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Isolation and characterization of *Acetobacter* and *Gluconobacter* spp from sugarcane and rotten fruits

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ABSTRACT

Potential acetic acid bacteria were investigated from different readily available sources. Seven different samples (sugarcane bagasse, sugarcane juice, sugarcane juice processing water, soil, rotten apples, rotten red grapes and rotten white grapes) were collected from local market. After processing and enrichment, samples were inoculated on Glucose Yeast Calcium carbonate (GYC) agar plates and incubated at 30°C for four days. Nineteen different bacterial colonies were selected and isolated on the basis of clear zone formation on GYC medium. The bacterial isolates were identified on the basis of their morphological, biochemical and physiological characterization. Among nineteen isolates, one was identified as *Acetobacter aceti*, one as *Acetobacter pasteurianus*, one as *Acetobacter orleansis*, two were identified as *Acetobacter cibinongensis*, and the remaining fourteen isolates were identified as *Gluconobacter* spp. As potential acetic acid producers, only the *Acetobacter* isolates were further assessed for their acid production capability under different temperature and pH using 'Potency Index' as a potency determining parameter. Temperature 30°C and pH 5.5 were found to be the optimum temperature and pH respectively for maximum acetic acid production by most of the species. *Acetobacter pasteurianus* with the highest P.I. value of 3.78 was the most potent acetic acid producer among these isolates.

KEYWORDS

Acetobacter;
Gluconobacter;
Potency index.

INTRODUCTION

Acetic acid bacteria, are a large group of Gram negative bacteria, whose major noticeable and useful characteristics is the ability to oxidise various carbon substrates especially sugars and alcohols rapidly and incompletely. They are obligatory aerobic and some of them are industrially used for vinegar production[1].

Acetic acid bacteria have a strong ability to oxidize ethanol, sugar alcohols and sugars into different organic acids by aerobic fermentation traditionally called oxidative fermentation. Acetic acid fermentation is typically oxidative fermentation and is used industrially to produce vinegar[2]. Acetic acid bacteria have been classified into 25 different genera. The major genera include *Acetobacter, Gluconobacter, Gluconoacetobacter,*

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Asaia, Neoasaia, Saccharibacter, Frateuria and *Kozakia*[3]. Among acetic acid bacteria *Acetobacter* and *Gluconobacter* strains are the major bacteria that are used in the production of vinegar industrially[4]. As vinegar is an important ingredient in the production of pickles, jams and jellies etc., vinegar based food industries considerably realize the necessity of acetic acid bacteria in industrial sector[5].

Bangladesh is not an industrially well developed country and the food and beverage sector are recently being flourished. Vinegar is an important ingredients used by these food industries for the production and preservation of different kinds of food items. In Bangladesh, vinegar industries generally collect acetic acid or acetic acid producing bacterial strains from abroad because pure and potential cultures of acetic acid bacteria are not commercially available in our country. Therefore, they have to spend a handsome amount of money for this purpose. Fruits and even sugarcane can be a good source of many kind of bacteria[6-8]. Use of various kinds of fruits and specially over-ripened fruits as a source of potential acetic acid bacteria had already been described[8,9]. Sugarcane juice is very much available in all seasons in Bangladesh. Moreover, everyday huge amounts of rotten fruits are discarded by local fruit markets. These rotten or over-ripened fruits could provide us a good source of acetic acid bacteria. If potential acetic acid producing bacterial culture can be isolated and made available from these kind of cheap and readily available sources in Bangladesh, a huge amount of foreign currency can be saved and vinegar producing industries can be developed, which ultimately can contribute a lot in food and beverage sector in Bangladesh. As *Acetobacter* and *Gluconobacter* are two main acetic acid producing genera[3,10], considering these above points the present study was conducted with the aim of isolation and characterization of those acetic acid bacteria from sugarcane and fruits. As different environmental conditions might affect the production of acetic acid[11], the isolated *Acetobacter* species were further examined to determine the effect of different environmental conditions (viz. temperature and pH) on acetic acid production.

MATERIALS AND METHODS

Collection of samples

Seven different samples (sugarcane bagasse, sugarcane juice, sugarcane juice processing water, soil, rotten apples, red grapes and white grapes) were collected in sterile sampling bags from street vendors of sugarcane juice and fruits in Dhaka Export Processing Zone (DEPZ) area and transported immediately to the laboratory of Microbiology & Industrial Irradiation Division (MIID), Atomic Energy Research Establishment (AERE), Savar, Dhaka, Bangladesh

Isolation and identification of bacterial isolates

For enrichment, all samples were homogenized with a stomacher (Seward Stomacher 400, UK) and poured into different conical flasks containing enrichment medium composed of 1.0% glucose, 0.5% ethanol, 0.3% acetic acid, 1.5% peptone and 0.8% yeast extract. The flasks were incubated at 30°C for five days[12,13]. After serial dilution, 0.1 ml aliquot from different dilutions was then spreaded on plates of glucose solid GYC medium (10% glucose, 1.0% yeast extract, 2.0% calcium carbonate, 1.5% agar, pH 6.8)[4,8] supplemented with 100 mg/l of Nystatin[14] to prevent the growth of yeasts and moulds. All GYC agar plates were incubated at 30°C for 96 hours. After that the bacterial colonies producing clear halo on GYC agar plates were selected and presumptively identified as acetic acid bacteria.

Cultural characteristics of bacterial isolates were studied by inoculating the colonies on GYC agar plates and incubating at 30°C for 96 hours. Different cultural characteristics including colony size, pigmentation, shape, edge, elevation, and opacity were studied after incubation. On the other hand, morphological characteristics were determined by Gram staining technique and microscopic examination[15]. Growth at different temperatures (15°C, 25°C, 30°C and 37°C), growth at different pH (4.5, 5.0, 5.5, 6.0, 6.5, 7.0)[16] were also observed.

The isolates in this study were classified according to the ninth edition of Bergey's Manual of Systematic Bacteriology[17]. The following biochemical tests were performed to identify bacterial isolates: catalase, oxidase, production of acetic acid from ethanol[16], growth in peptone, carbohydrate fermentation test (glucose, lactose, fructose, sucrose, maltose, xylose, manitol, sorbitol), gelatin hydrolysis test, motility test[16], ketogenesis of glycerol and nitrate reduction test[18]. Overoxidation of ethanol to CO_2 and H_2O[19], oxidation of Lactate to CO_2 and H_2O[16] and pigmentation (Brown) on GYP agar[20] were carried out to distin-

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guish between *Acetobacter* and *Gluconobacter*. Carbohydrate fermentation pattern were observed to distinguish different species of acetic acid bacteria using fermentation media consisting of 0.5% yeast extract and 1% sugar to be tested as the sole carbon source with 0.002% bromocresol purple as the pH indicator[13].

Potency assessment (with respect to acetic acid production)

The identified *Acetobacter* species were then qualitatively assessed for acetic acid production capability under different environmental conditions using Potency Index (P.I.) as the key parameter. Formation of clear zone in the selective media indicated the production of acetic acid by *Acetobacter* spp.[8] and the size of diameters of clear zones revealed the potency of each strain. After incubation of ninety six hours, diameters of the colonies formed by the isolates and the respective clear zones were measure and Potency Index was determined according to the following formula:

$$\text{Potency Index (P.I.)} = \frac{\text{Diameter of the clear zone formed (mm)}}{\text{Diameter of the bacterial colony (mm)}}$$

For this, pure cultures of the isolated *Acetobacter* spp. were obtained and then they were spot inoculated on the GYC agar plates using a sterile needle. For each isolate, three different temperatures (25°C, 30°C and 37°C) were taken at consideration for comparing the favorable temperature. The most favorable temperature was used to determine the effect of different pH, on the growth of *Acetobacter* spp. In both cases the plates were incubated for ninety six hours.

The higher P.I. value would indicate the greater production of acetic acid by the isolates. Thus the PI values of each isolate indicated the respective potency level of acetic acid production.

RESULT

Nineteen different bacterial isolates were selected from seven different collected samples based on the

TABLE 1 : Physiological and biochemical characteristics of the isolates:

Parameters Tested	Bacterial Isolates																	
	SB1	SB2	SB3	SB4	SJ1	SJ2	SJ3	SJ4	SJ5	W1	W2	A1	A2	A3	A4	GR1	GR2	GR3	GW1
Morphological Characteristics																			
Shape	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod
Gram reaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Biochemical Characteristics																			
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Pigmentation (Brown) on GYP	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+
Growth in peptone	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+
Ketogenesis of glycerol	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+
Nitrate Reduction	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Gelatin liquid faction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Physiological Characteristics																			
Growth at 15^0C	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+
Growth at 25^0C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 37^0C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH 4.5	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH 7.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

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characteristics shown on the GYC agar plates and were designated as SB1-4, SJ1-3, S1-2, W1-2, A1-4, GR1-3, GW1. All the isolates produced clear haloes surrounding their colonies. All isolated bacterial strains were found to be Gram negative, rod shaped, catalase positive and oxidase negative (TABLE 1) and were thus presumptively identified as acetic acid bacteria.

Among nineteen isolates five isolates (SB1, SB2, SJ1, GR1, GR2) showed the capability of overoxidation of ethanol to CO_2 and H_2O and were identified as *Acetobacter* spp. The remaining fourteen isolates were incapable of overoxidation and were thus selected as *Gluconobacter* spp. All the isolates showed positive growth at temperature 25°C and 37°C. Among those, isolates finally selected as *Gluconobacter* spp. could grow at 15°C. Isolates of both genera maintained positive growth at pH range 4.5 to 7.0.

Bacterial isolates were also tested for their characteristic sugar fermentation pattern. Isolates identified as *Acetobacter* spp. were able to utilize glucose, arabinose, xylose, ribose, galactose, mannose, mellibiose and trehalose as the carbon source and were unable to utilize lactose, sucrose, fructose, maltose, mannitol, sorbitol (TABLE 2). In case of *Gluconobacter* spp. all the isolates were able to ferment arabinose, xylose, ribose, glucose, galactose, mannose and mellibiose but could not ferment lactose, mannose, trehalose. Some species of *Gluconobacter* could utilize sucrose, fructose, maltose, mannitol, sorbitol but maximum species could not utilize the sugars. Isolated *Acetobacter* spp. were able to oxidize lactate to CO_2 and H_2O where as *Gluconobacter* strains were unable to oxidize. In case of ketogenesis of glycerol test, all isolated *Gluconobacter* species gave positive result and among the four isolated *Acetobacter* spp., two isolates gave positive results and which is the characteristic of *A. aceti* and *A. orleansis*. The other three isolates gave negative results of ketogenesis and were identified as *A. cibinongensis* and *A. pasteurianus*. In the case of nitrate reduction test all *Gluconobacter* isolates showed negative result. In case of suspected *Acetobacter* isolates three (SB1, SJ1, GR1) showed negative result for nitrate reduction similar to *A. aceti* and *Acetobacter cibinongensis* and the remaining two with positive results appeared as *A. orleansis* and *A. pasteurianus*. Finally identified isolates were shown in TABLE 3 along with their respective sources.

In this study, as potential acetic acid producer, emphasis was given on *Acetobacter* spp. and potency assessment was carried out by means of Potency Index. The P.I. values of the four *Acetobacter* spp. (*A. aceti, A. pasteurianus, A. cibinongensis* and *A. orleansis*) were determined at different temperatures after 4 days of incubation are shown in Figure 1. All the strains showed maximum P.I. values at 30°C. The maximum P.I. value of *A. aceti, A. pasteurianus, A. cibinongensis, A. orleansis* were 3.58, 3.78, 3.37 and 3.30 respectively.

The effect of pH on acetic acid production, expressed as P.I value was observed at the most favorable temperature (i.e. 30°C) (Figure 2). In the pH range, maximum P.I values of *A. aceti, A. pasteurianus* and *A. orleansis* were obtained at pH 5.5 which were 3.60, 3.78 and 3.30 respectively. In case of *A. cibinongensis*

TABLE 2 : Utilization of common carbohydrates by the isolates:

Sugars	Bacterial Isolates																		
	SB1	SB2	SB3	SB4	SJ1	SJ2	SJ3	SJ4	SJ5	W1	W2	A1	A2	A3	A4	GR1	GR2	GR3	GW1
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
fructose	-	-	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	+	-	-	-	-	-	-	-	+	+	-	+	-	-	-	+	+
mannitol	-	-	-	+	-	-	-	-	-	-	+	+	-	+	-	-	-	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	+
Sucrose	-	-	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

TABLE 3 : Identified bacteria with their sources.

Source	Code of isolate	Identified bacteria
Sugarcane bagasse	SB1	*Acetobacter aceti*
	SB2	*Acetobacter orleansis*
	SB3	*Gluconobacter sp.*
	SB4	*Gluconobacter sp.*
Sugarcane juice	SJ1	*Acetobacter cibinongensis*
	SJ2	*Gluconobacter sp.*
	SJ3	*Gluconobacter sp.*
	SJ4	*Gluconobacter sp.*
	SJ5	*Gluconobacter sp.*
Sugarcane juice processing water	W1	*Gluconobacter sp.*
	W2	*Gluconobacter sp.*
Apple	A1	*Gluconobacter sp.*
	A2	*Gluconobacter sp.*
	A3	*Gluconobacter sp.*
	A4	*Gluconobacter sp.*
Grape (red)	GR1	*Acetobacter cibinongensis*
	GR2	*Acetobacter pasturianus.*
	GR3	*Gluconobacter sp.*
Grape (white)	GW1	*Gluconobacter sp.*

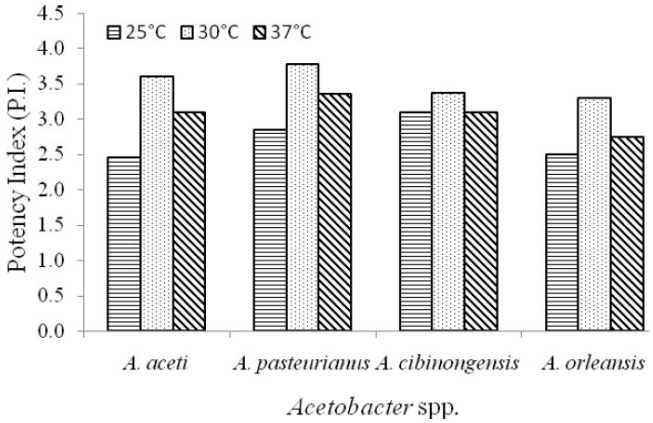

Figure 1: Effect of different temperature on acetic acid production of the isolated *Acetobacter* spp. in terms of Potency Index (P.I.).

the highest production of acetic acid was obtained at pH 6.5 with the P.I value 3.62. Among four isolated *Acetobacter* spp. *A. pasteurianus* showed the maximum acetic acid production with the P.I value 3.78 at pH 5.5.

DISCUSSION

Use of acetic acid in different food industries is increasing day by day worldwide including developing

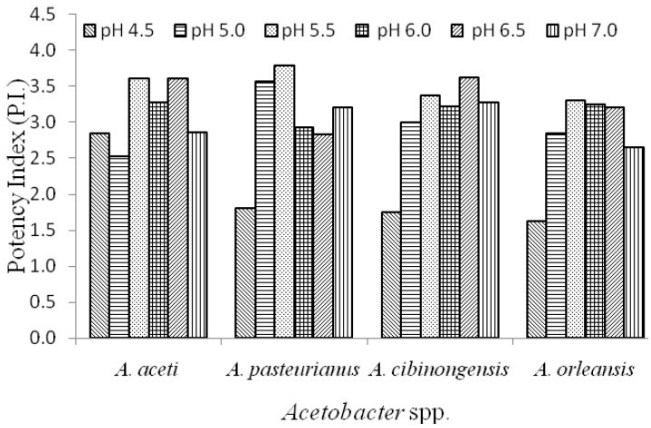

Figure 2: Effect of different pH on acetic acid production of the of the isolated *Acetobacter* spp. in terms of Potency Index (P.I.) at 30°C.

countries. A potential bacterial strain with high yield is the prerequisite for commercial production of acetic acid to meet the demand. Bangladesh is a developing country and there is nice scope to establish acetic acid production industries to meet the local demand. In this study an effort was exerted to explore such a potential strain of acetic acid producing bacteria from different natural sources.

Different fruit samples were selected for the isolation of acetic acid bacteria because they could be a good source of acetic acid as well as acetic acid producing bacteria[21]. This selection was done on the basis of clear zone formation around the bacterial colony due to the disappearance of $CaCO_3$. The disappearance of $CaCO_3$ and formation of clear zone around the growing colony was due to the production of acetic acid which reacts with $CaCO_3$ and produced calcium acetate which is water soluble. The similar selection procedure was used by Sharafi *et al*., (2008), Hanmoungjai *et al*.(2007)[8,13]. All the isolates were identified and classified according to the procedures described in the ninth edition of Bergey's Manual of Systematic Baceriology[18].

Gram reaction, microscopic observation, catalase and oxidase reaction were the primary selection criteria of two major acetic acid producing genera according to the ninth edition of Bergey's Manual of Systematic Bacteriology[17]. All isolated bacterial strains produced acetic acid from ethanol and thus were primarily identified as acetic acid bacteria.

Acetobacter strains were differentiated from *Gluconobacter* strains by the method described by[19], which is based on the fact that *Acetobacter* strains were

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able to over-oxidize ethanol to acetic acid and finally to CO_2 and H_2O through tricarboxylic acid cycle in neutral and acidic conditions (pH 7.0 and 4.5 respectively). Because of non-functional tricarboxylic acid cycle in *Gluconobacter*, the genera is unable to oxidize most organic acids such as acetic, citric, lactic, malic, pyruvic and succinic[22]. Upon incubation, all *Acetobacter* strains were able to change the medium from blue to yellow and further incubation resulted in the reversion of blue color which indicates that the acetic acid was converted into CO_2 and H_2O. This not only confirms *Acetobacter* strains, but also differentiates them from the *Gluconobacter* strains which only turned the media color to yellow and remained unchanged.

It was reported that the optimum temperature for the growth of acetic acid bacteria was in the range of 25°C to 35°C[23,24]. The temperature of incubation during isolation was also maintained 30°C, but during biochemical characterization, all the bacterial isolates were allowed to grow at 15°C, 25°C and 37°C for differentiating isolates. It was observed that all isolated *Acetobacter* and *Gluconobacter* strains were able to grow at 25°C and 37°C. *Gluconobacter* strains were able to grow at 15°C but *Acetobacter* strains were unable to grow at 15°C. The pH range for the optimum growth of acetic acid bacteria was 5.0-6.5. Therefore, the pH was adjusted to 5.5 during isolation[20]. But during biochemical test it was seen that all isolated *Acetobacter* and *Gluconobacter* strains were able to grow at pH 4.5 and pH 7.0.

In case of carbohydrate utilization test, results obtained were compared with other references[8,17,25] where no contradiction was observed.

On the basis of standard cultural, morphological and biochemical tests, among nineteen different bacterial isolates, one was identified as *A. aceti*, one was identified as *A. pasteurianus*, one was identified as *A. orleansis*, two were identified as *A. cibinongensis*, and the remaining fourteen isolates were identified as *Gluconobacter* spp.

Thus all the isolates of the present study belonged to one of the two genera of acetic acid bacteria which are *Acetobacter* spp. *and Gluconobacter* spp. of which *Acetobacter* spp. are the main tool and are generally involved for vinegar production[10,26]. So the isolates belonged to the genera *Acetobacter* were further investigated for determining the capability of acetic acid production in terms of Potency Index.

Most acetic acid bacteria are known to be mesophilic with optimum growth temperature of 30°C[27]. However, some of them are also able to grow at 37°C and 40°C which are thermotolerant strains[1,4]. In this study the highest amount of acetic acid in terms of P.I value was produced by the strains of *Acetobacter* spp. at 37°C which decreased with the increase of temperature. Similar effect of temperature was observed by other researches[3]. pH can be a crucial factor in case of production of desirable products by *Acetobacter*[28]. pH 5.0 resulted in the highest cell yield[11]. In this study all the *Acetobacter* isolates except *A. cibinongensis* exhibited highest amount of acetic acid production at pH 5.5.

Different enrichment media used for isolation of acetic acid bacteria[27,29] resulted in some differences in the isolated strains. In this study only one enrichment medium was used and thus some varieties of acetic acid bacteria might not be recovered. It has already been proved that different types of fruits and flowers[8] are good source of acetic acid bacteria. As an indissoluble tool for vinegar fermentation, further investigation regarding the feasibility and assessment of fermentation capability at both laboratory and industrial scale is to be carried out on these isolated *Acetobacter* spp. and *Gluconobacter* spp.

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