Indian Journal of Microbiology

Acetic acid bacteria: physiology and carbon sources oxidation

--Manuscript Draft--

Manuscript Number:	INJM-D-12-00772R1
Full Title:	Acetic acid bacteria: physiology and carbon sources oxidation
Article Type:	Review article
Keywords:	acetic acid bacteria; oxidative fermentation; cellulose; Acetobacter; Gluconacetobacter; Gluconobacter oxydans
Corresponding Author:	maria gullo, PhD University of Modena and Reggio Emilia ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Modena and Reggio Emilia
Corresponding Author's Secondary Institution:	
First Author:	Dhouha Mamlouk, PhD
First Author Secondary Information:	
Order of Authors:	Dhouha Mamlouk, PhD
	maria gullo, PhD
Order of Authors Secondary Information:	
Abstract:	Acetic acid bacteria (AAB) are obligately aerobic bacteria within the family Acetobacteriaceae, widespread in sugary, acidic and alcoholic niches. They are known for their ability to partially oxidise a variety of carbohydrates and to release the corresponding metabolites (aldehydes, ketones and organic acids) into the media. Since a long time they are used to perform specific oxidation reactions through processes called "oxidative fermentations", especially in vinegar production. In the last decades physiology of AAB have been widely studied because of their role in food production, where they act as beneficial or spoiling organisms, and in biotechnological industry, where their oxidation machinery is exploited to produce a number of compounds such as L-ascorbic acid, dihydroxyacetone (DHA), gluconic acid and cellulose. The present review aims to provide an overview of AAB physiology focusing carbon sources oxidation and main products of their metabolism.
Response to Reviewers:	Answers to Reviewers Thank you very much for all your comments and suggestions, stronghly contributed to improve the manuscript. Below a detailed response for each point raised out is provided. Reviewer #1: This review describes acetic acid bacteria focusing on their metabolism. It is a concise overview of the acetic acid bacteria involved in food fermentation, particularly vinegar production. A few things that should be considered to improve the review: - Emphasis on recent taxonomy (a lot of research has been done on AAB taxonomy in the last 5-10 years) - Figure would be good (eg reaction of ethanol to acetic acid with the various enzymes) - Biotechnology needs to be expanded more - VBNC needs to be brought out more in the cultivation section - English expression and language should be checked We have fully considered the criticisms raised out by the rewiever and accordingly the

	manuscript was revised. Recent taxonomy has been added to the paragraph “General characteristics” that in this new version of the manuscript has been renamed as follow: “General phenotypic characteristics and taxonomic aspects of acetic acid bacteria” As suggested, a figure (Fig.1) showing ethanol oxidation by ADH and ALDH and location within the cell has been added. Biotechnological applications of AAB has been expanded more (p. 2 lines 1-23). As suggested, the paragraph “Isolation and cultivability” was modified and VBNC concept added (p.5, lines 11-15) The whole manuscript has been revised and a thoroughly English revision has been done. Specific comments: Abstract - use biotechnology rather than biotech The term was corrected P3, L7 - assigned rather than allocated Allocated has been replaced by “assigned” (p.3, line 13), as suggested. P3, L12 & 14 - write ethanol (EtOH) or acetic acid (AcOH) and then use abbreviation The correction has been done. P3, L23 - surface layer Superficial has been replaced by “surface” (p. 1, line 14) P4, L1 - acetic acid production is not a fermentation The sentence was corrected: AcOH fermentation was replaced by “ AcOH production” P4, L5 - AAB are involved in these other food productions, not only implicated Implicated was replaced by involved, as suggested. Page 5 - what is the significance of 1996 in regards to these various foods and the bacteria isolations? The whole sentence was removed. Reference is needed for 'Early researchers....' The reference was added [35] : Dupuy P (1952) Recherche d'une technique d'isolement des Acetobacter du vin. Ann Technol Agric 1:107-112 (p.5. L.19) Page 6 L 10-15 - this section a few more sentences to explain these statements better. Lines 14-15: why the difference? Explain the significance between the two versions. To clarify, we removed the second part of the sentence: “Whereas previous edition of Bergey's Manual [25] recommended the use of a simple medium containing 0.5% yeast extract, 1.5% EtOH, and 2.5% agar.” In the new version of the manuscript, the sentence reports only the medium of the last version of bergey’s manual (p. 8, L: 5-9) “The last edition of Bergey's Manual of Systematic Bacteriology [1] proposed standard medium for enrichment and isolation of AAB, with the exception of Ga. europaeus (which strains are AcOH-dependent), containing: D-glucose, 0.05%; EtOH, 1.5%; yeast extract, 0.5%; peptone, 0.3%; cycloheximide, 0.01%; and agar, 0.12%.” Page 7 L 6-11 - this whole section needs a bit of rearranging. These lines should be at the start of the section

	The paragraph “Isolation and cultivation” was rearranged. The sentence was simplified and put at the start of paragraph “Isolation and cultivation of AAB strains, especially from fermented beverages, have been described to be problematic, giving rise to an underestimation of species richness when culture dependent methods are applied [31]”. Lines 6-14 - this needs to expanded to explain these observations and statements. It is suggesting that now all bacteria are growing up/being cultivated. Is this viable but not culturable (VBNC). Or poor growth medium? Highlight the difficulties in cultivating AAB and all the AAB present. The section was rearranged as follow: p. 5, lines 11-17 “ Low recovery of strains due to the fraction of population that could reach a viable but non-culturable (VBNC) state has been stated. For instance in wine it was shown that VBNC status of spoiling AAB is induced by O2 deprivation [32]. Whereas in vinegars some studies developed by no culture approaches, such as PCR/DGGE, revealed higher species diversity respect to that detected by culturing methods [33, 34, 3]. Limitations of culturing were in part overcame with the formulation of appropriate media, which allowed the cultivation of no growing strains or slow growing strains.” Page 8 L 1-8 : it would be a good idea to have a figure here for the reaction and enzymes, their location .... . It is always beneficial in a review to have a figure. As suggested, a figure (Fig.1) showing ethanol conversion and location of enzymes within the cell has been added. The figure rapresents the ethanol oxidation to acetic acid by the membrane-bound ADH and ALDH and cytoplasmic respiration of ethanol. P9 L 15 - ... were present in the strain. .... The genes were present, but was it shown that the pathways were functioning. Genomics show that genes are present but do not confirm that reactions are occurring. The sentence was removed. P 10 L 7 - High in what sense? Concentration? Structure? P 10 L 9 - ..... wide range of applications. ..... This does not make sense or belong here. To avoid repetition the sentence (p.10, L.6-9) was removed. The sentence reported in the new version of manuscript is at page 9, lines: 14-18 “The most characteristic reaction is the direct oxidation of glucose into Glucono-δ-lactone, which is oxidized into gluconic acid. This reaction is particularly active in Gluconobacter growing at high concentrations of sugars. D-gluconate can be further oxidized to 2-ketogluconate and 2,5-diketogluconate by the gluconate dehydrogenase and 2-ketogluconate dehydrogenase [52].” P 10 L 14 - last sentence Is this important for high production of vitamin C? The sentence was removed. P 11 L 1-5 - another sentence or two to expand this concept would be beneficial for the reader. To clarify the sentence was rewritten as follow (p. 10, Lines 14-17): “In G. oxydans it was reported that oxidization of glycerol is catalized by the membrane-bound glycerol/sorbitol dehydrogenase. This quinoprotein is considered the main polyol-dehydrogenase of G. oxydans that exhibits a broad substrate specificity. It catalyzes the oxidation of D-sorbitol, gluconate, and glycerol to L-sorbose, 5 ketogluconate and DHA, respectively [54]. “

	P 13 L 6-7: this sentence needs to be reworded and explained better. What has been reached? P 13 L 11 - sugared is not a good word here. Rewrite using high sugar content. In the new Conclusion section, the sentences were rewritten as follow: “AAB are food-associated microorgansims that have a long history in oxidative fermentation processes, where they are spoiling or beneficial organisms. The physiological uniqueness of AAB is due to their ability to partially oxidize carbon sources and quantitatively excrete the corresponding compounds in the surrounding media. This feature, besides vinegar, is exploited for the industrial production of a number of compounds from alcohols, sugar and sugar alcohols oxidation. Moreover they are considered promising for the production of the pure form of cellulose. Although the valuable potential of AAB in biotechnological applications, their industrial exploitation is not full developed. Perspectives to enhance their role as biocatalysts include the availability of genetic stable strains and further advance on their metabolic potential, in order to obtain high carbon substrates conversion efficiency.” Table 1. In the legend write AAB in full. At the top of the table, it would be much better to have at least abbreviations of the genera rather than numbers. It would make it much easier for the reader. AAB was replaced by acetic acid bacteria, as suggested. Number were replaced by abbreviations.

Acetic acid bacteria: physiology and carbon sources oxidation

Dhouha Mamlouk, Maria Gullo

Department of Life Sciences, University of Modena and Reggio Emilia, Via Amendola, 2, 42122, Reggio Emilia, Italy

Corresponding Author: maria.gullo@unimore.it

Telephone: +39052252057/63

Fax +390522522027

Abstract

Acetic acid bacteria (AAB) are obligately aerobic bacteria within the family *Acetobacteriaceae*, widespread in sugary, acidic and alcoholic niches. They are known for their ability to partially oxidise a variety of carbohydrates and to release the corresponding metabolites (aldehydes, ketones and organic acids) into the media. Since a long time they are used to perform specific oxidation reactions through processes called "oxidative fermentations", especially in vinegar production.

In the last decades physiology of AAB have been widely studied because of their role in food production, where they act as beneficial or spoiling organisms, and in biotechnological industry, where their oxidation machinery is exploited to produce a number of compounds such as L-ascorbic acid, dihydroxyacetone (DHA), gluconic acid and cellulose. The present review aims to provide an overview of AAB physiology focusing carbon sources oxidation and main products of their metabolism.

Keywords: acetic acid bacteria, oxidative fermentation, cellulose, *Acetobacter*, *Gluconacetobacter*, *Gluconobacter oxydans*

Introduction

Acetic acid bacteria (AAB) are strictly aerobic bacteria occurring in sugary, alcoholic and acidic niches such as fruits, flowers and particularly fermented beverages [1, 2, 3, 4]. Although foods are the most known sources of AAB, they play role as plant-associated bacteria (N_2 fixing), symbionts of insects and human pathogens [5, 6].

The metabolic potential of AAB in these environments is expressed by the partial oxidation of carbohydrates releasing the corresponding products (aldehydes, ketones and organic acids) into the surrounding media. Through processes called "oxidative fermentations", AAB perform specific oxidation reactions and channel the released electrons to molecular oxygen. Due to these ability they are known since a long time especially for their role in vinegar production [7].

Vinegar, an aqueous solution of acetic acid (AcOH) that is produced by AAB from a dilute ethanol (EtOH) solution [8], was the first investigated environment concerning the biological formation of AcOH. Early researches [9] allowed to recognize that the surface layer during vinegar formation, commonly known as "mother of vinegar", was a mass of living microorganisms causing AcOH production.

AAB are also involved in the production of other foods, like palm wine [10], cocoa powder [11], *nata de coco* (a fermented food from coconut), *pulque* (a beverage from agave) and *kombucha*, a slightly acid and sparkling beverage obtained from tea fermentation by a symbiotic culture of AAB and yeasts [8].

They can spoil fermented beverages such as wine, cider and beer, where the production of AcOH is undesired [12], whereas in others foods, such as sourdough for bread production [13], AAB can occasionally occur contributing to the acidification of dough.

Besides fermented foods, some AAB are used as biocatalysts for the industrial production of a range of compounds, making them important biocatalysts for the development of eco-friendly fermentation processes as an alternative to the chemical synthesis. Strains of *G. oxydans* produce enzymes involved in amino acids synthesis e.g. glutamic and aspartic acids thanks to the incomplete set of tricarboxylic acid (TCA) enzymes which could function primarily for glutamate, aspartate and succinate biosynthesis [14].

One of the most important biotechnological application of *G. oxydans* is the production of L-ascorbic acid (vitamin C) precursors such as L-sorbose from D-sorbitol and 2-keto-L-gluconic acid from 2,5-Diketo-D-gluconic acid or L-Sorbosone [15].

Strains of *G. oxydans* are also exploited for the microbial production of DHA that is used in the pharmaceutical industry as a cosmetic tanning agent and also as an intermediate for the synthesis of various organic chemicals and surfactants [16].

Among organic acids, gluconic acid, used as a bulk chemical in the food, textile, medical and construction industries can be produced by *G. oxydans* which oxidize glucose to gluconate by the membrane bound glucose dehydrogenase [17].

Other applications of *G. oxydans* are the production of miglitol's precursors, used as a therapeutic drug for the treatment of non-insulin-dependent diabetes mellitus; D-tagatose, used as a bulking agent in food and a non-calorific sweetener; and shikimate, which is a key intermediate for a number of antibiotics [16].

Species of *Acetobacter* and *Gluconacetobacter* genera, besides vinegar, are of interest in agricultural field, where especially strains of *Ga. diazotrophicus* have been proved useful for their role as N_2 fixing bacteria [5]. Finally *Ga. xylinus* is well known for the ability to produce high amount of pure cellulose [18].

On the basis of the increasing prospective on AAB for food, beverages and other biotechnological applications, this work aims to review physiology of AAB including carbon sources oxidation and metabolites production.

General phenotypic characteristics and taxonomic aspects of acetic acid bacteria

AAB are gram negative or gram-variable, non-spore forming, ellipsoidal to rod-shaped cells that can occur in single, pairs or in short chains. They could be motile due to the presence of peritrichous or polar flagella. Catalase positive and oxidase negative, AAB have an obligate aerobic metabolism, with oxygen as the terminal electron acceptor. The optimum pH for the growth is 5-6.5, while they can grow at lower values (3-4) [1]. Their optimum temperature vary between +28 and +30°C although some species are recognized as thermotolerant [19, 20]. They can produce pigments and also different kinds of exopolysaccharides [21]. Main distinctive traits of AAB are reported in Table 1.

AAB are assigned to the family *Acetobacteriaceae* that belongs to the order *Rhodospirillales* as part of the *Alphaproteobacteria*, within the family *Acetobacteraceae.* At present they are represented by the following genera: *Acetobacter, Acidomonas, Ameyamaea, Asaia, Gluconacetobacter, Gluconobacter, Granulibacter, Kozakia, Neoasaia, Neokomagataea, Saccharibacter, Swaminathania* and *Tanticharoenia.*

The most updated data on valid published species of each genus are reported by the List of Prokaryotic names with Standing in Nomenclature [22].

Since their first description as “vinegar bacteria”, about 150 years ago [9], classification of AAB has undergone robust changes, with scission, renaming, restoration and emendation of genera and species [23].

During the last decades studies on AAB identity and their phylogenetic relationships have been achieved by polyphasic approaches combining phenotypic, chemotaxonomic and

genotypic data of strains. Both the use in a polyphasic strategy of 16S rRNA gene as molecular marker, and the formulation of more suitable isolation media, reflect the increased number of new described genera and species.

However, identification at the species level is often difficult due to the low resolution power of phenotypic characterization and the high sequence similarity (≥ 99,5%) of 16S rDNA of phylogenetically closely related species [24].

Weaknesses of phenotypic characterization are generated by a difficult standardisation of tests, in some cases by low number of discriminant characters and by instability of physiological traits of preserved strains [25].

For instance physiological deficiencies caused by inactivation of enzymes, such as the membrane-bound alcohol dehydrogenase (ADH) and cellulose synthase, deriving from genetic instability, can affect reliability of phenotypic assays. The main source of genetic instability has been attributed to mobile genetic elements, mainly transposons, widely distributed in the genome. Insertion sequences (IS) responsible for EtOH oxidation deficiency, like those of the family IS 12528, were found in the chromosome of *A. pasteurianus* NCIB 7214 (5 copies) [26], *A. aceti* 1023 (1 copy) and *G. oxydans* IFO 12528 (10 copies). Likewise 100 copies of IS 1380 occur in *A. aceti* 1023 and 74 copies in *A. pasteurianus* NBRC 3283 [1, 27]. Also spontaneous cellulose deficient mutants due to the IS 1031 were detected in *Ga. xylinus* ATCC 23769 [28].

Advances in taxonomy of AAB derive from the availability of full genome sequences especially of type strains, that allowed the application of new genomic approaches.

To solve ambiguities of phylogenetically closely related species, recently the use of different genes as phylogenetic markers, such as housekeeping genes (*dnaK*, *groEL* and *rpoB*) has been proved useful for AAB species differentiation [29]; whereas protein-coding

genes, such as those involved in the metabolism of AcOH, have been applied to investigate phylogenetic relationships among *Acetobacter, Gluconacetobacter* and *Gluconobacter* genera [30]. Since multigene analysis can resolve ambiguities in phylogenetic reconstructions when a single gene is not enough, it is expected that the availability of more complete genome sequences will increase the application of these approaches.

Isolation and cultivability

Isolation and cultivation of AAB strains, especially from fermented beverages, have been described to be problematic, giving rise to an underestimation of species richness when culture dependent methods are applied [31].

Low recovery of strains due to the fraction of population that could reach a viable but non-culturable (VBNC) state has been stated. For instance in wine it was shown that VBNC status of spoiling AAB is induced by O_2 deprivation [32]. Whereas in vinegars some studies developed by no culture approaches, such as PCR/DGGE, revealed higher species diversity respect to that detected by culturing methods [33, 34, 3].

Limitations of culturing were in part overcame with the formulation of appropriate media, which allowed the cultivation of no growing strains or slow growing strains.

A number of conventional culture media to isolate AAB from different sources are reported in literature, which carbon source are mainly glucose, mannitol and EtOH. Early researches proposed a variety of media containing yeast-glucose agar (pH 5.5-6.0), peptone-glucose agar fortified with yeast extract and tomato juice, and media containing EtOH (1.5%) as single carbon source, yeast extract (0.5%) and agar (2.5%) [35]. To control the growth of other bacteria and yeasts, these media can be acidified and/or supplemented with antibiotics and cycloheximide [36].

A medium that allows successful isolation from different niches is glucose yeast extract carbonate (GYC) composed by D-glucose, 10%; yeast extract, 0.5%; peptone 0.3%; $CaCO_3$, 0.12%; and agar, 0.12% [37]. After incubation, colonies of AAB are recognized by the surrounding zones of $CaCO_3$ clearing. $CaCO_3$ neutralizes AcOH generated by AAB, preventing physiological stress and cell death [25]. The last edition of Bergey's Manual of Systematic Bacteriology [1] proposed standard medium for enrichment and isolation of AAB, with the exception of *Ga. europaeus* (which strains are AcOH-dependent), containing: D-glucose, 0.05%; EtOH, 1.5%; yeast extract, 0.5%; peptone, 0.3%; cycloheximide, 0.01%; and agar, 0.12%.

Important advances in recovering AAB strains from industrial vinegar have been reached introducing a double agar layer (0.5% in the lower layer and 1% in the upper layer) and media containing EtOH and AcOH, in an attempt to simulate the environment of the acetification tanks. Using this approach the new species *Ga. europaeus* isolated from industrial wine vinegar was described [38].

It was also stated that isolates from cider or wine vinegar grew readily in Reinforced AE-Medium (RAE), containing D-glucose, 4.0%; peptone, 1.0%; yeast extract, 1%; citric acid, 0.15%; disodium hydrogen phosphate (Na_2HPO_4), 0.38%; glacial AcOH, 1 ml; absolute EtOH, 1 ml; agar, 0.5% upper layer; 1% bottom layer); pH 3.8. The simpler AE medium (glucose, 0.5%; yeast extract, 0.3%; peptone, 0.4%; agar, 0.9%; absolute EtOH, 3 ml; glacial AcOH, 3 ml) has been proved suitable for the isolation of strains from spirit vinegars [39, 40].

Actually, some of the most widely used isolation media are: GYC, AE; YPM medium (yeast extract, 0.5%; peptone, 0.3%; mannitol, 2.5%; agar, 1.2%) and MYA medium (malt extract, 1.5%; yeast extract, 0.5%; agar, 1.5%; and EtOH, 60 ml) [41].

Carbon sources

Ethanol oxidation

AAB partially oxidize EtOH by two successive catalytic reactions of the ADH and a membrane-bound aldehyde dehydrogenase (ALDH) that are bound to the periplasmic side of the cytoplasmic membrane. The complete oxidation of EtOH occurs at cytoplasmic level by a NAD-ADH and NAD-ALDH. The AcOH produced can be further utilized by acetyl CoA synthase and via TCA cycle (Fig. 1).

The membrane-bound ADH and ALDH complexes are tightly linked to the respiratory chain, which transfers electrons via ubiquinone (UQ) and a terminal ubiquinol oxidase to oxygen as final electron acceptor [42]. ADH oxidizes EtOH to acetaldehyde, which is further oxidized to AcOH by ALDH as follows:

$$CH_3CH_2OH + PQQ \rightarrow CH_3CHO + PQQH_2 \text{ (alcohol dehydrogenase)}$$

$$CH_3CHO + PQQ + H_2O \rightarrow CH_3COOH + PQQH_2 \text{ (aldehyde dehydrogenase)}$$

ADH of many AAB is composed by three subunits. Subunit I is the largest (72-78 kDa) and it is encoded in the *adhA* gene. It possesses a heme *c* and a pyrroloquinoline quinone (PQQ) as cofactors and requires Ca^{2+} to be active, according to the catalytic mechanism given by Anthony (1996) [43] and Goodwin and Anthony (1998) [44]. The subunit II which molecular size range from 44-45 kDa contains three heme *c* moieties and it is encoded in the *adhB* gene. The third and smallest subunit (20 kDa), encoded in the *adhS*, helps the two functional subunits with their association to the membrane protecting the catalytic subunit from proteolysis and it contribute to the correct conformation of the ADH complex for electron transport on the periplasmic surface [26, 45]. Oxidation of EtOH occurs at PQQ site that acts as two-electron redox mediator; electrons are initially transferred to UQ, which will be re-oxidized by a membrane-associated oxidase.

Eventually, oxygen is the final electron acceptor, resulting in formation of H_2O and a proton motive force necessary for energy production through a membrane-bound ATPase.

AAB possesses also an inactive form of ADH with the same subunit composition of the active form, but having 10 times lower Q-1 reductase activity. However, it exhibits an ubiquinol:ferricyanide oxidoreductase activity, an ethanol:Q-1 and ethanol: ferricyanide oxidoreductase activities. The ubiquinol:ferricyanide oxidoreductase activity is higher in the inactive ADH than in the active enzyme suggesting that it play a role for the regulation of redox levels of UQ/ubiquinol in the cytoplasmic membrane contributing to the functionality of AAB under acidic and high aeration conditions [45].

The ALDH complex of AAB is composed of two or three subunits of different molecular masse organized as an operon. It has been reported that the ALDH enzymes from *A. aceti* and *Ga. europaeus* contain three subunits, whereas two subunits were detected in *G. suboxydans*, *A. rances*, and *A. polyoxogenes* [1]. Its optimum pH is between 4 and 5, although it can catalyses the oxidation of acetaldehyde to acetate at lower pH values [46]. ALDH is sensitive to oxygen concentrations, and when these are low its activity decreases, accumulating acetaldehyde into the media. It is also more sensitive to the presence of EtOH than ADH [47].

Sugars oxidation

AAB are known to have a high oxidative ability against sugars, mainly glucose but also arabinose, fructose, galactose, mannitol, mannose, ribose, sorbose and xylose (Table 1) [48]. They can catabolize sugars through the cytoplasmic hexose monophosphate pathway (Warburg-Dickens pathway) [36]. The Entner-Doudoroff pathway occurs only in cellulose-synthesizing *Acetobacter* and *Gluconacetobacter* strains, where it appears to be more active than the hexose monophosphate cycle [49].

The oxidative pentose-phosphate pathway was reported to be the most important route for phosphorylative breakdown of sugars and polyols to CO_2 in *G. oxydans*. Therefore it was predicted that *G. oxydans* has the capability to take up and to channel many polyols, sugars and sugar derivatives into the oxidative pentose phosphate pathway: polyols are first oxidized by soluble dehydrogenases; these products and other ketoses and aldoses are further modified by isomerases and epimerases. Finally, the compounds are phosphorylated by specific or unspecific kinases forming intermediates of the oxidative pentose phosphate pathway [50].

Acetobacter species can use sugars through the hexose monophosphate pathway and also through the Embden-Meyerhof-Parnas and Entner-Doudoroff pathways [51]. Sugars are further metabolised to CO_2 and H_2O via the TCA pathway, which is not functional in *Gluconobacter*. Sugar is more preferred as carbon source by *Gluconobacter* than by *Acetobacter* because the species of this genus can obtain energy more efficiently by the metabolisation of the sugars via pentose phosphate pathway [48]. The most characteristic reaction is the direct oxidation of glucose into Glucono-δ-lactone, which is oxidized into gluconic acid. This reaction is particularly active in *Gluconobacter* growing at high concentrations of sugars. D-gluconate can be further oxidized to 2-ketogluconate and 2,5-diketogluconate by the gluconate dehydrogenase and 2-ketogluconate dehydrogenase [52].

At industrial scale, massive gluconic acid production by *G. oxydans* requires high glucose concentrations, low pH and high aeration rate. The further oxidation to ketogluconic acids is potentially undesirable reaction when using *Gluconobacter* strains for gluconic acid production. Suppression of ketogluconates formation has been achieved performing processes at low pH values [53].

Sugar alcohols oxidation

AAB can oxidize several sugar alcohols like glycerol to dihydroxyacetone (DHA), D-mannitol to D-fructose, D- sorbitol to L-sorbose, D-arabitol to xylulose, D and meso-erythritol to L-erythrulose [1, 7]. Particularly in winemaking, AAB can use glycerol as carbon source which is converted into DHA by the glycerol dehydrogenase; an enzyme bound with the cellular membrane inducing a high accumulation of DHA in the media. In wine, DHA can react with proline producing a "crust-like" aroma and it can bind SO_2 reducing its anti-microbial activity [36].

In *G. oxydans* it was reported that oxidization of glycerol is catalized by the membrane-bound glycerol/sorbitol dehydrogenase. This quinoprotein is considered the main polyol-dehydrogenase of *G. oxydans* that exhibits a broad substrate specificity. It catalyzes the oxidation of D-sorbitol, gluconate, and glycerol to L-sorbose, 5 ketogluconate and DHA, respectively [54].

In *G. oxydans* can oxidize glycerol by the enzyme glycerol/sorbitol dehydrogenase that is considered the major polyol-dehydrogenase with a broad substrate specificity. It catalyzes the oxidation of D-sorbitol, gluconate, and glycerol, producing L-sorbose, 5 ketogluconate and DHA, respectively [54].

The metabolic pathway of glycerol in *A. pasteurianus*, predicted from *Ga. xylinus*, showed that glycerol utilization is accompanied by the formation of DHA, cellulose, CO_2, and small amounts of acetate. In this species, DHA phosphate from glycerol can be produced by two pathways, one is via DHA catalyzed by glycerol dehydrogenase and the other one is via glycerol 3-phosphate by glycerol kinase. The DHA phosphate may be converted to D-glyceraldehyde 3-phosphate by triosephosphate isomerase and thus enter into the gluconeogenesis pathway [27].

Organic acid oxidation

The ability of *Acetobacter and Gluconacetobacter* strains to oxidize AcOH (Fig 1) generating the so-called acetate overoxidation occurs via TCA cycle [42]. Other acids such as lactic, pyruvic, malic, succinic, citric, and fumaric are similarly metabolized. In contrast, strains of *Gluconobacter* do not have a functional TCA cycle because of deficiencies in the two key enzymes, *alpha*-ketoglutarate dehydrogenase and succinate dehydrogenase. Consequently, they are unable to metabolize AcOH and other organic acids. Although the optimum pH for the oxidation of organic acids by AAB is near 6.0, there is evidence that it occur at lower values (3.5 to 4.0). In vinegar, for instance, *Acetobacter* species exhibits a biphasic growth curve, where the first corresponds to an EtOH oxidation with AcOH production, and the second to an overoxidation [42].

Several strains of *Acetobacter* and *Gluconobacter,* particularly strains of *A. pasteurianus,* can oxidize lactate to acetoin. Acetoin has a characteristic "butter-like" aroma and flavor occurring in spoiled wine [36].

Production of exopolysaccharides

Dextrans, levans and cellulose are the main exopolysaccharides produced by AAB glucose metabolism [1]. *Ga. xylinus* species have been regarded as model system for the study of biochemistry and genetics of cellulose biogenesis. The rate of cellulose production in *Ga. xylinus* is proportional to the rate of cell growth, and the yield is dependent on the carbon sources. Activators for bacterial cellulose production are compounds like caffeine and related xanthines [1]. *Ga. xylinus* synthesizes a cellulose mat that covers the surface of the growth medium in static cultures, whereas round balls of cellulose are formed in shaking cultures. The key enzyme in cellulose synthesis by *Ga. xylinus* is the membrane bound

cellulose synthase which uses UDP-glucose as substrate. The pathway from glucose to cellulose consists of the following four enzymatic steps:

Glucose → glucose-6-phosphate → glucose-1- phosphate → UDP-glucose → cellulose

Cells of cellulose-producing AAB are entrapped in the polymer matrix, supporting the population at the liquid-air interface. This facilitates oxygen and nutrient supply, since the concentration of nutrients in the cellulose matrix is enhanced by its absorptive properties, in contrast to the surrounding aqueous environment. Aeration of cultures gives rise to the formation of spontaneous non-cellulose-producing mutants. Most *Ga. xylinus* and *Ga. intermedius* strains produce besides the water-insoluble cellulose also a water-soluble polysaccharide called "acetan," a heteropolymer containing glucose, mannose, glucuronic acid and rhamnose in a molar ratio of 4: 1: 1: 1. Acetan formation seems to influence the degree of polymerization and crystallinity of the cellulose fibrils [55].

Production of exopolysaccharides especially cellulose from AAB seems to be a promising area of application because of the increasing need of pure cellulose in medical and engineering fields [18].

Conclusion

AAB are food-associated microorgansims that have a long history in oxidative fermentation processes, where they are spoiling or beneficial organisms. The physiological uniqueness of AAB is due to their ability to partially oxidize carbon sources and quantitatively excrete the corresponding compounds in the surrounding media. This feature, besides vinegar, is exploited for the industrial production of a number of compounds from alcohols, sugar and sugar alcohols oxidation. Moreover they are considered promising for the production of the pure form of cellulose. Although the

valuable potential of AAB in biotechnological applications, their industrial exploitation is not full developed.

Perspectives to enhance their role as biocatalysts include the availability of genetic stable strains and further advance on their metabolic potential, in order to obtain high carbon substrates conversion efficiency.

References

1. Sievers M, Swings J (2005) Family *Acetobacteraceae*. In: Garrity GM (ed) Bergey's Manual of Systematic Bacteriology, 2nd edn. Springer, New York, pp 41-95
2. Wu J, Gullo M, Chen F, Giudici P (2010) Diversity of *Acetobacter pasteurianus* strains isolated from solid-state fermentation of cereal vinegars. Curr Microbiol 60:280-286
3. Mamlouk D, Hidalgo C, Torija MJ, Gullo M (2011) Evaluation and optimization of bacterial genomic DNA extraction for no-culture techniques applied to vinegars. Food Microbiol 28:1374-1379
4. Gullo M, Giudici P (2008) Acetic acid bacteria in traditional balsamic vinegar: phenotypic traits relevant for starter cultures selection. Int J Food Microbiol 125(1):46-53
5. Pedraza RO (2008) Recent advances in nitrogen-fixing acetic acid bacteria. Int J Food Microbiol. 125(1):25-35
6. Kim EK, Kim SH, Nam HJ, Choi MK, Lee KA, Choi SH, Seo YY, You H, Kim B, Lee WJ (2012) Draft genome sequence of *Gluconobacter morbifer* G707T, a

pathogenic gut bacterium isolated from *Drosophila melanogaster* intestine. J Bacteriol 194(5):1245

7. Adachi O, Moonmangmee D, Toyama H, Yamada M, Shinagawa E, Matsushita K (2003) New developments in oxidative fermentation. Appl Microbiol Biot 60(6):643-653
8. Kersters K, Lisdiyanti P, Komagata K, Swings J (2006) The family *Acetobacteraceae*: the genera *Acetobacter, Acidomonas, Asaia, Gluconacetobacter, Gluconobacter and Kozakia*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt (ed) The Prokaryotes, 3rd edn. Springer-Verlag New York pp 163-200
9. Pasteur L (1864) Mémoire sur la fermentation acétique. Annales Scientifiques de l'E.N.S, Paris, 1, 113-158
10. Swings J, De Ley J (1977). The biology of *Zymomonas*. Bacteriol Rew 41:1-46
11. Camu N, De Winter T, Verbrugghe K, Cleenwerck I, Vandamme P, Takrama JS, Vancanneyt M, De Vuyst L (2007) Dynamics and Biodiversity of Populations of Lactic Acid Bacteria and Acetic Acid Bacteria Involved in Spontaneous Heap Fermentation of Cocoa Beans in Ghana. Appl Environ Microbiol 73(6):1809-1824.
12. Bartowsky EJ, Henschke PA (2008) Acetic acid bacteria spoilage of bottled red wine-a review. Int J Food Microbiol 125(1):60-70
13. Gullo M, Romano AD, Pulvirenti A, Giudici P (2003) *Candida humilis*-dominant species in sourdoughs for the production of durum wheat bran flour bread. Int J Food Microbiol 80(1):55-59

14. Greenfield S, Claus GW (1972) Nonfunctional tricarboxylic acid cycle and the mechanism of glutamate biosynthesis in *Acetobacter suboxydans.* J Bacteriol 112:1295-1301

15. Gupta A, Singh VK, Qazi GN, Kumar A (2001) *Gluconobacter oxydans*: Its Biotechnological Applications. J Mol Microbiol Biotechnol 3(3): 445-456

16. De Vero L, Gullo M, Giudici P (2010) Acetic Acid Bacteria, Biotechnological Applications In Flickinger MC (ed) Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation and Cell Technology, Wiley, New York, pp 9-25

17. Singh OV, Kumar R (2007) Biotechnological production of gluconic acid: future implications. Appl Microbiol Biotechnol 75(4):713-772

18. Czaja W, Young D, Kawecki M, Brown RM (2007) The future prospects of microbial cellulose in biomedical applications. Biomacromolecules 8:1-12

19. Saeki A, Theeragol G, Matsushita K, Toyama H, Lotong N, Adachi O (1997) Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at higher temperatures. Biosci Biotechnol Biochem 61:138-145

20. Ndoye B, Lebecque S, Dubois-Dauphin R, Tounkara L, Guiro AT, Kere C, Diawara B, Thonart P (2006) Thermoresistant properties of acetic acids bacteria isolated from tropical products of Sub-Saharan Africa and destined to industrial vinegar. Enzyme Microb Tech 39:916-923

21. De Ley J, Gillis M, Swings J (1984) Family VI. *Acetobacteraceae*. In: Krieg, NR Holt JG. (eds.) Bergey's Manual of Systematic Bacteriology, Baltimore, pp 267-278

22. Euzéby JP (1997) List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. Int J Syst Bacteriol 47:590-592. List of prokaryotic names with standing in nomenclature. http://www.bacterio.net

23. Ludwig W (2008) Nucleic acid techniques in bacterial systematics and identification. Int J Food Microbiol 125: I–XII

24. Cleenwerck I, de Vos P (2008) Polyphasic taxonomy of acetic acid bacteria: an overview of the currently applied methodology. Int J Food Microbiol 125(1):2-14

25. Gullo M, Mamlouk D, De Vero L, Giudici P (2012) *Acetobacter pasteurianus* strain AB0220: cultivability and phenotypic stability over 9 years of preservation. Curr Microbiol 64:576-580

26. Kondo K, Horinouchi S (1997) Characterization of the genes encoding the three-component membrane-bound alcohol dehydrogenase from *Gluconobacter suboxydans* and their expression in *Acetobacter pasteurianus.* Appl Environ Microbiol 63:1131-1138

27. Azuma Y, Hosoyama A, Matsutani M, Furuya N, Horikawa H, Harada T, Hirakawa H, Kuhara S, Matsushita K, Fujita N, Shirai M (2009) Whole-genome analyses reveal genetic instability of *Acetobacter pasteurianus*. Nucleic Acids Res 37(17):5768-5783

28. Coucheron DH (1991) An *Acetobacter xylinum* insertion sequence element associated with inactivation of cellulose production. J Bacteriol 173:5723-5731

29. Cleenwerck I, De Vos P, De Vuyst L (2010) Phylogeny and differentiation of species of the genus *Gluconacetobacter* and related taxa based on multilocus sequence analyses of housekeeping genes and reclassification of *Acetobacter*

xylinus subsp. sucrofermentans as *Gluconacetobacter sucrofermentans* (Toyosaki et al. 1996) sp. nov., comb. nov. Int J Syst Evol Microbiol 60(10):2277-2283

30. Matsutani M, Hirakawa H, Yakushi T, Matsushita K (2011) Genome-wide phylogenetic analysis of *Gluconobacter*, *Acetobacter,* and *Gluconacetobacter*. FEMS Microbiol Lett 315(2):122-1228

31. Kittelman M, Stamm WW, Follmann H, Truper HG (1989) Isolation and classification of acetic acid bacteria from high percentage vinegar fermentations. Appl Bicrobiol Biotech 30:47-52

32. Millet V, Lonvaud-Funel A (2000) The viable but non-culturable state of wine microorganisms during storage. Lett Appl Microbiol 30:136-141

33. De Vero L, Gala E, Gullo M, Solieri L, Landi S, Giudici P (2006) Application of denaturing gradient gel electrophoresis (DGGE) analysis to evaluate acetic acid bacteria in traditional balsamic vinegar. Food Microbiol 23:(8):809-813

34. Gullo M, De Vero L, Giudici P (2009) Succession of selected strains of *Acetobacter pasteurianus* and other acetic acid bacteria in traditional balsamic vinegar. Appl Environ Microbiol 75:2585-2589

35. Dupuy P (1952) Recherche d'une technique d'isolement des *Acetobacter* du vin. Ann Technol Agric 1:107-112

36. Drysdale GS, Fleet GH (1988) Acetic Acid Bacteria in Winemaking: A Review. Am J Enol Vitic 39(2):143-154

37. Carr JG, Passmore SM (1979) Methods for identifying acetic acid bacteria. In: Skinner FA, Lovelock DW (ed) Identification Methods for Microbiologists. Academic Press, UK, pp 33-47

38. Sievers M, Sellmer S, Teuber M (1992) *Acetobacter europaeus sp. nov*., a main component of industrial vinegar fermenters in central Europe. Syst Appl Microbiol 15:386-392

39. Entani E, Ohmori S, Masai H, Suzuki KI (1985) *Acetobacter polyoxogenes* sp. nov., a new species of an acetic acid bacterium useful for producing vinegar with high acidity. J Gen Appl Microbiol 31:475-490

40. Sokollek SJ, Hertel C, Hammes WP (1998) Cultivation and preservation of vinegar bacteria. J Biotechnol 60:195-206

41. Gullo M, Caggia C, De Vero L, Giudici P (2006) Characterization of acetic acid bacteria in "traditional balsamic vinegar. Int J Food Microbiol 106:209-212

42. Matsushita K, Toyama H, Adachi O (2004) Respiratory chains in acetic acid bacteria: membrane bound periplasmic sugar and alcohol respirations. In Zannoni D (ed) Respiration in Archaea and Bacteria, Advances in Photosynthesis and Respiration. Springer, Dordrecht, pp 81-99

43. Anthony C (1996) Quinoprotein-catalysed reactions. Biochem J 320:697-711

44. Goodwin PM, Anthony C (1998) The Biochemistry, Physiology and genetics of PQQ and PQQ-containing enzymes. Adv Microb Physiol 40:1-80

45. Yakushi T, Matsushita K (2010) Alcohol dehydrogenase of acetic acid bacteria: structure, mode of action, and applications in biotechnology. Appl Microbiol Biotechnol 86(5):1257-1265

46. Adachi O, Matsushita K, Shinagawa E, Ameyama M (1980) Crystallization and properties of NADP-dependent d-glucose dehydrogenase from *Gluconobacter suboxydans*. Agric Biol Chem 44:301-308

47. Muraoka H, Watabe Y, Ogasawara N, Takahashi H (1983) Trigger damage by oxygen deficiency to the acid production system during submerged acetic acid fermentation with *Acetobacter aceti*. J Ferment Technol 61:89-93

48. De Ley J, Gillis M, Swings J (1984). In: Krieg, N.R., Holt, J.G. (Eds.), Family VI. *Acetobacteraceae*, 1th Ed. Bergey's Manual of Systematic Bacteriology, Williams and Wilkins Co, Baltimore, pp 267-278

49. White GA, Wang CH (1964) The dissimilation of glucose and gluconate by *Acetobacter xylinum.* The origin and the fate of triose phosphate. Biochem J 90(2):408-423

50. Deppenmeier U, Ehrenreich A (2009) Physiology of acetic acid bacteria in light of the genome sequence of *Gluconobacter oxydans*. J Mol Microbiol Biotechnol 16: 69-80

51. Attwood M, van Dijken J P, Pronk JT (1991) Glucose metabolism and gluconic acid production by *Acetobacter diazotrophicus* J Ferment Bioeng 72(2):101-105

52. Klasen R, Bringer-Meyer S, Sahm H (1995) Biochemical characterization and sequence analysis of the gluconate: NADP 5-oxidoreductase gene from *Gluconobacter oxydans*. J Bacteriol 177(10):2637-2643

53. Roehr M, Kubicek CP, and Kominek J (1996) Gluconic acid. In: Roehr M (ed) Biotechnology, 2nd edm. VCH, New York, pp 348-362

54. Prust C, Hoffmeister M, Liesegang H, Wiezer A, Fricke WF, Ehrenreich A, Gottschalk G, Deppenmeier U (2005) Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat Biotechonol 23:195-200

55. Ross P, Mayer R and Benziman M (1991) Cellulose biosynthesis and function in Bacteria. Microbiol Mol Biol Rev 55:135-158

56. Yukphan P, Malimas T, Muramatsu Y, Potacharoen W, Tanasupawat S, Nakagawa Y, Tanticharoen M, Yamada Y (2011) *Neokomagataea* gen. nov., with descriptions of *Neokomagataea thailandica* sp. nov. and *Neokomagataea tanensis* sp. nov., osmotolerant acetic acid bacterium of the a *Proteobacteria*. Biosci Biotechnol Biochem 75(3):419-426

57. Yukphan P, Malimas T, Muramatsu Y, Takahashi M, Kaneyasu M, Potacharoen W, Tanasupawat S, Nakagawa Y, Hamana K, Tahara Y, Suzuki K, Tanticharoen M, Yamada Y (2009) *Ameyamaea chiangmaiensis* gen. nov., sp. nov., an acetic acid bacterium in the alpha proteobacteria. Biosci Biotechnol Biochem 73(10):2156-2162

Table

Table 1 Distinctive characteristics of acetic acid bacteria genera

Characteristic	*G.*	*A.*	*Ga.*	*Ac.*	*As.*	*K.*	*Sw.*	*Sa.*	*N.*	*Gr.*	*Am.*	*T.*	*Ne.*
Production of AcOH	+	+	+	+	−	+	+	v (w/−)	+	v (w/−)	+	−	−
Oxidation of:													
Acetate to CO_2 and H_2O	−	+	+	+	w	w	w	−	−	w	+	−	−
Lactacte to CO_2 and H_2O	−	+	+	v (−/w)	w	w	w		−	+	w	−	−
Growth on 0.35% AcOH (pH 3.5)	+	+	+	+	−	+	+	−	+	nd	+	+	−
Growth in the presence of 1% KNO_3	−	−	−	+	−	−	+	nd	−	nd	−	−	−
Production of keto-d-gluconic acid from d-glucose:													
2,5-diketo-d-gluconic acid	v	−	v	−	−	−	nd	nd	nd	nd	−	+	nd
5-keto-d-gluconic acid	+	v	v	−	+	+	nd	+	+	nd	+	+	nd
2-keto-d-gluconic acid	+	v	v	−	+	+	nd	+	+	nd	+	+	nd
Production of DHA from glycerol	+	v	v	−	v	+	+	−	w	−	w	+	−
Growth on methanol as carbon source	−	v	−	+	−	−	−	−	−	+	vw	−	nd
Production of water soluble brown pigment(s)	v	−	v	−	−	−	+	−	−	nd	−	+	−
Production of γ-pyrones from:													
d-glucose	v	−	v	nd	−	−	nd	nd	nd	nd	nd	nd	nd
d-fructose	+	−	−	nd	v (+/w)	v	nd	nd	nd	nd	nd	nd	nd
Acid production from:													
l-arabinose	+	v	v	+	+	+	+	+	+	nd	nd	nd	nd
d-arabinose	+	−	−	v	+	v	nd	−	w	nd	nd	nd	nd
d-xylose	+	v	v	+	+	+	v	+	+	−	nd	nd	nd
l-rhamnose	−	−	−	−	v	−	−	−	w	nd	nd	nd	nd
d-glucose	+	v	+	+	+	+	+	+	+	w	nd	nd	nd
d-galactose	+	v	+	+	+	+	+	+	+	nd	nd	nd	nd
d-mannose	+	v	v	+	+	+	+	+	+	nd	nd	nd	nd
d-fructose	+	−	+	−	+	−	v	v	+	nd	nd	nd	nd
l-sorbose	+	−	v	nd	+	−	nd	−	−	nd	nd	nd	nd
Melibiose	+	−	−	v	+	+	nd	+	+	nd	nd	nd	nd
Sucrose	+	−	−	−	+	v	nd	+	+	−	nd	nd	nd
Raffinose	−	−	−	−	−	+	nd	−	+	nd	−	w	−
d-mannitol	+	−	v	−	v	−	−	+	w	−	−	−	−
d-sorbitol	+	−	−	−	v	−	+	−	+	−	−	−	−

Dulcitol	−	−	−	−	v	−	v	−	w	−	−	−	−
Glycerol	+	−	+	+	+	+	+	−	+	v (w/−)	w	+	−
EtOH	+	+	+	+	−	+	+	−	+	+	+	+	−
Production of cellulose	−	−	v	−	−	−	nd	−	nd	nd	nd	nd	nd
Production of levan-like mucous substance(s) from sucrose	−	v	−	−	−	+	nd	−	−	nd	−	−	−
Growth in the presence of 30% d-glucose	−	v	v	+	+	−	nd	+	+	nd	−	+	+
Motility and flagellation	N-m (mostly) or po	N-m or pe	N-m or pe	N-m or pe	N-m or pe	N-m	pe	N-m	N-m	N-m	pe	N	N
Major ubiquinone	Q10	Q9	Q10	Q10	Q10	Q10	Q10	Q10	Q10	nd	Q10	Q10	Q10
G + C content (mol%)	54–64	52–64	56–67	62–63	59–61	56–57	57–60	52–53	63.1	59	66.0	65.6	56.8

Adapted from [24, 56, 57]. *G.*: *Gluconobacter*; *A.*: *Acetobacter*; *Ga.*: *Gluconacetobacter*; *Ac.*: *Acidomonas*; *As.*: *Asaia*; *K.*: *Kozakia*; *Sw.*: *Swaminathania*; *Sa.*: *Saccharibacter*; *N.*: *Neoasaia*; *Gr.*: *Granulibacter*; *Am.*: *Ameyamaea*; *T*: *Tanticharoenia*; *N.*: *Neokomagataea*. +: positive, −: negative, w: weak, v: variable, nd: not determined, N-m: non-motile, po: polar, pe: peritrichous.

Figure

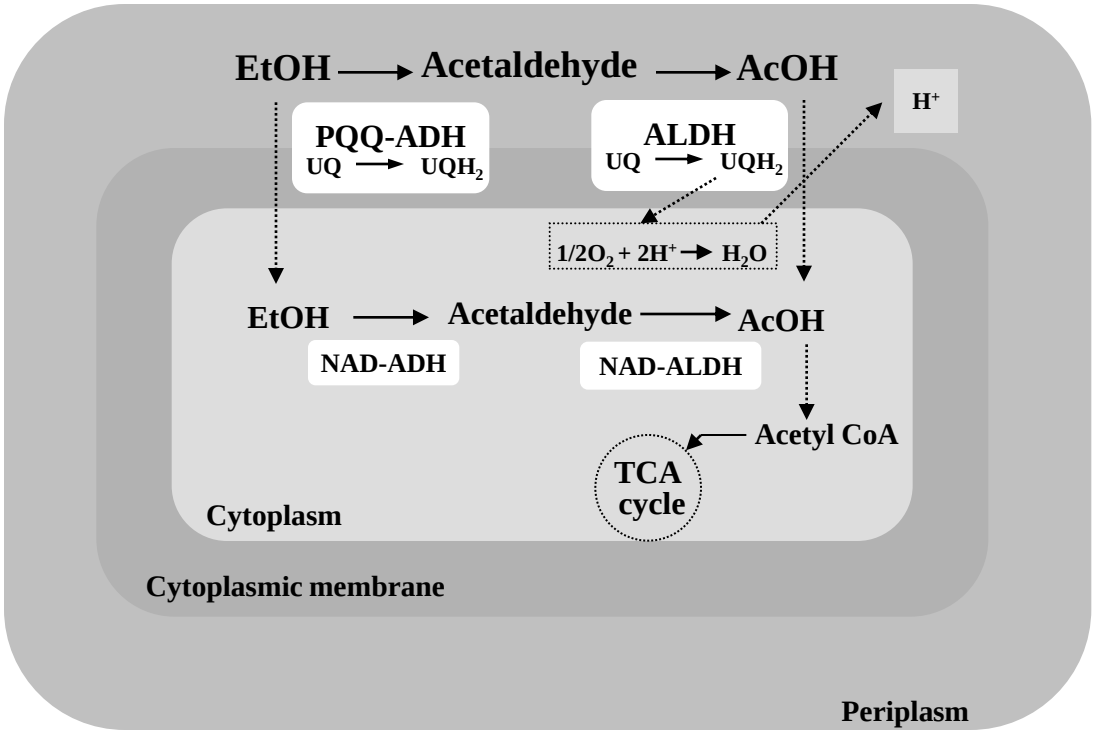

Fig.1 Ethanol oxidation by PQQ-ADH and ALDH at the outer surface of cytoplasmic membrane and by NAD-ADH and NAD-ALDH in the cytoplasm

Answers to Reviewers

Thank you very much for all your comments and suggestions, stronghly contributed to improve the manuscript. Below a detailed response for each point raised out is provided.

Reviewer #1:
This review describes acetic acid bacteria focusing on their metabolism. It is a concise overview of the acetic acid bacteria involved in food fermentation, particularly vinegar production.
A few things that should be considered to improve the review:

- Emphasis on recent taxonomy (a lot of research has been done on AAB taxonomy in the last 5-10 years)
- Figure would be good (eg reaction of ethanol to acetic acid with the various enzymes)
- Biotechnology needs to be expanded more
- VBNC needs to be brought out more in the cultivation section
- English expression and language should be checked

We have fully considered the criticisms raised out by the rewiever and accordingly the manuscript was revised.

Recent taxonomy has been added to the paragraph "General characteristics" that in this new version of the manuscript has been renamed as follow:
"General phenotypic characteristics and taxonomic aspects of acetic acid bacteria"

As suggested, a figure (Fig.1) showing ethanol oxidation by ADH and ALDH and location within the cell has been added.

Biotechnological applications of AAB has been expanded more (p. 2 lines 1-23).

As suggested, the paragraph "Isolation and cultivability" was modified and VBNC concept added (p.5, lines 11-15)

The whole manuscript has been revised and a thoroughly English revision has been done.

Specific comments:
Abstract
- use biotechnology rather than biotech
The term was corrected

P3, L7 - assigned rather than allocated
Allocated has been replaced by "assigned" (p.3, line 13), as suggested.

P3, L12 & 14 - write ethanol (EtOH) or acetic acid (AcOH) and then use abbreviation
The correction has been done.

P3, L23 - surface layer
Superficial has been replaced by "surface" (p. 1, line 14)

P4, L1 - acetic acid production is not a fermentation
The sentence was corrected:
AcOH fermentation was replaced by " AcOH production"

P4, L5 - AAB are involved in these other food productions, not only implicated
Implicated was replaced by involved, as suggested.

Page 5 - what is the significance of 1996 in regards to these various foods and the bacteria isolations?
The whole sentence was removed.

Reference is needed for 'Early researchers....'
The reference was added [35] : Dupuy P (1952) Recherche d'une technique d'isolement des Acetobacter du vin. Ann Technol Agric 1:107-112 (p.5. L.19)

Page 6 L 10-15 - this section a few more sentences to explain these statements better.

Lines 14-15: why the difference? Explain the significance between the two versions.

To clarify, we removed the second part of the sentence: "Whereas previous edition of Bergey's Manual [25] recommended the use of a simple medium containing 0.5% yeast extract, 1.5% EtOH, and 2.5% agar."
In the new version of the manuscript, the sentence reports only the medium of the last version of bergey's manual (p. 8, L: 5-9) "The last edition of Bergey's Manual of Systematic Bacteriology [1] proposed standard medium for enrichment and isolation of AAB, with the exception of *Ga. europaeus* (which strains are AcOH-dependent), containing: D-glucose, 0.05%; EtOH, 1.5%; yeast extract, 0.5%; peptone, 0.3%; cycloheximide, 0.01%; and agar, 0.12%."

Page 7 L 6-11 - this whole section needs a bit of rearranging. These lines should be at the start of the section

The paragraph "Isolation and cultivation" was rearranged. The sentence was simplified and put at the start of paragraph "Isolation and cultivation of AAB strains, especially from fermented beverages, have been described to be problematic, giving rise to an underestimation of species richness when culture dependent methods are applied [31]".

Lines 6-14 - this needs to expanded to explain these observations and statements. It is suggesting that now all bacteria are growing up/being cultivated. Is this viable but not culturable (VBNC). Or poor growth medium? Highlight the difficulties in cultivating AAB and all the AAB present.

The section was rearranged as follow:

p. 5, lines 11-17 " Low recovery of strains due to the fraction of population that could reach a viable but non-culturable (VBNC) state has been stated. For instance in wine it was shown that VBNC status of spoiling AAB is induced by O_2 deprivation [32]. Whereas in vinegars some studies developed by no culture approaches, such as PCR/DGGE, revealed higher species diversity respect to that detected by culturing methods [33, 34, 3].
Limitations of culturing were in part overcame with the formulation of appropriate media, which allowed the cultivation of no growing strains or slow growing strains."

Page 8 L 1-8 : it would be a good idea to have a figure here for the reaction and enzymes, their location .... . It is always beneficial in a review to have a figure.

As suggested, a figure (Fig.1) showing ethanol conversion and location of enzymes within the cell has been added. The figure rapresents the ethanol oxidation to acetic acid by the membrane-bound ADH and ALDH and cytoplasmic respiration of ethanol.

P9 L 15 - ... were present in the strain. .... The genes were present, but was it shown that the pathways were functioning. Genomics show that genes are present but do not confirm that reactions are occurring.

The sentence was removed.

P 10 L 7 - High in what sense? Concentration? Structure?
P 10 L 9 - ..... wide range of applications. ..... This does not make sense or belong here.
To avoid repetition the sentence (p.10, L.6-9) was removed.
The sentence reported in the new version of manuscript is at page 9, lines: 14-18
"The most characteristic reaction is the direct oxidation of glucose into Glucono-δ-lactone, which is oxidized into gluconic acid. This reaction is particularly active in *Gluconobacter* growing at high concentrations of sugars. D-gluconate can be further oxidized to 2-ketogluconate and 2,5-diketogluconate by the gluconate dehydrogenase and 2-ketogluconate dehydrogenase [52]."

P 10 L 14 - last sentence Is this important for high production of vitamin C?

The sentence was removed.

P 11 L 1-5 - another sentence or two to expand this concept would be beneficial for the reader.
To clarify the sentence was rewritten as follow (p. 10, Lines 14-17):
"In *G. oxydans* it was reported that oxidization of glycerol is catalized by the membrane-bound glycerol/sorbitol dehydrogenase. This quinoprotein is considered the main polyol-dehydrogenase of *G. oxydans* that exhibits a broad substrate specificity. It catalyzes the oxidation of D-sorbitol, gluconate, and glycerol to L-sorbose, 5 ketogluconate and DHA, respectively [54]. "

P 13 L 6-7: this sentence needs to be reworded and explained better. What has been reached?

P 13 L 11 - sugared is not a good word here. Rewrite using high sugar content.

In the new Conclusion section, the sentences were rewritten as follow:

"AAB are food-associated microorgansims that have a long history in oxidative fermentation processes, where they are spoiling or beneficial organisms. The physiological uniqueness of AAB is due to their ability to partially oxidize carbon sources and quantitatively excrete the corresponding compounds in the surrounding media. This feature, besides vinegar, is exploited for the industrial production of a number of compounds from alcohols, sugar and sugar alcohols oxidation. Moreover they are considered promising for the production of the pure form of cellulose. Although the valuable potential of AAB in biotechnological applications, their industrial exploitation is not full developed.

Perspectives to enhance their role as biocatalysts include the availability of genetic stable strains and further advance on their metabolic potential, in order to obtain high carbon substrates conversion efficiency."

Table 1.
In the legend write AAB in full. At the top of the table, it would be much better to have at least abbreviations of the genera rather than numbers. It would make it much easier for the reader.

AAB was replaced by acetic acid bacteria, as suggested.
Number were replaced by abbreviations.

*Copyright Transfer Form

Indian Journal of Microbiology

Association of Microbiologists of India

Copyright Transfer and Financial Disclosure/Conflict of Interest Statement

The copyright to this article is transferred to the Association of Microbiologists of India (for U.S. government employees: to the extent transferable) effective if and when the article is accepted for publication. The author warrants that his/her contribution is original and that he/she has full power to make this grant. The author signs for and accepts responsibility for releasing this material on behalf of any and all co-authors. The copyright transfer covers the exclusive right to reproduce and distribute the article, including reprints, translations, photographic reproductions, microform, electronic form (offline, online) or any other reproductions of similar nature.

An author may self-archive an author-created version of his/her article on his/her own website and or in his/her institutional repository. He/she may also deposit this version on his/her funder's or funder's designated repository at the funder's request or as a result of a legal obligation, provided it is not made publicly available until 12 months after official publication. He/she may not use the publisher's PDF version, which is posted on www.springerlink.com, for the purpose of self-archiving or deposit. Furthermore, the author may only post his/her version provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The original publication is available at www.springerlink.com".

The author is requested to use the appropriate DOI for the article. Articles disseminated via www.springerlink.com are indexed, abstracted and referenced by many abstracting and information services, bibliographic networks, subscription agencies, library networks, and consortia. After submission of the agreement signed by the corresponding author, changes of authorship or in the order of the authors listed will not be accepted.

I, the undersigned corresponding author, also certify that I/we have no commercial associations (e.g., consultancies, stock ownership, equity interests, patent-licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted article, except as disclosed on a separate attachment. All funding sources supporting the work and all institutional or corporate affiliations of mine/ours are acknowledged in a footnote. *Please mention if a separate attachment is enclosed.*

Title of the article

ACETIC ACID BACTERIA : PHYSIOLOGY AND CARBON SOURCES OXIDATION

Author (s)

NHOUHA HAITOUK MARIA GULLO

Author(s)' signature (s)

Marie Pello

Date

23/12/2012

Please sign this form, scan and upload to:
www.editorialmanager.com/injm while submitting your manuscript.