | AB4 | Panadura | *A. pomorum* |
| | AB5 | Panadura | *A. pasteurianus* |
| **Nelli (*Phyllanthus emblica*)** | AN6 | Panadura | *A. pomorum* |
| | AN7 | Panadura | *A. aceti* |
| | AN8 | Panadura | *A. aceti* |
| | AN9 | Panadura | *Gluconacetobacter* sp. |
| **Mango (*Mangifera indica*)** | MN10 | Panadura | *G. frateurii* |
| | MN11 | Panadura | *Gluconacetobacter* sp. |
| | MN12 | Panadura | *G. frateurii* |
| | MN13 | Panadura | *A. pomorum* |
| | MN14 | Panadura | *Gluconacetobacter* sp. |
| | MN18 | Ambilipitiya | *G. frateurii* |
| **Banana (*Musa spp.*)** | SB15 | Ambilipitiya | *A. pasteurianus* |
| | SB16 | Ambilipitiya | *A. aceti* |
| | SB17 | Ambilipitiya | *A. pomorum* |
| | SB19 | Ambilipitiya | *A. aceti* |
| **Surinam cherry (*Eugenia uniflora*)** | CH26 | Panadura | *A. pomorum* |
| **Bitter orange (*Citrus aurantium*)** | SO27 | Ampara | *A. aceti* |
| | SO28 | Ampara | *G. frateurii* |
| **Avacado (*Persia americana*)** | AV29 | Bandarawela | *A. aceti* |
| | AV30 | Bandarawela | *Gluconacetobacter* sp. |
| **Suagarcane (*Saccharum officinarum*)** | SC31 | Ampara | *A. pomorum* |
| | SC32 | Ampara | *A. pomorum* |
| **Molasses** | MS33 | Ampara | *A. aceti* |
| | MS34 | Ampara | *Gluconacetobacter* sp. |
| | MS35 | Ampara | *A. pomorum* |
| **Rose apple (*Syzygium jambos*)** | RA38 | Kamburupitiya | *Gluconacetobacter* sp. |
| | RA39 | Kamburupitiya | *A. pomorum* |
| **Cannonball flower (*Couroupita guianensis*)** | CB40 | Kamburupitiya | *G. frateurii* |
| **Coconut toddy** | CT41 | Panadura | *Gluconacetobacter* sp. |
| | CT42 | Panadura | *A. pasteurianus* |
| **Guava (*Psidium guajava*)** | GV43 | Horowpathana | *Gluconacetobacter* sp. |

**Table 4:** Growth properties of isolated strains.

| Strain No. | Growth on the potato agar plate | | Ethanol tolerance at 37 ℃ on the potato agar plate | | | | | Acetic Acid tolerance on the potato agar plate | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 37 ℃ | 40 ℃ | | | | | | 30 ℃ | | 37 ℃ | |
| | | | 2% | 4% | 6% | 8% | 10% | 1% | 2% | 1% | 2% |
| **SL13E-2** | +++ | + | +++ | +++ | +++ | +++ | +++ | +++ | – | – | – |
| **PP1[A]** | + | – | +++ | +++ | +++ | +++ | ++ | – | – | – | – |
| **PP2[A]** | + | – | +++ | +++ | +++ | + | + | – | – | – | – |
| **SF3[A]** | +++ | + | +++ | +++ | +++ | +++ | +++ | ++ | – | + | – |
| **AB4[A]** | +++ | + | +++ | +++ | +++ | +++ | +++ | +++ | – | – | – |
| **AB5[A]** | +++ | + | +++ | +++ | +++ | +++ | +++ | +++ | – | ++ | – |
| **AN6[A]** | ++ | + | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **AN7[A]** | ++ | + | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **AN8[A]** | +++ | + | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **MN13[A]** | ++ | + | +++ | +++ | +++ | +++ | +++ | +++ | – | – | – |
| **SB15[A]** | + | – | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **SB16[A]** | ++ | + | +++ | +++ | +++ | +++ | +++ | + | – | – | – |
| **SB17[A]** | +++ | + | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **SB19[A]** | ++ | – | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **PP21[A]** | +++ | + | +++ | +++ | +++ | +++ | +++ | +++ | – | ++ | – |
| **PP22[A]** | ++ | + | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **PP24[A]** | ++ | – | +++ | +++ | +++ | +++ | ++ | +++ | – | – | – |
| **CH26[A]** | +++ | + | +++ | +++ | +++ | +++ | +++ | + | – | – | – |
| **SO27[A]** | +++ | ++ | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **AV29[A]** | +++ | +++ | +++ | +++ | +++ | – | – | – | – | – | – |
| **SC31[A]** | ++ | + | +++ | +++ | +++ | +++ | +++ | +++ | – | – | – |
| **SC32[A]** | +++ | + | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **MS33[A]** | +++ | + | +++ | +++ | +++ | +++ | +++ | +++ | – | ++ | – |
| **MS35[A]** | + | – | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **SF37[A]** | ++ | + | +++ | +++ | +++ | +++ | +++ | +++ | – | – | – |
| **RA38[A]** | +++ | + | + | + | + | – | – | – | – | – | – |
| **RA39[A]** | + | – | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **CT41[A]** | +++ | + | ++ | ++ | ++ | ++ | ++ | – | – | – | – |
| **CT42[A]** | +++ | + | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **Gv43[A]** | +++ | + | + | + | + | – | – | – | – | – | – |
| **AN9[GA]** | +++ | + | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **MN11[GA]** | + | + | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **MN14[GA]** | ++ | – | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **PP20[GA]** | +++ | + | +++ | +++ | +++ | +++ | ++ | – | – | – | – |
| **PP23[GA]** | +++ | – | +++ | +++ | +++ | +++ | +++ | +++ | – | ++ | – |
| **PP25[GA]** | + | + | +++ | +++ | +++ | +++ | ++ | – | – | – | – |
| **AV30[GA]** | +++ | + | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **MS34[GA]** | +++ | + | +++ | +++ | +++ | +++ | +++ | – | – | – | – |
| **MN10[G]** | + | + | +++ | – | – | – | – | +++ | – | ++ | – |
| **MN12[G]** | + | – | +++ | – | – | – | – | – | – | – | – |
| **MN18[G]** | +++ | – | +++ | – | – | – | – | – | – | – | – |
| **SO28[G]** | +++ | + | +++ | – | – | – | – | ++ | – | + | – |
| **SF36[G]** | +++ | + | +++ | – | – | – | – | +++ | – | – | – |
| **CB40[G]** | + | – | +++ | – | – | – | – | +++ | – | ++ | – |

Note: +++: Good growth; ++: Moderate growth; +: Poor growth; -: Negative growth; A: *Acetobacter* type; GA: *Gluconoacetobacter* type; G: *Gluconobacter* type.

10% ethanol at 37 ℃. But in contrast, acetic acid tolerance of isolated *Acetobacter* strains was very poor where a very few numbers of strains could grow only at 1% acetic acid concentration even at 30 ℃ and none of the isolates showed their growth at 2% acetic acid even at 30 ℃. But, the strain MS33, showed good growth at 1% acetic acid at 37 ℃, and by considering these growth characters and ethanol and acetic acid tolerance, it can be considered as a thermotolerant strain. On the other hand, all the *Gluconobacter* isolates of the current study showed very poor resistance to both acetic acid and ethanol, which is typical to the Genus *Gluconobacter*.

**Acetate production of the isolated strains**

Then the acetic acid production of isolated *Acetobacter* strains was studied at 30 ℃, 36 ℃ and 37 ℃ with 4% and 6% initial ethanol concentrations using *A. pasteurianus* SL13E-2 isolated from Sri Lankan coconut water vinegar vat (Perumpuli *et al*., 2014) as the reference strain. After several preliminary studies, and considering the growth characters of the isolates at different temperatures, *A. pateurianus* AB5, PP21 and *A. pomorum* SC31 isolated from *Ambarella* (*Spondias dulcis*), Papaya (*Carica papaya*), and Sugarcane (*Saccharum officinarum*) respectively, were selected for further examination of their acetic acid production at different temperatures. Thus, those strains were statically grown in YPGD media supplemented with 4% and 6% ethanol at 30 ℃, 36 ℃ and 37 ℃ (Figure 1 and Figure 2 respectively).

When compared the growth characters of the test strains, all the isolates except for the reference SL13E-2 strain, showed a poor growth at 37 ℃ either with 4% and 6% initial ethanol (data not shown). As it is shown in Fig.1, as well as the SL13E-2 strain, all three tested strains gave beyond 3.5% (w/v) of acetic acid production either at 30 ℃ or 36 ℃ without any considerable lag phase. At 37 ℃, even after five days of lag phase *A. pasteurianus* PP21 gave a maximum acetic acid production of 2.2% (w/v) with a 4% initial ethanol level, and it was significantly higher than that of the other two isolates. But in contrast, both *A. pasteurianus* AB5 and *A. pomorum* SC31 strains showed very poor growth (data not shown) and no significant acid production which is due to high-stress conditions and inhibiting enzyme activity at elevated temperatures. However, with a 4% initial ethanol level, SL13E-2 reference strain showed an acid production of 2.95% (w/v) with a very short lag phase even at 37 ℃ due to its high thermotolerant properties. According to Perumpuli *et al*. (2014) both ADH and ALDH activities of SL13E-2 strain were found to be 3.2-fold and 1.5-fold greater at 37 ℃ than those of 30 ℃. This is the main reason for good acid production by SL13E-2 even at 37 ℃ at 6% ethanol.

Furthermore, as it is shown in Figure 2, any of the strains isolated in the current study did not show any acid production at 37 ℃ with 6% (w/v) initial ethanol which could be due to high-stress conditions to the isolated strains gained by a high percentage of initial ethanol and high-temperature conditions. But, when compared the acetic

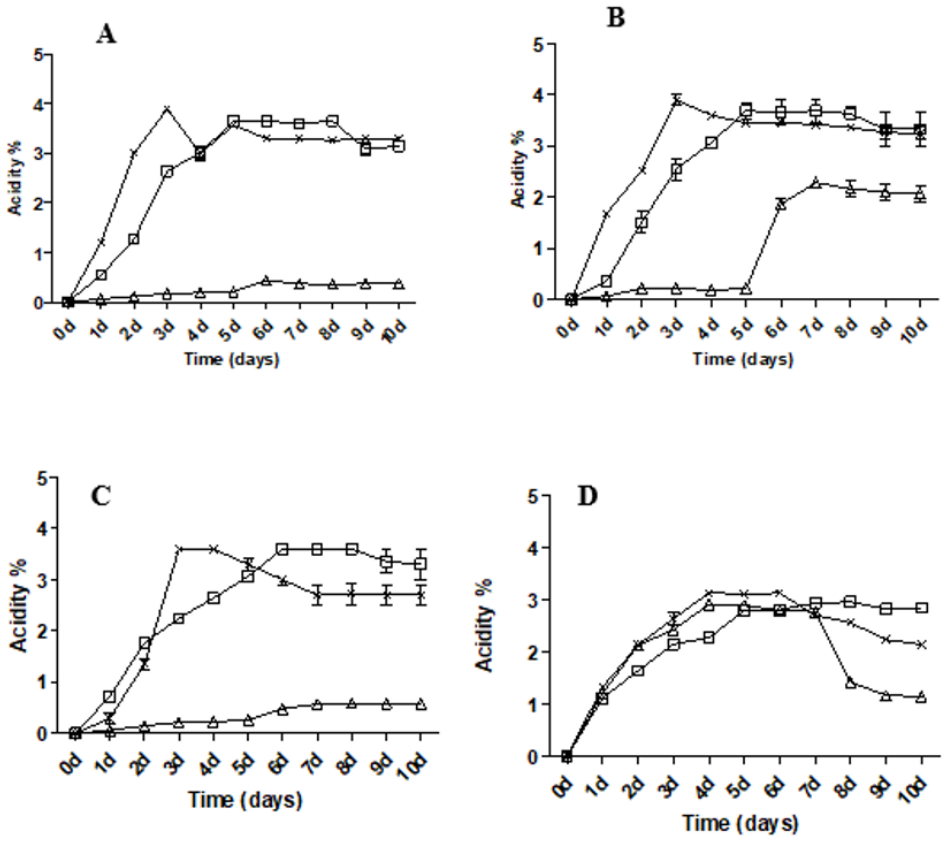


**Figure 1:** Comparison of acetic acid production of *A. pasteurianus* AB5 (A) PP21; (B) *A. pomorum* SC31; (C) with *A. Pasteurianus* SL13E-2 and (D) the reference strain at 30 ℃. (Squares), 36 ℃ (Crosses), and 37 ℃ (Triangles) with 4% initial ethanol level.

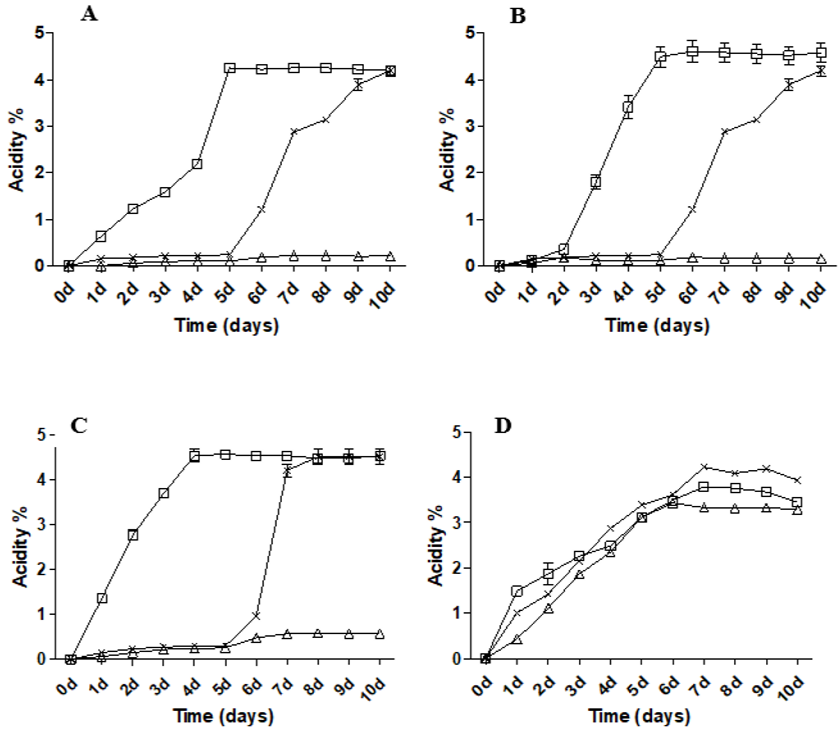


Figure 2: Comparison of acetic acid production of *A. pasteurianus* AB5 (A) PP21; (B) *A. pomorum* SC31; (C) with *A. Pasteurianus* SL13E-2 and (D) the reference strain at 30 ℃ (Squares), 36 ℃ (Crosses), and 37 ℃ (Triangles) with 6% initial ethanol level.

acid production of all three isolates with both 4% and 6% initial ethanol, it was found that *A. pasteurianus* PP21 showed the highest acid production of 4.53% (w/v) at 30 ℃ with 6% (w/v) initial ethanol (Fig.2). Moreover, when compared with the other two isolates, *A. pasteurianus* PP21 gave a significantly higher level of acetic acid production of 4.2% (w/v) at 36 ℃ with a 6% initial ethanol level. In contrast, as it is shown in Figure 2, the reference strain, *A. pasteurianus* SL13E-2 strain gave an acid production of 3.7% (w/v) at 37 ℃ even with 6% ethanol. These results suggest that all three strains are showing less thermotolerant properties when compared to SL13E-2. But when compared to SL13E-2 at 30 ℃ and 36 ℃ all the tested isolates of the current study showed a higher acetic acid production using 6% (v/v) initial ethanol.

Further, when comparing the acid production and growth pattern of the isolated strains with 6% initial ethanol, all isolates except for SL13E-2 showed a five days lag period at 36 ℃, and finally, they all gave 4% (w/v) acetic acid production, which is more or less similar that of 30 ℃. According to Saeki *et al*. (1997) acquiring a lag phase means taking a long time by the organisms to adapt to higher ethanol concentrations at higher temperatures. Furthermore, when compared to acetic acid production of all four strains with 4% and 6% initial ethanol at any temperature level, acetate over-oxidation was observed by all the strains with 4% initial ethanol level, and it was comparatively higher in SL13E-2. But, in contrast, such over-oxidation of acetic acid was not observed by all the isolates with 6% (w/v) initial ethanol, where they produced over 4% (w/v) acetic acid during fermentation. These results are even in accordance with previously stated results by Perumpuli *et al*. (2014). Furthermore, as it was reported by Saeki *et al*. (1997), acetate over-oxidation was not perceived when the acetic acid level of the culture media exceeded 4.5% (w/v). Consequently, since the acetate production by selected isolates with 6% (w/v) initial ethanol in the current study was also found to be around 4.5% (w/v), acetate over-oxidation was not observed during acetate fermentation by all four strains. In addition, the presence of a remaining amount of oxidizable ethanol and other carbon sources in the culture media is also interrupted over-oxidation of acetic acid (Yamada *et al*., 2008). Thus, the selected strains can be used in vinegar fermentation successfully without any acetate over-oxidation.

Moreover, currently, coconut vinegar production is mostly depending on the mother vinegar, where the responsible organisms are not being identified clearly. Thus, use of pure strains with acetic acid production at higher temperatures would be advantageous in the development of the fermentation industry.

## CONCLUSION

During the current study, 43 acetic acid bacteria were isolated successfully from different sources in Sri Lanka and, they were found to be belonging to the genus *Acetobacter*, *Gluconobacter*, and *Gluconacetobacter.* According to their growth characters, ethanol and acetic acid tolerance, *A. pasteurianus* AB5, PP21, and *A. pomorum* SC31 were found to be some potential candidates for vinegar production of temperature up to 36 ℃. Furthermore, these results suggest *A. pasteurianus* PP21 as a good candidate for the production of fruit vinegar with higher acetic acid concentrations for culinary purposes while the other two strains can be used in the production of vinegar with lower

acid concentrations that is ideal for drinking purposes at higher temperatures. However, further studies on the development of fruit vinegar and vinegar for culinary purposes using locally available fruits and vegetables respectively, using the isolated AAB are needed.

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## DECLARATION OF CONFLICT OF INTEREST

The authors declare no competing interests.

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