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A Comparative Study between Two Local Isolates of Acetic Acid Bacteria For Acetic Acid Production in Sulaymaniyah

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Abstract

Two local isolates of acetic acid bacteria belonging to the genus Acetobacter sp. Were isolated from natural sources. The first isolate (Acetobacter sp. N1) was isolated from soil sample taken from garden of biology department and the second isolate (Acetobacter sp. N2) was isolated from raw vinegar by using yeast extract agar and nutrient broth, (pH 5.0 & 5.5) respectively.
Both isolates were identified according to morphology of colonies, type of cells according to Gram-staining, catalase, oxidase, moility test, and also their production of acetic acid as Acetobacter sp.
Production of acetic acid was detected from both isolates by using fermentation medium (apple extract medium supplemented with other nutrient). The total acidity was 14% and 16% for both N1 and N2 respectively.

Key words: Acetobacter, Acetic acid, Fermentation, Acid producers

Introduction

Acetobacter sp. especially A. aceti is a begin microorganism that is ubiquitous in the environment, existing in alcoholic ecological niches such as flowers, fruits, honey bees, as well as in water and soil. It has a long history of safe use in the fermentation industry for the production of acetic acid from alcohol. There are no reports in the literature suggesting that A. aceti is a pathogen of humans or animals. It also is not considered a plant pathogen. The potential risks to human health or the environment associated with the use of this bacterium in fermentation facilities are low. Since the taxonomy of the genus was recently revised, some older production strains in use for acetic acid production may, in fact, not meet the current taxonomic designation of A. aceti. {1}

The history of safe use for this bacterium is predominately for food grade acetic acid (vinegar) production. Members of the genus Acetobacter have been used industrially since the 1850's {2}. A. aceti has also been reported in the literature as being used for cellulose production for specialty papers or headphones (3,4}; however, strains capable of cellulose production are classified as A. pasteurianus or A. hansenii under the new taxonomic system {5}.

There are a number of TSCA (Toxic Substances Control Act) applications for acetic acid. These include manufacturing of acetate rayon, plastics production, rubber production, and photographic chemicals. {1}
Vinegar is a sour liquid consisting mainly of acetic acid and water, produced by the action of bacteria on dilute solutions of ethyl alcohol derived from previous yeast fermentation. The colouring and flavoring are characteristic of the alcoholic liquor from which the vinegar is made. The metabolic process involves conversion of ethanol to acetaldehyde by alcohol dehydrogenase and conversion of hydrated acetaldeyde to acetic acid by acetaldehyde dehydrogenase {7}.

Cider vinegar is produced by the alcoholic fermentation of apple juice and may require nutrient supplementation. Modern fermenations are highly aerated, submerged processes. Typical commercial processes, involving production of 12-15% acetic acid are carried out in a semi-continuous manner {7}.
Acetic Acid (CH3COOH) is a colourless liquid with a sharp, irritating odour and sour taste. In aqueous solution, it functions as a weak acid. It is miscible in all proportions with water and with many organic solvents {8}.

Acetic acid can be prepared by the action of air on solutions of alcohol in the presence of certain strains of bacteria. Dilute solutions (4 to 8 %) prepared in this way from wine, cider, or malt is called vinegar. Concentrated acetic acid is prepared industrially by several synthetic processes, such as the reaction of methyl alcohol and carbon monoxide (CO) in the presence of a catalyst, or the oxidation of acetaldehyde {8}.
Acetic acid bacteria, which oxidize ethanol to acetic acid and can exist at low pH values, come from the closely related genera Acetobacter and Gluconobacter. Acetobacter are a genus of aerobic ellipsoidal to rod-shaped bacteria that grow in the presence of alcohol, securing energy by oxidizing organic compounds to organic acids (ie: alcohol to acetic acid) {6}.

Pure cultures of these organisms are characterized by their high degree of variability and in industrial fermentations mixed cultures will consequently develop from a pure culture. Industrial cultures are selected to tolerate high acidity and to yield high acetate production rates. These bacteria are extremely sensitive. They die as a result of lack of oxygen and of ethanol and are also damaged by acetate and ethanol concentration gradients. The sensitivity to lack of oxygen increases with increasing total concentration of acetic acid plus ethanol. An oxygen utilization of 80% can be achieved without adverse effects on the fermentation via efficient aeration. Maintaining acetic acid concentrations above 6% and avoiding total depletion of ethanol can avoid the conversion of acetic acid to CO2 and H2O, or over-oxidation {7}.
Acetic acid bacteria are gram-negative bacteria and well known as vinegar producers since more than hundred years ago {9}.
They belong to the family Acetobacteraceae, which having an obligately aerobic (respiratory) metabolism. Members of the family are recognized by their unique ability to oxidize ethanol to acetic acid. Recent identification of acetic acid bacteria revealed that they can be divided into six genera; Acetobacter, Gluconobacter, Gluconacetobacter, Asaia, Kozakia, and Acidomonas {10,11}.

Acetobacter species have a high ability to oxidize ethanol to acetic acid, which is absolutely dependent on the enzyme located on the outer surface of cytoplasmic membrane so called membrane-bound dehydrogenase; alcohol dehydrogenase (ADH) and acetaldehyde dehydrogena (ALDH). Alcohol dehydrogenase has pyrroloquinoline quinone (PQQ) as the prosthetic groups, where as neither PQQ nor flavin adenine dinucleotide (FAD) functions as the primary coenzyme of acetaldehyde dehydrogenase (12,13,14}
Vinegar has been known as food preservative, food seasoning, mixture in cosmetics, solvent, and some bacterial diseases inhibitor{15}. Thailand has several traditional alcoholic beverages; Satoh, Ou, and Krachae, which can be used as the starting materials for vinegar fermentation. Because

production cost of these alcoholic beverages are inexpensive, therefore, it is very much attractive for using in vinegar production {15}.

In Sulaymaniyah province there is no factory for producing vinegar, which is an important industrial and economic product of widely usable in daily life as food additives, food supplements, dinking liquid, and preservatives.
The aims of this study were to isolate and identified acetic acid bacteria from alcoholic ecological niches such as flowers, fruits, as well as in water, soil and raw vinegar, and produced vinegar from the soil isolate, and the raw vinegar isolate; and also comparison between both isolates through acetic acid production.

Methods

In order to prepare 1L of Yeast extract agar
3.0 gm Yeast extracts, 3.0 gm Peptone, 7.0 gm Glucose, 1.0 gm Dipotassium phosphate, 1.0 gm CaCO3, 20 gm Agar were dissolved in 1L D.W and pH was adjusted to 5.0.
To prepare 1L of 8 gm/L Nutrient broth 5.0 gm Peptone, 5.0 gm NaCl, 2.0 gm Yeast extracts, 1.0 gm Beef extract
were dissolved in 1L D.W and pH was adjusted to 5.5

To prepare Fermentation medium (Cider extract medium) 250 gm Apple, 90 gm Sucrose, 2% Yeast extract were dissolved in 160 mL D.W and pH was adjusted to 4.5
Total acidity: 0.3865
Total solidity: 24.5

The primary stain (Gram stain) consists of
20 ml of solution A and 80 ml of solution B.
Solution A was prepared by dissolving 2.0 gm
crystal violet (90% dye content) in 20 ml of Ethanol (95% v/v).
Solution B was prepared by dissolving 0.8 gm of Ammonium oxalate in 80 mL of D.W.

The primary stain was prepared by combining 20 ml of solution A and 80 ml of solution B and mixing thoroughly. The mixture allowed to stand for 24 hours, and filtered before use.
lodine (Mordant Solution) prepared by grinding 2.0 gm KI with 1.0 gm lodine in a mortar and pestle then distilled water is added solely with continuous grinding until the iodine dissolved. The volume is completed to 300 ml with D.W and stored in a dark bottle.

To prepare Safranin (Counter Stain) 0.25 gm Safranin is dissolved in 10 ml of Ethanol (95% v/v), then 100 ml of D.W was added and mixed thoroughly. The solution is allowed to stand for several days and filtered before use.

Sodium hydroxide NaOH solution (0.1N) was prepared by dissolving 0.4 gm of NaOH in 100 ml of D.W.
Phenolphthalein indicator was prepared by manufacturer and ready to use.

Hydrogen peroxide solution (3% H2O2) was prepared by diluting 3 ml of H2O2 in 97 ml of D.W.
To prepare Oxidase test reagent 1.0 gm of tetra methyl-p-phenylene diamine was dissolved in 100 ml of D.W, then stored in dark container.

The culture media were sterilized by autoclave at 121C, 15 Bar for 15 minutes.
The glassware was sterilized by oven at 160-180 C for 20 min.

Yeast extract agar was inoculated from raw vinegar, by streak plate method in order to gain single colonies and other plates were inoculated from suitable dilution of soil sample by spreading method.
The inoculated plates are then incubated at 28C for 24-48 hours.
After growth single colonies were isolated and sub-cultured to get a pure isolate.

The two isolates were identified according to Gram staining and some biochemical tests, such as catalase, oxidase; and also by motility test.

Gram stain procedure was achieved according to James and Tod, 1952 then the slide was examined under oil immersion lens.
The Acetobacter and Gluconobacter are typically Gram-negative (in a few cases Gram-variable), ellipsoidal to rod-shaped bacteria. Occuring singly, in pairs, or in chains.

To achieve Catalase test a loopful of isolated separate colonies were transferred to the center of clean slide. Few drops of 3% H2O2 were added on to the cells, and bubbles were observed immediately.

Oxidase test was achieved by putting a piece of filter paper in a clean Petri dish and 2-3 drops of freshly prepared oxidase reagent were added to the filter paper, by using a glass rod, a colony of isolated bacteria was removed and smeared on the filter paper.
The positive result was indicated by blue-purple color formation after few seconds.

To perform motility test inoculate by stabbing semisolid culture with isolated bacteria and then incubate the tube at 25 C for 24 hours and observe for growth away from the stab line which indicates positive test or motility was examined by placing a drop of broth culture medium on the center of a slide then place a cover slip on it, after that the slide was examined under microscope.

For Maintenance of the bacterial isolates, the isolates were cultured on nutrient agar slant, and incubated at 30C for 3 days, after that the slants were preserved at 4C in refrigerator.

To produce acetic acid by bacterial isolates, two flasks (1L volume) were prepared for this experiment, each flask was contained approximately 500 ml of the fermentation medium. The two flasks were inoculated with the bacterial isolates. Flask 1 was inoculated with the isolate N1, and flask 2 was inoculated with the isolate N2). The flasks were incubated at 30 C for two weeks.

The acetic acid produced by the two isolates was determined by titration with Sodium hydroxide (NaOH) 0.1N solution, in the presence of phenolphthalein indicator. After the end point was reached, the total acidity was calculated by the following equation:
Total Acidity% = N NaOH x V (ml) NaOH x eq. wt. of acetic acid x 100 / weight of sample x 1000.

Results and Discussion

The two isolates of acetic acid bacteria belonging to the genus Acetobacter (N1 and N2) were isolated from soil sample and raw vinegar respectively. Both isolated bacterial cells were Gram- negative, rod-shaped according to Gram staining method; and also both isolates were catalase positive, through production of bubbles after adding few drops of 3% H2O2; oxidase negative; and they were motile.

The cells of Acetobacter sp. (Gram stained smear) 100X

Acetic acid bacteria are all Gram-negative, rod-shaped, motile with peritrichous flagella, catalase positive, and oxidase negative Strains produced acid mainly from D-glucose, ethanol, and L-arabinose. They grew well at pH 4.5 {12}.

All of tested strains grew at 30 C: Most strains produced 2-ketogluconic acid and 5-ketogluconic acid, whereas isolates from fermented vinegar did not produced both two ketoacids. {12}

Test of acetic acid production:

Both local isolates (N1, N2) were tested for production of acetic acid by using submerged process (batch culture) applied in flasks (1000 ml in volume).

The biomass levels changed significantly over the early stages of the experiment, with a rapid decline over the first day, possibly due to the high temperature levels present in the system. It appears that once the temperature was stabilized the biomass reached a peak before a gradual decline was recorded over the subsequent days. As steady state conditions are reached, the rate of inflow is equal to outflow, and biomass growth is constant due to steady conditions in the reaction vessel, for example, substrate concentration, temperature and oxygen supply. {6}

The concentration of acetic acid remains quite steady over the initial days of the experiment. This may be related to the organism adjusting to the conditions of the system or could be linked to the fact that the reaction vessel was run as a batch system to increase cell numbers. Interestingly a significant increase in acetic acid concentration is observed after Day 3, where there is also a noticeable decline in biomass concentration. The concentration of acetic acid does however appear to plateau after this period. {8} For industrial comparison, the fermentation medium was inoculated from both N1 (isolated from soil sample) and N2 (isolated from raw vinegar) isolates separately, and the result of 14% and 16% total acidity was obtained for both N1 and N2 isolates respectively.

The resulting higher acidity obtained from N2 isolate may belong to the reason that the N2 culture may not pure, and there is a possibility of existing other ethanol oxidizers (acid producers) for example Gluconobacter (the nearest genus from Acetobacter), Lactobacillus and Streptococcus.

While the N1 culture does not contain such interacting bacteria, so its total acidity was lower than N2 culture.

The determination of total acidity may not accurate due to the fact that the systems used in this study were not at steady state.

Recombinant DNA technology may help further understanding of fermentation industry, which, in turn, will aid the future development of industrial processes. Further research is required for the stability and specificity of biosensors before they can be included as regular analytical tools for fermentation processes.

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