BIOTECHNOLOGICAL APPLICATIONS OF ACETIC ACID BACTERIA IN FOOD PRODUCTION

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Contents

Summary

Acetic acid bacteria are producers of certain foods and drinks, such as vinegar, kombucha beverage, and cocoa but they can spoil other products such as wine, beer, soft drinks, and fruits as well. Vinegar has been produced as long as winemaking has been practiced and therefore dates back to at least 10000 BC. Vinegar is the product of acetic acid fermentation of dilute alcoholic solutions. Aerobically, food-grade acetic acid is produced by the two-step vinegar process. The first step is the production of ethanol from a carbohydrate source such as glucose which is carried out by yeasts and the second step is the oxidation of ethanol to acetic acid carried out by acetic acid

bacteria. Although a variety of bacteria can produce acetic acid, mostly members of *Acetobacter* and *Gluconobacter* are used commercially. Submerged fermentation has almost completely replaced surface fermentation methods and industrial fermentation processes have evolved from the simple "let-alone" method to the more sophisticated and controlled "field" fermentation. The Frings Acetator® was the first submerged culture process and it is still the most commercially successful system today. The common types of vinegar in a region often reflect the local alcoholic beverage. Cocoa is classed into the category of foods whose characteristic flavor is developed through a fermentation process. Fermentation methods of cocoa vary between different countries and even between different processors. Kombucha is a traditional fermented beverage obtained by the fermentation of sugared tea with a symbiotic culture of acetic acid bacteria and yeasts. One of the greatest hurdles when studying acetic acid bacteria is their cultivation and maintenance in pure culture. Recently, the acetic acid bacteria taxonomy has been strongly rearranged.

1. Introduction

Acetic acid bacteria are producers of certain foods and drinks, such as vinegar, kombucha beverage, and cocoa but they can spoil other products such as wine, beer, soft drinks, and fruits. Acetic acid bacteria are also used for the production of cellulose. The main characteristic of the acetic acid bacteria is the oxidation of alcohols and sugars to different kinds of acids. People have benefited from the actions of acetic acid bacteria long before they were recognized as the causative agent in acetous fermentation. Recently, the acetic acid bacteria taxonomy has been strongly rearranged as new techniques using 16S rRNA sequence analysis have been introduced.

2. Traditional and current bio/technology using acetic acid bacteria

2.1 Vinegar and acetic acid production

Vinegar is a clear aqueous liquid, colorless or the color of the raw material or colored by caramel with a prescribed content of acetic acid between 40 and 150 g/l. Worldwide acetic acid is called vinegar, if obtained by oxidative fermentation of ethanol-containing solutions by acetic acid bacteria. Although vinegar does not entirely exclude diluted chemically produced acetic acid in every country of the world, this term is used here to describe acetic acid which is produced by primary microbial metabolism, the so called acetic acid fermentation or vinegar fermentation. The first vinegar was probably a result of spoiled wine, considering that the Latin word *acetum* means sour or sharp wine. Thus, it has been produced as long as wine making has been practiced and therefore dates back to at least 10000 BC. Acetic acid was used as a medicinal agent and was probably the first known antibiotic. Vinegar is traditionally the product of acetic acid fermentation of dilute alcoholic solutions. At the present time it is produced microbiologically from natural alcoholic solutions (10-15% by volume of ethyl alcohol) or by dilution of acetic acid. For most of human history, acetic acid was produced by fermentation of sugar to ethyl alcohol and its subsequent oxidation to acetic acid by microorganisms. This process was supplemented in the nineteenth century by wood distillation. In 1916, the first dedicated plant for the production of acetic acid by chemical rather by biological means became commercial. Fermentation production

routes have traditionally been aimed at the food market. Currently, acetic acid is produced chemically and biologically. Biological routes include an aerobic route using acetic acid bacteria, mostly species of the genera *Acetobacter* and anaerobic route using *Clostridia*. Aerobically, food-grade acetic acid is produced by the two-step vinegar process. The first step is the production of ethanol from a carbohydrate source such as glucose. This is carried out at 30-32°C using the yeast *Saccharomyces cerevisiae*. The second step is the oxidation of ethanol to acetic acid. The alcohol-containing solution is called "mash". Its alcohol concentration is given in percent per volume. Usually, it also contains some acetic acid, expressed in grams of acetic acid per 100 ml (% w/v). The sum of ethanol (vol %) and acetic acid (g per 100 ml) is called "total concentration" because the sum of these rather incommensurable values gives the maximal concentration of acetic acid that can be obtained by complete fermentation. The quotient of the total mash concentration indicates the concentration yield. Acetic acid fermentation is accompanied by secondary fermentation which combines to produce the flavor and typical aroma. Small quantities of volatile substances are formed during secondary fermentation, which include ethane, acetaldehyde, ethyl formate, ethyl acetate, isopentyl acetate, butanol, methylbutanol, and 3-hydroxi-2 butanone, which vary from vinegar to vinegar depending on the starting material and which, because of their individual characteristics, produce vinegars with a variety of odor, taste, color, and other properties. The fermentation is usually stopped at a minimum residual ethanol level to avoid overoxidation, the oxidation of acetic acid to water and CO_2. The most important properties of a production strain in the vinegar industry are tolerance to high concentrations of acetic acid and total concentration, low nutrient requirements, inability to overoxidize the formed acetic acid, high production rate, and resistance to phage infections. Although a variety of bacteria can produce acetic acid, mostly members of *Acetobacter* and *Gluconobacter* are used commercially, typically the aerobic bacterium *Acetobacter aceti* at 27-37°C. Other species frequently isolated from vinegar fermentations include *A. pasterianus*, *A. polyoxogenes*, *A. europaeus*, *A. xylinus*, *A. hansenii*, *A. obodiens* and *A. intermedius*. In the production of traditional Chinese vinegars cultures of *Acetobacter lovaniensis* are used. Pure cultures are not widely used in the acetic acid fermentation industry. *Acetobacter* spp. are better acid producers and are more commonly used in commercial vinegar production. However, they can oxidize acetic acid to CO_2 and H_2O (overoxidation) which is not a problem with *Gluconobacter* spp. Ethanol is dehydrogenated to acetic acid and the reduced co-substrates are oxidized via the respiratory chain. This fermentation is an incomplete oxidation because the reducing equivalents generated are transferred to oxygen and not to carbon dioxide. To improve strains of *Acetobacter* genetically, recombinant DNA techniques are considered to be useful. Host-vector systems and an efficient transformation method for *Acetobacter* spp. have been developed. Genes have been characterized which encode indispensable components of the acetic acid fermentation, such as alcohol dehydrogenase and aldehyde dehydrogenase. In addition, spheroplast fusion of the *Acetobacter* strains has been applied to improve their properties for use in vinegar production. Submerged fermentation has almost completely replaced surface fermentation methods. Industrial fermentation processes have evolved from the simple "let-alone" method involving a partially filled open container of wine exposed to air to the "field" fermentation in which a series of casks are filled with wine and inoculated in series by the vinegar produced in the previous casks. A certain amount of vinegar is still manufactured following the centuries-old empirical methods of the small producer, but

since the last century a flourishing vinegar-manufacturing industry has developed.

2.1.1 Fruit substrate vinegars

Wine vinegar

Wine vinegar, which is obtained from acetous fermentation of wine, is largely produced in continental Europe. The wines used for acetification are those with too low an alcohol content (7-9% v/v*)* or those in which volatile acidity is too high. Spoilt wines cannot be used. If wines with high alcohol content are to be used, they need to be appropriately diluted with water, since a high concentration of alcohol will inhibit the development of acetic acid bacteria. For the same reason, the wine must be free of sulfur dioxide. Both white and red or rose wines can be used to produce white or red vinegar, respectively. In the domestic or small-scale production of wine vinegar, the wine is poured into small wooden barrels, together with the vinegar starter, which consists of colonies of *Acetobacter* taken from barrels in which vinegar has already been produced. The barrel must contain air, so for this reason it is not filled completely. Acetification is slow and stops spontaneously when acidity reaches 7-8%. The slow transformation of wine into vinegar leads to the formation of many substances which impart excellent organoleptic qualities to the end product. The most important of these are acetaldehyde and ethyl acetate. The vinegar is then partly withdrawn for use and replaced with fresh wine to be acetified. Vinegar produced in this way is bound to vary in composition and character as it may be more or less cloudy and its acidity may vary according to the degree of alcohol in the wine and the nature of the fermentation. Wine vinegars contain the same spectrum of amino acids as spirit vinegar, but in larger amounts. Polyphenol compounds have been shown to be of great interest regarding the stability of wine vinegars. The phenolic compounds are generally contributed by the solid parts of the grapes. Therefore, in the case of wines kept in longer contact with the grapes a larger amount of polyphenols will be extracted. To avoid instability due to enzymes and microorganisms wine vinegar is pasteurized prior to bottling or afterwards. At lower temperatures, only the enzymes are inactivated, and at higher temperatures, the microorganisms as well.

Cider vinegar

Cider vinegar is prepared from apple wine that has undergone acetous fermentation and is widely used as table vinegar. It is yellowish in color and may be darkened with caramel. Its acidity is not very high and its acidic, astringent flavor recalls the fruit of origin. Many of the compounds found in cider vinegar were not found in the cider from which the vinegar was produced. The compounds correspond approximately to those found in wine vinegar.

Honey vinegar

This vinegar is obtained from honey, to which the right quantity of water is added. It is then subjected to alcoholic fermentation to produce ethanol. At the right temperature conditions and oxygen levels acetic bacteria produce acetic acid. The vinegar is then clarified by bland filtration or by decanting, so that all the qualities of honey remain

unaltered.

Fruit vinegars

Fermented juices from other fruits such as peaches and berries are also used to produce vinegar. Since these alcoholic liquids are not distilled, they maintain the subtle flavors and aromas of the raw ingredients.

2.1.2 Starch substrate vinegars

Malt vinegar

Malt vinegar is produced from malted barley with or without the addition of other cereals. Malt vinegar manufacture involves mashing, fermentation, and acetification. During mashing, the malted barley, sometimes mixed with other cereals such as maize and rice, is milled and mixed with hot water in mash casks, where the starch is converted by α-amylase into maltose, dextrose, and dextrins. The sweet liquor drains off the mash through the perforated false bottom of the cask and is collected in vessels where it is fermented by the addition of yeasts, which convert the fermentable sugars to ethanol and carbon dioxide. When fermentation is complete, the alcoholic liquor is separated from the yeasts and acetified by inoculation with *Acetobacter* cultures. The resultant alcohol is thus oxidized to acetic acid in the presence of atmospheric oxygen. The process lends itself to the different systems in current use. The vinegars are more or less aromatic, the superior varieties are those produced by the old and slow Orleans process. Malt vinegar is straw-colored and must in any case contain 4% w/v of acetic acid.

Rice vinegar

In the Far East, where rice is the staple cereal, vinegar is prepared from rice as such, from sake, or from the byproducts of sake manufacture. Traditional methods, similar to the Orleans process, are still in use but have been largely superseded by modern submersion techniques. This vinegar has a fairly low acidity and high amino acid content. It is light in color and has a clean, delicate flavor. It is therefore highly prized in oriental cooking since it does not significantly alter the taste of the food. The Chinese vinegars are rich in amino acids and, pyroglutamic acid and lactic acid predominate besides acetic acid.

Molasses vinegar

This vinegar is prepared from sugar syrup or molasses. It serves to make use of the byproducts of the sugar industry, but is not widely used.

2.1.3 Spirit vinegar

By far the largest percentage of vinegar is spirit vinegar, sometimes referred to as white, distilled, or alcohol vinegar, which is produced from diluted purified ethanol. In countries where it is permitted by law, wide use is made of synthetic ethanol, diluted to

10-14% v/v. Mashes obtained by alcoholic fermentation of natural sugar-containing liquids may also serve as raw materials. Spirit vinegar is strongly acid but not aromatic. It contains a small amount of amino acids which are mainly products of autolysis of the acetic acid bacteria. This vinegar is less expensive and is the most widespread in the world. In a number of countries, spirit vinegar is sold completely colorless which requires a treatment with activated carbon. In other countries it is colored yellow with caramel or other colorants admitted for food.

2.1.4 Special vinegars

Balsamic vinegar

A particular type of highly prized vinegar has been produced for centuries in Northern Italy. The raw material is grape must, preferably Trebbiano. When alcoholic fermentation begins, about 24 hours after pressing, the must is boiled gently until it has reduced to about one-half or one-third of its starting volume. The result is a liquor with a high sugar concentration (about 30%) in which alcoholic and acetous fermentation take place together very slowly. The sugar tolerance is an important trait of acetic acid bacteria for the production of traditional balsamic vinegar, since it is made from cooked must with a high sugar concentration. In particular few species are able to grow at elevated sugar concentration e.g. *Gluconobacter diazotrophicus* species is able to grow at 30% of D-glucose. Yeasts (*Saccharomyces* and *Zygosaccharomyces*) and acetic acid bacteria (*Acetobacter* and *Gluconobacter*) are needed for the formation of this vinegar. Traditional balsamic vinegar takes many years to produce. During the process of fermentation, maturing, and aging, the product becomes highly concentrated and the sugars, alcohols, aldehydes, and organic acids undergo gradual chemical transformation. The vinegar battery consists of a variable number of barrels (between five and twelve or even more) of different woods and decreasing in size. They are set up in well-ventilated areas which are hot and dry in summer and cold in winter. According to the traditional method, part of the contents of the smallest barrel is withdrawn annually for consumption, and is replaced with an equal volume from the next largest barrel in the battery. This is in turn replenished from its neighbor, and so on up the line. Finally, the largest barrel is topped up with the season's boiled must. The process takes at least 12 years, though it is not uncommon to find vinegars that are 50 or more years old, and the yield is very low (no more than 1 l of vinegar from 100 kg of fresh must). The end product, however, is of exceptionally high quality, dark brown in color, of a syrupy consistency, sweet and sour to the taste, and with a characteristically pleasant aromatic smell. In traditional balsamic vinegar the total solids are very high (20-70%), acidity varies between 6 and 18 % w/v acetic acid and there are large amounts of sugars, essentially glucose and fructose, as well as numerous aromatic substances that have gradually been formed over the years.

Chinese vinegar

A very detailed description of vinegar making was recorded in an ancient Chinese book, *Qimin Yaoshu*. Just like soy sauce, vinegar is another important condiment in Chinese cuisines. In China, Shanxi aged vinegar and Zhenjiang scented vinegar are considered the best among the traditional Chinese vinegars. Both were fermented using a mixed

solid culture. Shanxi aged vinegar is a typical example of Big-Qu fermentation. Big-Qu is a fermentation starter brick shaped, and is made of wheat, barley, or green peas. During fermentation, saccharification and alcoholic fermentation occur at low temperature. The freshly obtained vinegar is then placed outdoors, heated in summer by the sun and frozen in the winter. The Shanxi aged vinegar is viscous in texture, dark purple in color, sweet in taste, and has a long shelf life. The Zhenjiang scented vinegar presents a typical example of Small-Qu fermentation. Regular rice or waxy rice is used and the whole process normally takes about 60 days. The finished product is famous for its delicate combination of color, fragrance, sourness, mellowness, and richness.

2.2 Cocoa production

Cacao beans, the starting material for cocoa and chocolate manufacture, are the seeds from fruits (pods) of the *Theobroma cacao* tree. The popularity of cocoa is not its nutritional value but rather its unique flavor, color and aroma. To obtain these sensory characteristics the beans must be fermented, as roasting unfermented dried cacao does not result in the desired flavor, aroma or color. Cocoa is therefore classed into that category of foods whose characteristic flavor is developed through a fermentation process. The fermentation and drying process are also referred to as "curing". In the intact seed, enzymes and their substrates are separated by biological barriers which, during fermentation, break down allowing enzymes and substrates to freely mix to produce the flavor and aroma precursors of chocolate. The principal objectives of fermentation are the removal of mucilage to provoke aeration of the fermenting seeds and to facilitate drying later on, and to provide heat and acetic acid necessary for killing (preventing germination) and curing the seeds. It is recommended that the curing process commence immediately following pod breaking. The biochemical changes that affect curing may be divided into those that occur in the external pulp owing to microbial action and those initiated within the seed as a consequence of the former. To induce fermentation the pods are opened following harvesting to reveal the beans, covered with pulp. The pulp is predominantly water with 10-15% sugars and has a low pH, between pH 3.6-4.0. Fermentation methods vary between different countries and even between different processors. The fermentation is natural and therefore, the microbial species present also differ between batches and different geographical locations. Occasionally a more controlled situation is required and starter cultures are used, however, these generally do not compare favorably with the natural fermentations. The sterile pulp is inoculated with a variety of microorganisms during handling and storage which perform the fermentation. Fresh cocoa pulp contains sugars and citric acid which makes it an excellent medium for the growth of microorganisms. The beans are fermented for 2-12 days. During this time the sticky pulp becomes a turbid broth from which the beans absorb flavors. The events taking place during pulp fermentation correspond to a succession of microorganisms metabolizing the pulp. When starting fermentation, the low pH value and the high sugar content of the pulp allow anaerobic fermentation by yeasts and lactic acid bacteria. In addition the yeasts hydrolyze the pectin that covers the beans. Without pectin, the bitter alkaloids are able to leach out of the bean or be altered by alcohol that can enter the beans. As the microorganisms ferment the sugars they produce heat and high temperatures, up to 45-50°C develop. As the temperature rises and alcohol accumulates the proportion of yeasts falls rapidly and acetic acid bacteria predominate. Lactic acid bacteria start to grow, although their

numbers and activity is thought be subordinate to that of the acetic acid bacteria. The pulp is stirred and drained which increases the level of aeration. The presence of oxygen and the low pH favor the growth of acetic acid bacteria, mostly *Acetobacter* spp. Acetic acid and lactic acid are the most significant metabolites penetrating the beans and are responsible for the acidic taste. When present in high concentrations, acetic acid may mask the impression of cocoa aroma. A number of species of the genus *Acetobacter* which have been found in fermented cocoa not only oxidize ethanol to form acetic acid but also oxidize this acid (overoxidation), causing a decrease in acetic acid during late stages of fermentation. Additionally, *Gluconobacter* spp. were found. When fermentation is complete the beans are sun- or air-dried to reduce the water content to below 7.5%. The beans are then roasted at 121°C to obtain the characteristic smell and flavor of chocolate.

2.3 Kombucha beverage production

Kombucha is a traditional fermented beverage obtained by the fermentation of sugared tea with a symbiotic culture of acetic acid bacteria and yeasts, which together form the so called “tea fungus”. Kombucha has a history of several thousand years in the East and yet is quite popular today in the West because of its beneficial effects on human health. Analysis of the fermented liquid has revealed the presence of acetic, lactic and gluconic acids as major chemical compounds.

A diverse range of flavor compounds, including a range of alcohols, aldehydes, ketones, esters, and amino acids have also been identified. Bacteria and yeasts present in Kombucha form a powerful symbiosis able to inhibit the growth of potential contaminating bacteria. The main acetic acid bacteria found in the tea fungus are: *Acetobacter xylinus*, *Acetobacter xylinoides*, *Acetobacter aceti*, *Acetobacter pasterianus* and some *Gluconobacter* species. *Acetobacter xylinus* has the ability to synthesize a floating cellulose network which enhances the association formed between bacteria and yeasts. Caffeine and related xanthines of the tea infusion stimulate the cellulose formation by *A. xylinus*.

The yeasts convert sucrose into fructose and glucose and produce ethanol. Acetic acid bacteria convert glucose to gluconic acid and ethanol to acetic acid. The resultant low pH and presence of antimicrobial metabolites reduces the competition of other bacteria, yeasts and filamentous fungi. Black tea and white sugar are the best substrate for the preparation of Kombucha, although green tea can also be used.

Tea leaves are added to boiling water and allowed to infuse for about 10 minutes after which the leaves are removed. Sucrose (50 g/l) is dissolved in the hot tea and the preparation is left to cool. Tea is poured into a wide-mouthed clean vessel and is acidified by the addition of vinegar or already prepared Kombucha. The tea fungus is laid on the tea surface, and the jar is carefully covered with a clean cloth. The preparation is left to incubate at room temperature for 1-8 weeks. When the fermentation is completed, the beverage is filtered and stored in capped bottles at 4°C. The taste of the Kombucha changes during fermentation from a pleasant fruit sour-like flavor after a few days, to a mild vinegar-like taste with prolonged incubation.

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Biographical Sketches

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