Research Article

Isolation and Identification of Thermotolerent Acetic Acid Bacteria from Waste Fruits

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ABSTRACT

In the present study, thirteen thermotolerant acetic acid bacteria were isolated from waste pomegranate and apple fruit in Carr agar medium following the dilution plate technique method at 37°C. The bacterial strain capable to change the colour of the medium from green to yellow were considered as *Acetobacter* sp. Most of the bacterial isolate could able to tolerate 3-4% of ethanol concentration, change the colour and pH of the medium and produce acetic acid in the range of 1.1-15.2 g/L. Among thirteen bacterial strains, the bacterial strain PAAB-3 could able to tolerate maximum ethanol (5%) and produce maximum acetic acid (15.2g/L) at 37°C. On the basis of morphological and biochemical characteristics the bacterial isolate, PAAB-3 is tentatively identified as *Acetobacter aceti*.

Key words: Acetic acid, Overoxidation, Pomegranate, Thermo tolerant Waste fruits.

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INTRODUCTION

Acetic acid bacteria (AAB) are a group of micro-organisms included in the *Acetobacteraceae* family. Aceticacid bacteria plays very important role in the industrial production of vinegar.[1] In the natural ecosystem, AAB founds in fruits or flowers where as naturally spoiled fruits are also considered as an excellent medium for the growth of AAB which might be due to the partial fermentation of the rotten food into alcohols.[2] Their name acetic acid bacterium is due to their very unique ability of oxidising alcohol into acetic acid, through this oxidative fermentation.[2] Two membrane-bound enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) present in the outer surface of the cytoplasmic membrane of acetic acid bacteria are responsible for the catalysis of ethanol. PQQ-dependent alcohol dehydrogenase (ADH) at first oxidized ethanol to acetaldehyde which is further oxidized to acetic acid by aldehyde dehydrogenase (ALDH).[3] AAB are mostly gram-negative, catalase-positive, oxidase-negative, obligate aerobes, non-spore forming, motile, rod and can grow in the presence of 0.35% acetic acid.[4] The most important in genera of *Acetobactaceae* involved in fermentation of foods are *Acetobacter* and *Gluconobacter*. The major characteristic that can easily differentiate between these two close genera are that *Acetobacter* can oxidize acetate to CO_2 and H_2O, while *Gluconobacter* cannot as they have non-functional α-ketoglutarate and succinate dehydrogenase of TCA cycle.[5] Acetic acid bacteria are mostly mesophilic, however, strains that can grow at a temperature of 42°C have been also reported as thermotolerant acetic acid bacteria.[6] During industrial vinegar production, generation of temperature during fermentation process poses a challenge for most of the industrial strain.

Keeping the above literature in sight, the present research investigation is aimed to isolate and identify potential thermotolerent AAB from rotten fruits that could grow at higher temperature and could tolerate higher ethanol concentration to carter the future need of food and beverage industry.

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MATERIALS AND METHODS

Sample collection and enrichment: For isolation of acetic acid producing bacteria, rotten apples and

pomegranates were collected aseptically in a sterile zip pouch plastic bag from local market of Bhubaneswar, Odisha. The collected samples were washed in sterile distilled water and were subsequently crushed. For enrichment of the sample approximately 5g of samples were submerged in 3% acetic acid and 4% ethanol solution and incubated at 37°C for 2-3 days.[7,8]

Isolation of acetic acid bacteria: From each enrichment broth medium mentioned above, 1ml of sample was taken out and mixed with 9 ml of sterile distilled water following serial dilution method. 0.1 ml of diluted samples from last tube were transferred with sterile pipette and pour plated on Carr agar medium (ethanol 2.0 %, yeast extract 3.0%, bromocresol green 0.002%, agar 2.0% and pH 6.8)[9] in aseptic condition inside the laminar flow. After solidification, the plates were taken out and incubated at 37°C for 24 h.[10] Bacterial colony could able to turn the blue-green colour of the Carr agar medium into yellow and blue-green again were considered as acetic acid bacteria[11] and were isolated for further study

Screening for alcohol tolerance ability: All the thirteen bacterial isolates were screened for their ability to grow in Carr broth medium (with bromocresol green) and supplemented with 3-8% (v/v) ethanol. In 50 ml medium, 100 µl of pre-grown respective bacterial culture were inoculated in aseptic condition and incubated at 37°C for 24 h. After 24 hr of incubation, pH and colour change of the growth medium was observed. The growth medium without inoculation was kept as control.

Acetic acid production: For acetic acid production all the thirteen isolates were incubated at various time points at 37°C, 120 RPM for 120 h in GYC broth medium (g/L: Glucose 20; $CaCO_3$ 20; yeast extract 10) added with 5% ethanol aseptically after sterilization. Further the amount acetic acid was estimated in triplicate by titration of the fermented sample against 0.5 N sodium hydroxide (NaOH) with phenolphthalein indicator as described earlier by Beheshti and Shafiei.[10] The amount of (in gm) of acetic acid produced in 100 ml of medium was calculated using the following formula:

Acetic acid (g/100 ml) = Volume of NaOH (ml) used in titration × 0.03 × 20.

Identification of selected acetic acid bacteria: The selected bacterial isolate was identified by means of morphological examination and some biochemical characterisation. The standard parameters investigated included colony characteristics, shape, size, motility, Gram's reaction, catalase, oxidase, MR test, Voges-Proskauer (V-P) reaction, indole production, nitrate reduction, carbohydrate metabolism (acid-gas production), oxidation of lactate to CO_2 and H_2O, oxidation acetate to CO_2 and H_2O, growth in the presence of ethanol, growth at different pH, ketogenesis from glycerol, cellulose production and water-soluble brown pigment production test were carried out following the standard methods described.[12-14] Identification of the bacterial isolate was carried out according to ninth edition of the Bergey's Manual of Determinative Bacteriology.[15]

Statistical analysis: The results obtained were subjected to statistical analysis as mean and standard deviation.[16] The mean values and standard deviations were calculated from the data obtained from three different experiments.

RESULTS

Isolation of acetic acid bacteria: A total thirteen bacterial isolates were isolated using Carr agar medium. Out of the thirteen, seven bacterial isolated were isolated from rotten apple enrichment culture and named as AAAB-1 to AAAB-7. The rest six bacteria were isolated from rotten pomegranate enrichment culture and named as PAAB-1 to PAAB-7. All the thirteen bacterial isolates were isolated from Carr agar medium on the basis of change of the colour of the medium from green to yellow after 24 h and green again after 72 h due to over oxidation (Figure 1).

Thirteen bacterial isolates were tested for their ability to grow in the medium supplemented with different concentration (3-8%) of ethanol. It has been found that most of the bacterial isolates showed moderate growth, change in colour and pH of the medium between 3-4% of alcohol concentration in the growth medium (Table 1). Among the thirteen bacterial isolates, PAAB-3 showed highest (5%) ethanol tolerance ability with moderate change in pH and colour of the medium and hence selected as a most efficient strain for further study. Though some of the bacterial isolates PAAB-1, PAAB-3 and PAAB-5 could able to grow at 7% of alcohol concentration in the growth medium but their growth, change of pH and colour of the medium is very negligible. In the control medium, neither the colour nor the change of pH has been observed at any alcohol concentration (Table 1)

Acetic acid production: Acetic acid production abilities of the thirteen bacterial isolates have been examined at 37°C, 5% ethanol, 120 RPM for 120 h in GYC medium. Acetic acid production rate was estimated by titration method. It has been observed that the acetic acid production ability of the thirteen bacterial isolates were in the range of 1.1 g/L(AAAB-

5) – 15.2 g/L (PAAB-3). The maximum production was observed between 72-96 h of incubation and marginal decrease or increase thereafter (Figure 2). As maximum citric acid production (15.2 g/L) has been observed by the bacterial isolate PAAB-3, hence selected for further identification study

Identification of the selected bacterial isolates: Based on the morphological and biochemical analysis (Table 2), the bacterium, PAAB-3, was found to be smooth, small, round, off-white and convex colony, rod shaped with a diameter of 0.7–0.9 µm, positive for, catalase, motility, acid-gas from arabinose, galactose, glucose, mannose, ribose, xylose, growth on ethanol, production of acetic acid from ethanol, over oxidation of ethanol to CO_2 and H_2O, oxidation of lactate to CO_2 and H_2O, Ketogenesis of glycerol, MR test and growth at pH 4.5 whereas found negative for Gram staining, production of oxidase, indole, cellulose, brown pigment, VP test, nitrate reduction, growth in peptone and growth at pH> 8.0. Based on these above morphological and biochemical tests (Table 2), the isolates PAAB-3 was tentatively identified as *Acetobacter aceti*.

DISCUSSION

In the present study thirteen acetic acid bacteria have been isolated using enrichment culture of ethanol, acetic acid and rotten fruit waste such as apple and pomegranate at 37°C. Food industry generates huge amount of agro-industrial waste during the manufacturing of juice, jellies, jam and pickles, which creates a huge environmental problem and can be recycled to produce valuable commercial products. Hence, in the present

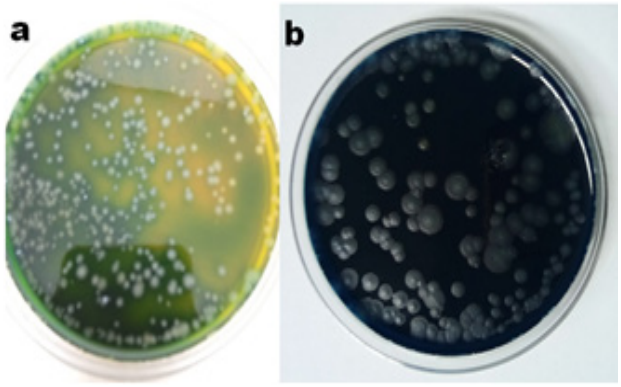

Figure 1: Carr agar medium (a) After acetic acid production (b) After over oxidation.

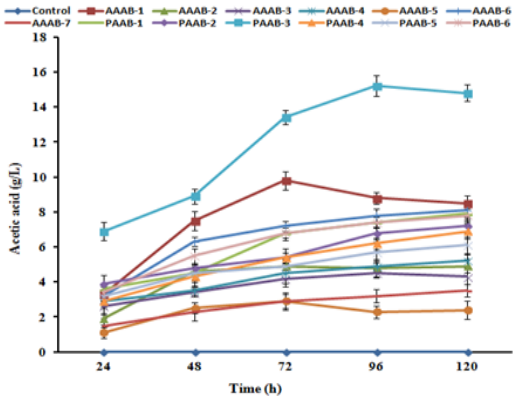

Figure 2: Acetic acid production by thirteen bacterial isolates at 37ºC in GYC broth medium.

Table 1: Alcohol tolerance ability of the bacterial isolates.

Strain No	3% alcohol	4% alcohol	5% alcohol	6% alcohol	7% alcohol	Maximum colour change at (%) of alcohol	Maximum Change of pH at (%) of alcohol
Control	-	-	-	-	-	-	6.8
AAAB-1	++	++	++	+	-	++ (4%)	5.3 (4%)
AAAB-2	++	+++	++	-	-	++ (4%)	5.5 (4%)
AAAB-3	+++	++	+	-	-	++ (3%)	5.7 (3%)
AAAB-4	++	+++	++	+	-	+++ (4%)	4.9 (4%)
AAAB-5	+++	++	+	-	-	++ (3%)	5.3 (3%)
AAAB-6	+++	+	+	-	-	++ (3%)	5.9 (3%)
AAAB-7	+++	++	+	-	-	++ (3%)	5.4 (3%)
PAAB-1	++	+++	++	+	+	++ (4%)	4.8 (4%)
PAAB-2	++	+++	+	+	-	++ (4%)	5.1 (4%)
PAAB-3	++	++	+++	++	+	+++ (5%)	4.5 (5%)
PAAB-4	+++	++	+	-	-	++ (3%)	5.7 (3%)
PAAB-5	++	+++	+	+	+	++ (4%)	5.5 (4%)
PAAB-6	++	+++	++	+	-	++ (4%)	5.7 (5%)

-ve = No change, +ve = Low growth and change of colour, ++ve = Moderate growth and change of colour, +++ve = Strong positive growth and change of colour

Table 2: Identification of the selected bacterial isolate.

Test	Results
Shape	Rod
Size	0.7-0.9µm
Gram's stain	-ve
Spore	-ve
Catalase	+ve
Oxidase	-ve
Growth on ethanol	+ve
Motility	+ve
Production of acetic acid from ethanol	+ve
Over oxidation of ethanol to CO_2 and H_2O	+ve
Oxidation of lactate toCO_2 and H_2O	+ve
Brown pigmentation	-ve
Growth in peptone	-ve
Ketogenesis of glycerol	+ve
Nitrate Reduction	-ve
Growth at pH 4.5	+ve
Growth at pH > 8.0	-ve
Cellulose production	-ve
Indole production test	-ve
MR test	+ve
VP test	-ve
Sugars	
Arabinose	+ve
Galactose	+ve
Glucose	+ve
Mannose	+ve
Ribose	+ve
Xylose	+ve
fructose	-ve
Lactose	-ve
Maltose	-ve
mannitol	-ve
Sorbitol	-ve
Sucrose	-ve
Growth at NaCl > 2%	-ve
Growth at NaCl < 2%	+ve
D-glucose concentration> 30%	-ve

study the waste fruits were collected and recycled for isolation of acetic acid bacteria can effectively reduce the environmental problem as well as the production cost of acetic acid. In addition, these agro-industrial residues are very well adapted to fermentation cultures due to their cellulosic and starchy nature, as well as little risk of bacterial contamination. Many reports have also addressed the usefulness of the enrichment culture technique in selective isolation of acetic acid bacteria.[7,17]

Most of the acetic acid bacteria are mesophilic with optimum growth temperature of 30°C.[7] However, some of them are also able to grow at 37°C and 40°C and known as thermotolerant strains.[10] All the thirteen acetic acid bacterial isolates (Figure 1) were isolated from Carr agar medium at 37°C on the basis of change of the colour of the medium from green to yellow and green again due to overoxidation. Carr agar contains ethanol as a carbon source and bromocresol green as a pH indicator. The oxidation of ethanol generates acetic acid and thus, the medium turns from green to yellow. However, as acetic acid is overoxidised to CO_2 and H_2O, the green appearance returns after an extended incubation period[11,18] and used to distinguished between the member of the genus *Acetobacter* and *Gluconobacter*.[15] Hence the present finding confirmed that all the bacterial isolates are belongs to *Acetobacter* sp and thermotolerent. Thermotolerent *Acetobacter* sp isolated at 37°C has been also reported earlier by other researcher.[17,19]

Thermotolerance properties of acetic acid bacteria in the presence of high ethanol concentration is an outstanding characteristic.[20,21] All the thirteen isolates were screened for their ability to grow and tolerate different concentration of ethanol in the medium. In the presence of higher concentration of ethanol, they could able to produce acetic acid by changing the colour and dropping the pH of the medium (Table 1). It has been observed that most of them were able to grow between 3%-4% of ethanol where as the bacterial isolate PAAB-3 showed maximum (5%) tolerance to ethanol (Table 1) with marked increase in acetic acid production (Figure 2). The present finding is related to findings of Holt *et al.*[15] who reported the maximum levels of alcohol tolerance by *Acetobacter aceti* was 5 percent. Ability of acetic acid bacteria of tolerating 5-9% of the ethanol concentration and high temperature has been also reported by Beheshti and Shafiei.[10]

It has been observed that the acetic acid production rate of all the isolated thirteen bacterial isolates are in the range of 1.1 g/L– 15.2 g/L. The maximum production (15.2 g/L) has been observed by the bacterial isolate PAAB-3. The present finding is higher than Moryadee and Pathom-Aree,[17] who observed acetic acid production of 8.7 g/L at 37°C but at lower ethanol concentration (2%). Acetic acid production higher than the present finding was also reported earlier.[8,18]

On the basis of morphological and biochemical characterization, the selected bacterial isolates PAAB-3, was identified as *A. aceti*. The present finding is very similar to others who also reported acetic acid production by thermotolorent *A. acetic,* isolated from fruit waste.[8,17,19,20]

CONCLUSION

On the basis of the results it is concluded that all the thirteen thermotolerent bacterial strains isolated in this study could successfully produce acetic acid at 37°C and could be served as potential microorganisms in the production of vinegar. Since the identified *Acetobaceter aceti* (PAAB-3) could able to grow at higher temperature and higher alcohol concentration. it may be useful for production of acetic acid at industrial scale relatively at higher temperature. However, a detail study is required for optimization of growth medium and factors affecting the production path way to enhance the production process at large industrial scale. Optimization of acetic acid production by thermtolerant *Acetobacter aceti* isolated and identified in this study may be further used to enhance the acetic acid production study.

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CONFLICT OF INTEREST

The authors declare no Conflict of interest.

ABBREVIATIONS

AAB: Acetic acid bacteria; **ADH:** alcohol dehydrogenase; **ALDH:** aldehyde dehydrogenase; TCA cycle: Tricarboxylic acid cycle; **GYC broth medium:** Glucose yeast extract calcium carbonate broth medium; **RPM:** Rotation per minute; **AAAB:** Apple acetic acid bacteria; **PAAB:** Pomegranate acetic acid bacteria; **VP reaction:** Voges- Proskauer reaction; MR test: Methyl red test

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