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Title: Aerobic submerged fermentation by acetic acid bacteria for vinegar production: process and biotechnological aspects

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Abstract: Strictly aerobic acetic acid bacteria (AAB) have a long history of use in fermentation processes, and the conversion of ethanol to acetic acid for the production of vinegar is the most well-known application.
At the industrial scale, vinegar is mainly produced by submerged fermentation, which refers to an aerobic process in which the ethanol in beverages such as spirits, wine or cider is oxidized to acetic acid by AAB. Submerged fermentation requires robust AAB strains that are able to oxidize ethanol under selective conditions to produce high-titer acetic acid. Currently submerged fermentation is conducted by unselected AAB cultures, which are derived from previous acetification stocks and maintained by repeated cultivation cycles.
In this work, submerged fermentation for vinegar production is discussed with regard to advances in process optimization and parameters (oxygen availability, acetic acid content and temperature) that influence AAB activity. Furthermore, the potential impact arising from the use of selected AAB is described.
Overcoming the acetification constraints is a main goal in order to facilitate innovation in submerged fermentation and to create new industry-challenging perspectives.

UNIVERSITÀ DEGLI STUDI
DI MODENA E REGGIO EMILIA

Reggio Emilia,19/06/2014

Dear Professor Joseph Boudrant,
Please, find enclosed the revised version of the manuscript Ref. No.: PRBI-D-14-00238R1 entitled "*Aerobic submerged fermentation by acetic acid bacteria for vinegar production: process and biotechnological aspects*" submitted by authors Maria Gullo, Elena Verzelloni and Matteo Canonico.
The manuscript was full revised according to suggestions provided by reviewers.
A detailed point by point response to the issues raised by the reviewers is included.

Concerning the question raised by the reviewers on the use of the term "total concentration", I would like to elucidate that this term expresses the maximal concentration of acetic acid that can be obtained by complete ethanol fermentation. Moreover since the quotient of the total vinegar concentration produced over the total mash concentration indicates the production yield (%), total concentration is commonly used both in the industry and in researches dealing with acetic acid fermentation. This issue was detailed in the manuscript (par. 2. Aerobic submerged fermentation). Moreover, in addition to values of total concentration, single concentrations of ethanol and acetic acid were provided, if available from the literature. For reviewers guidance, some references (quoted on the manuscript) have been listed also in the response letter.

I confirm that all authors agree to resubmit the manuscript to *Process Biochemistry*. We declare that this manuscript has not been published or submitted or being submitted to another journal.
I thank you very much for your cooperation and guidance.

Sincerely,

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Title: Aerobic submerged fermentation by acetic acid bacteria for vinegar production: process and biotechnological aspects
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Reviewers' comments

Reviewer #3: This manuscript had been revised according to the reviewers' suggestions. However, there are still lots of mistakes, which make it difficult to understand. These are listed below:

1.In abstract, page 2, line 39-40, what is the meaning "the potential impacts arising from is selected starter are described"?

The sentence was wrongly modified in the previous revision, it is now corrected as follow:

"Furthermore, the potential impact arising from the use of selected AAB is described."

2.page 6, line 135-136, I am wondering if the words "However, very few improvements have been introduced at the industrial level for the use of AAB as effective cultures." have the logistic relation in the paragraph?

Lines 135-136 of the manuscript refer to the sentence: "Current industrial vinegar production has robust process control tools and strategies to conduct fermentation". As suggested (lines 135-136) are not consistent with the paragraph.
To clarify, we removed the sentence (Lines 135-136) and we modified the next sentence.
The revised sentence is:

"The difficulty of cultivating AAB is one of the reasons why vinegar fermentation is still performed using unselected cultures".

3.page 9-10, the paragraphs about acetic acid bacteria oxidation have no sense. It is not necessary to write individually.

We agree with this observation, so the paragraph 2.2. "Acetic acid bacteria oxidation" was removed. To clarify, we integrated the content of this paragraph within paragraph 2. "Aerobic submerged fermentation".

4.page 12, line 275-286, these paragraphs are too short and difficult to follow.

As suggested, these sentences were modified.

"AAB are obligate aerobic bacteria, and oxygen deprivation during SF causes a rapid loss of productivity. During SF, the level of oxygen consumption is directly related to the substrate-to-product conversion and it is linked to the AAB growth phase. Therefore, during the lag phase, the quantity of consumed oxygen and the acetic acid produced is low; during the exponential growth phase, the oxygen consumption is high and is proportional to a high production rate of acetic acid [48,49]. It has been stated that the effect of oxygen deprivation is directly proportional to the total

concentration, the acetic acid concentration, the rate of fermentation and the length of the interruption of aeration".

5.page 12, line 287, what is the meaning of the total concentration?

Total concentration is defined as:
"the sum of ethanol (ml per 100 ml) and acetic acid (g per 100 ml) is called 'total concentration' because it expresses the maximal concentration of acetic acid that can be obtained by complete fermentation. Ref.: [10].

Below some examples of literature elucidating the use of the total concentration is provided:

- Hromatka O, Ebner H. Vinegar by submerged Oxidative Fermentation. Ind Eng Chem 1959;51:1279-1280. . Ref.: [37]
- Ebner H, Sellmer S, Follmann H. Acetic acid. In: Rehm HJ, Reed, G editors. Biotechnology. Weinheim: Wiley-VCH; 1996 Vol 6, p. 381-401. Ref.: [10]
Rubio-Fernández et al., 2004. Ref.: [58]
Fregapane et al 2001. Ref.: [82]

Page 13, line 319-320, the unit of rate is mg/L? line 325, 5,76 mg/L?

Yes, we expressed oxygen consumption in mg/L. Where necessary ppm have been converted into mg/L.

6.page 15, line 371, there is no section of 5.2, 5.3, etc?

Since the paragraph 5.1 was too long, we decided to divide it in 2 parts. The new paragraph 5.2. "Acetic acid resistance and species" describes the behavior of different species in ethanol/acetate media.

7.page 16, line 382-387, these paragraphs are too short and difficult to follow.

To clarify, these sentences were modified as follow:

"Therefore the effect of acetic acid on AAB growth is a function of the concentrations of substrate (ethanol) and product (acetic acid) and of the growth conditions. For instance in shaking ethanol cultures of *Acetobacter* strains, it was found that 10 g/L of acetic acid has an activating effect on growth, and lower concentrations resulted in a significant decrease in the logarithmic growth phase [71].
Activation and inhibition effects on AAB growth as a function of the total concentration have been observed during the start-up phase in SF. In these conditions a total concentration of approximately 8% (ethanol between 35.5 and 47 g/L and acetic acid between 30 and 45 g/L) was determined to be optimal for a wine vinegar start-up, using an AAB culture of the prevailing *A. aceti* cells [48]".

Reviewer #4: The authors have substantially improved the review by considering the points raised by the reviewers.

The tables still require improvement.
Table 1. Title - ... total concentration of ethanol and acetic acid.
- Is it usual to refer to the total concentration do ethanol and acetic acid? It seems a bit odd since they are very different compounds.

As suggested the title of Table 1 was modified.
Table 1. Main acetic acid bacteria species in vinegars produced by different systems and their resistance to ethanol and acetic acid (expressed as total concentration).

As you commented it seems not correct to refer to concentration as the sum of ethanol (vol%) and acetic acid (g per 100 ml). However the "total concentration" is a commonly used calculation.

Definition of total concentration: "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". Ref.: [10]

Below some examples of literature elucidating the use of the total concentration is provided:
- Hromatka O, Ebner H. Vinegar by submerged Oxidative Fermentation. Ind Eng Chem 1959;51:1279-1280. . Ref.: [37]
- Ebner H, Sellmer S, Follmann H. Acetic acid. In: Rehm HJ, Reed, G editors. Biotechnology. Weinheim: Wiley-VCH; 1996 Vol 6, p. 381-401. Ref.: [10]
Rubio-Fernández et al., 2004. Ref.: [58]
Fregapane et al 2001. Ref.: [82]

Highlights

- Unselected acetic acid bacteria are applied to produce vinegar
- Submerged fermentation provides the highest acetic acid titer
- Robust industrial strains must be resistant to acetic acid and ethanol
- Overcoming acetification constraints by selected acetic acid bacteria

Aerobic submerged fermentation by acetic acid bacteria for vinegar production: process and biotechnological aspects

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Contributors

M.G. conceived of the study and its design, coordinated the study and helped to draft the manuscript. E.V. drafted and reviewed the manuscript. M.C. drafted the introduction and tables. M.G. and E.V. drafted figures in close collaboration. All authors read and approved the final manuscript.

Abstract

Strictly aerobic acetic acid bacteria (AAB) have a long history of use in fermentation processes, and the conversion of ethanol to acetic acid for the production of vinegar is the most well-known application.

At the industrial scale, vinegar is mainly produced by submerged fermentation, which refers to an aerobic process in which the ethanol in beverages such as spirits, wine or cider is oxidized to acetic acid by AAB. Submerged fermentation requires robust AAB strains that are able to oxidize ethanol under selective conditions to produce high-titer acetic acid. Currently submerged fermentation is conducted by unselected AAB cultures, which are derived from previous acetification stocks and maintained by repeated cultivation cycles.

In this work, submerged fermentation for vinegar production is discussed with regard to advances in process optimization and parameters (oxygen availability, acetic acid content and temperature) that influence AAB activity. Furthermore, the potential impact arising from the use of selected AAB is described.

Overcoming the acetification constraints is a main goal in order to facilitate innovation in submerged fermentation and to create new industry-challenging perspectives.

Keywords: acetic acid bacteria, *Gluconacetobacter*, *Acetobacter*, submerged fermentation, acetic acid resistance

Contents

1. Introduction

Acetic acid bacteria (AAB) are strict aerobes that belong to *Alphaproteobacteria* and have the ability to partially oxidize carbon sources into a corresponding organic compound, such as ethanol to acetic acid [1,2]. This feature makes them valuable biocatalysts for a number of useful applications, but at the same time AAB are also spoiling organisms in some fermentation processes [3].

Acetic acid is the primary metabolite of AAB and is produced from the bioconversion of ethanol through two reactions catalyzed by the membrane-bound pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). ADH oxidizes the ethanol to acetaldehyde, which is then converted to acetic acid by ALDH and released into the surrounding environment. These two dehydrogenase complexes are strictly connected to the respiratory chain, which transfers electrons through ubiquinone (UQ) to oxygen, which acts as the final electron acceptor. The acetic acid produced by the partial oxidation of ethanol can be further oxidized in the cytoplasm by a set of soluble $NaD(P)^+$-dependent dehydrogenases (ADH and ALDH) via the tricarboxylic acid cycle, resulting in so-called acetate oxidation (overoxidation) [4].

Acetic acid is the main component in vinegar and is also recognized as an effective antimicrobial compound that prevents the growth of pathogenic and spoilage organisms in fermented foods; it also causes spoiling in beverages such as wine, in which it is detrimental even at concentrations as low as 1.2-1.4 g/L [5].

Food-grade vinegar, which is used worldwide as a preservative and condiment for food [6], is a diluted solution of acetic acid and is produced through a microbial oxidation carried out by AAB [7]. In addition, vinegar has been demonstrated to possess healthful properties [8].

Vinegar brewing can be performed by two main systems. The first system is solid-state fermentation (SSF), which uses microorganisms grown on substrates in the absence of free water; this system is used to produce vinegar from grains in Asian countries. SSF includes three main biological steps: starch liquefaction and saccharification, alcohol fermentation and acetic acid fermentation [9]. The second system is liquid fermentation, which comprises a set of techniques developed in Western and European countries. Among these techniques, the submerged system is used to produce vinegar at industrial scale [10] (Fig. 1).

A submerged system has several advantages over other techniques (e.g. SSF and surface fermentation), including high yield and process speed. Over the last few decades, many studies have examined process variables (oxygen availability, temperature, acetic acid and ethanol content), and a number of strategies for process control have been developed. As a result, acetic acid fermentation systems and the modern vinegar industry benefit from robust processes and optimization tools [11-15].

Major studies have also been conducted to examine the prevalent microflora, in order to determine the role of AAB in vinegar fermentation [16-24]. Differences in the species detected correlate with the selective pressure exerted by the acetic acid concentration of collection sites. In particular, highly acidic vinegar environments (acetic acid > 6% (w/v)) favor the prevalence of *Gluconacetobacter* species, whose ADH shows a higher stability in high acetic acid content; in low acidity vinegars (acetic acid concentration $\leq$ 6% (w/v)) *Acetobacter* species are dominant, although *Gluconacetobacter* has also been found [25-27]. Although the aforementioned studies provided a good understanding of the ecophysiology of AAB in acidic niches, very little literature is available on the functionality of AAB in submerged processes relating to process parameters. The reasons for this lack of information can be mainly attributed to the difficulty of handling of AAB, resulting often in

slow growing cultures, especially those derived from highly acidic vinegars.

In addition, it is well known that a large fraction of microorganisms present in both natural and industrial environments are uncultivable under standard laboratory conditions. Environments in which viable but not cultivable microorganisms have been found include soil [28], activated-sludge process for waste-water treatment [29], clinical samples exhibiting mixed communities of biofilm-forming bacteria [30], vinegars [31] and paper mill [32]. The uncultivability phenomenon limits the understanding of species richness and diversity of these environments and consequently a broad-spectrum strategy to select efficient strains as starter culture is affected.

The difficulty of cultivating AAB is one of the reasons why vinegar fermentation is still performed using unselected cultures.

Vinegar consumption has been increasing yearly worldwide [33], and understanding the microbial composition and activity of AAB in submerged conditions can result in further processes optimization, positively impacting production yield. Moreover, consumer demand for high added-value products, including fermented and low sour beverages indicates potential applications for novel and functional starter cultures.

The present review aims to outline the main features of the aerobic submerged process for vinegar production at the industrial scale and to overcome acetification constraints in order to further enhance processes optimization.

2. Aerobic submerged fermentation

AAB are exploited for the commercial production of a variety of biomolecules including dihydroxyacetone [34], 2-keto-L-gulonic acid, D-sorbitol [35], gluconic acid [36], using submerged fermentation (SF) processes.

SF for vinegar production is an aerobic process by which the ethanol in liquids such as spirits, wine or cider is oxidized to acetic acid by AAB, in controlled stirring conditions [37].

The acetic acid fermentation proceeds according to the basic stoichiometric equation $C_2H_5OH + O_2 \rightarrow CH_3COOH + H_2O$; ($\Delta G° = -455$ kJ/mol).

The sum of ethanol (ml per 100 ml) and acetic acid (g per 100 ml) is called 'total concentration' because it expresses the maximal concentration of acetic acid that can be obtained by complete fermentation. This calculation, commonly used in the vinegar industry [10], is based on the fact that, according to the above equation, a 1 molar ethanol solution (4.6 g/100 ml) produces 6 g of acetic acid in 102 ml. 4.6 g/100 ml of ethanol corresponds (in volume) to 5.8 ml ethanol/100 ml; 6 g/102 ml of acetic acid are equivalent to 5.9 g of acetic acid per 100 ml.

The quotient of the total vinegar concentration produced over the total mash concentration indicates the yield (%) and expresses the relation between the input total concentration and the output total concentration of the mash [10]. Theoretically, the total concentration should remain constant throughout the process. Because ethanol is also a carbon source for the synthesis of cellular constituents, it can be depleted at the cytoplasmic level and it can be partially lost by evaporation, so the actual yield is lower than the theoretical one [38].

The basic requirements for submerged processes are the availability of suitable alcoholic stocks, uninterrupted aeration and AAB strains that tolerate high concentrations of acetic acid and ethanol, that are not sensitive to phage infections and that require small quantities of nutrients, to produce high amounts of acetic acid [10].

One of the most important features of the bioreactors used in these processes is the aeration system. This system consists of a hollow body turbine supported by a non-rotating stator. The turbine sucks air from the outside and releases it into radial holes that

open in the opposite direction of rotation; the action of turbines results in very fine air bubbles and homogenous air-liquid dispersion. The air-liquid emulsion is pushed upwards and diverted by deflectors. All of the mass is maintained in a constant state of agitation to prevent the formation of low oxygen tension areas, which are unfavorable for the metabolic activity of AAB [39,40].

The oxygen transfer is influenced by a high number of parameters including physical properties of gas and liquid, operational conditions and geometrical parameters of the bioreactor. Therefore, the oxygen transfer rate depends on stirring since it facilitates the disruption of large bubbles into smaller ones, on the surface tension of the solution, on the viscosity and on the fraction of gas retained in the bioreactor. A detailed description of the oxygen transfer rate and parameters that affect its efficiency in aerobic SF can be found in [41,42].

In stirred conditions, the liquid produces foam, which can lead to the formation of a reducing environment and compromise the acetification process. Generally, bioreactors are equipped with a mechanical skimmer, consisting of a rotating chamber with radial wings that turn very quickly; the foam entering into the chamber is centrifuged. The exhausted gases are eliminated from an upper opening, while the foam is connected to the exhaust duct [39].

The heat generation during SF is unavoidable because acetic acid fermentation is an exothermic reaction, producing approximately 8.4 MJ for every liter of oxidized ethanol [39]. Moreover, during charge of substrate and discharge of product, the temperature can vary greatly. Fermentation breakdown due to temperature variation is generally avoided by heating and cooling systems.

2.1 Fermentation mode

SF at the industrial scale is primarily performed in a semi-continuous mode (a repeated fed-batch process). In this operation mode, alcoholic substrates are added after the start of the acetification and then are added intermittently, depending on consumption.

Semi-continuous operation is reported to be the most advantageous for vinegar production, partly because it reduces the risk of substrate inhibition and catabolite repression. Moreover, it allows for the reuse of the acetifying culture in the subsequent cycle and to obtain products with a wide range of both acetic acid and ethanol concentrations [10,11] (Fig. 2). Other fermentation modes have been evaluated, especially for the production of high-titer acetic acid, which is one of the main demands of vinegar industry; so far, the highest yields are obtained by using the semi-continuous mode.

For example, in using continuous mode, a maximum of 9-10% (w/v) of acetic acid concentration is reached because the specific growth rate of AAB decreases at low ethanol concentration and a high acetic acid content. Previous comparative studies showed that in continuous culture at a total concentration of 12%, the specific growth decreased from 0.027 h^{-1} at 4.5% (v/v) ethanol to 0.006 h^{-1} at 1% (v/v) ethanol.

However, no decrease of the specific growth rate was observed by increasing the acetic acid concentrations in semi-continuous fermentation [43]. Further experiments have confirmed this behavior in both continuous and semi-continuous conditions [11].

The simplest semi-continuous operation mode is performed by two bioreactors arranged in series. The first bioreactor contains the inoculum derived from a previous cycle, to which wine or other alcoholic liquids (12-15% (v/v) ethanol, 1-2% (w/v) acetic acid) are added. When the acetifying mass reaches an ethanol content of approximately 2-3% (v/v), it is pumped into the second bioreactor where it will remain until the ethanol is depleted (0.2-0.3% (v/v)) and the required acetic acid content is achieved [10].

The duration of a fermentation cycle is between 18 and 30 hours. The length mainly depends on the initial concentration of ethanol, the efficiency of the aeration system and the duration of the bacterial lag phase. For wine, intervals of 24 hours for each cycle were optimal to obtain high acetic acid concentrations; shorter intervals provided higher acetification rates but significantly lower acetic acid concentrations. Intervals of more than 30 hours between each loading step correlate with instability of cycles [44].

3. Acetic acid bacteria in submerged fermentation

According to previous studies, the indigenous bacterial population during SF for vinegar production appears quite homogeneous, as it is mostly composed of the genus *Gluconacetobacter* and, in some cases, *Acetobacte*r. Moreover, the majority of studies identify the establishment of a single strain or only a few strains of the same species, suggesting the formation of a stable environment that exerts a strong selective pressure, due mainly to the presence of acetic acid [16-18].

However, a greater degree of heterogeneity has been observed in indigenous strains of cider vinegar produced by SF, when compared to strains of wine or spirit vinegars (Table 1). Reasons for this phenomenon could include differences in raw materials, processes or technical conditions during fermentation.

Cider vinegars display a wide range of acetic acid content (from 3.9 to 9.0% (w/v)), whereas wine and spirit vinegars generally have higher and restricted acetic acid content, ranging from 4.4-8.0% (w/v) and 11.5-12.2% (w/v), respectively [39]. The less stringent conditions of acetic acid concentration during SF could be responsible for the wider diversity of species detected in cider vinegars.

The relevance of the *Gluconacetobacter* genus in SF was first observed by Sievers and

co-workers [18], who identified *Ga. europaeus* (now *Komagataeibacter europaeus)* [45] as the main component of the microflora in industrial bioreactors. Some unique growth conditions for this species are: ability to grow at pH 2.5, an acetic acid concentration of 10-14% (w/v), a constant oxygen supply and the requirement of acetic acid. However, *Ga. europaeus* has also frequently been isolated from low acidity vinegars, during processing steps with constant acetic acid content [27,31].

A. pasteurianus and *A. aceti*, whose strains have a strong oxidative activity against ethanol, are mostly found as indigenous organisms in low acidity vinegars (~6%) [9,22,31,46]. In contrast, strains of *Ga. xylinus* species that are able to produce suitable amounts of acetic acid can have an opposing role in vinegar production due to their ability to synthesize cellulose, thus potentially causing drawbacks. However, in surface fermentation *Ga. xylinus* is reported to have a high acetic acid productivity; this is most likely because the structure of the cellulose membrane network supports cells close to the air-liquid interface, facilitating oxygen uptake. Conversely, in shake conditions, they exhibited slowed growth and lower substrate consumption [47].

4. Oxygen availability in submerged fermentation

AAB are obligate aerobic bacteria, and oxygen deprivation during SF causes a rapid loss of productivity.

During SF, the level of oxygen consumption is directly related to the substrate-to-product conversion and it is linked to the AAB growth phase. Therefore, during the lag phase, the quantity of consumed oxygen and the acetic acid produced is low; during the exponential growth phase, the oxygen consumption is high and is proportional to a high production rate of acetic acid [48,49]. It has been stated that the effect of oxygen deprivation is directly

proportional to the total concentration, the acetic acid concentration, the rate of fermentation and the length of the interruption of aeration. Previous experiments conducted on *A. aceti* under oxygen-deficient conditions, showed a strict correlation between the total concentration and cell damage. In particular, decreases in ethanol oxidation as well as of ADH and ALDH activities (20 and 50%, respectively) were observed after interruption of the oxygen supply. Moreover, enzymatic damage increased with increasing acidity; at an acetic acid content of greater than 4% (w/v) and ethanol lower than 4.9% (v/v), ADH and ALDH lost 60% and more than 90% of their enzymatic activity in crude preparation, respectively [50,51].

In submerged conditions of mixed AAB culture, it was noted that at a total concentration of 5%, an interruption of aeration for 2-8 minutes had the same effect as an interruption for 15-60 seconds when the total concentration was 10-12% [10], whereas in SF of a culture of A. *aceti*, an interruption of aeration for 10 s at 6% (w/v) acetic acid caused a total inhibition of acetic acid production [51].

Oxygen deprivation can be harmful not only during SF but also during the transfer of cultures from the precultivation flasks to the acetators and during any transfer from one acetator to another. To reduce bacteria death and to maintain a high respiration activity throughout the whole fermentation process, a so-called RAMOS (respiration activity monitoring system) device, that ensures a constant oxygen supply, was recently proposed [52]. With this method, cultures drained off from bioreactors can be transferred into an aerated bubble column and transported without an interruption of the oxygen supply. When comparing bacteria death and respiration activity, a higher number of living cells were present in cultures transferred by the RAMOS device than those managed with traditional procedures.

Several investigations have been conducted to evaluate the ability of AAB to grow with

limited oxygen concentrations [53,54]. The earliest research was performed using wine, where AAB causes spoilage with very low concentrations of dissolved oxygen. AAB were found in bottled wines where oxygen in the headspace was enough for growth as well as in wine stored in barrels, where oxygen permeates through the wood at rate of 30 mg/L per year, which is sufficient for AAB survival [55].

However, relatively little information is available regarding the optimal oxygen levels required by AAB when performing bioprocesses. In SF, it was found that during continuous culturing of *A. aceti* in the exponential phase, the optimal rate of oxygen consumption is about 1 mg/L, which corresponds to an acetic acid production of 45 g/L [56].

Similar results (2 mg/L of dissolved oxygen) have been found in semi-continuous mode using *A. aceti,* whereas the same culture in batch mode required less oxygen (0.7 mg/L of dissolved oxygen) [49,57]. Recent studies on a *Ga. xylinus* strain from rice vinegar confirmed the same behavior, with a greater concentration of dissolved oxygen present (5,76 mg/L) in shaking flask fermentation than in surface fermentation (0.3 mg/L) [47].

During SF, oxygen is generally supplied as a mixture of air at a high flow rate. As an alternative, the intermittent use of oxygen-rich air at a lower flow rate was suggested [58]. Oxygen-rich air is not used at the industrial scale because of the high cost of the equipment and safety issues for managing high-pressure oxygen. However, it could result in increases in process yield, improve the sensorial characteristics of the vinegar, and reduce the loss of volatile components, including ethanol. Trials conducted at the pilot scale showed improved acetic acid productivity (from 0.72 g/L/h with air to 1.35 g/L/h with oxygen-rich air) and a reduction in the total process time using 36% oxygen-rich air. Both lower (26%) and higher (over 40%) oxygen contents caused a decrease in acetic acid productivity [58]. The inhibition of acetic acid fermentation at high oxygen concentration may seem contradictory because oxygen is the substrate of the acetification reaction and

an increase in the oxygen partial pressure should improve the oxygen transfer rate and hence the productivity [40]. However, a high dissolved oxygen content can inhibit AAB growth by contributing to oxidative stress and protein damage in cells [59]. Moreover, during SF a correlation has been observed between exponential increases in acetaldehyde concentration with ALDH inhibition when the oxygen content is higher than 40% [58].

5. Growth on ethanol as carbon source and acetic acid resistance

Acetobacter and *Gluconacetobacter* species exhibit three growth phases in ethanol media. Although the diauxic growth-phase pattern shows some variations from species to species, it can be generally described as follows: first, they perform a rapid oxidation of ethanol to acetic acid, which is released from the periplasm into the surrounding environment (the ethanol oxidation phase). Then, a stationary phase occurs, resulting in a decrease in viable cell numbers or low growth yields (stationary phase). Finally, there is a second exponential phase (the acetate oxidation phase) in which acetic acid is catabolized by soluble ADH and ALDH in the cytoplasm, for both energy generation and carbon assimilation [4].

During ethanol oxidation and stationary phases AAB accumulated acetic acid in the environment without utilizing it, while during overoxidation phase (ethanol depleted) they oxidize acetic acid to CO_2 and H_2O.

The switch from acetate accumulation to acetate oxidation is controlled by changes in the metabolic flow through the tricarboxylic acid cycle [60]. In *Acetobacter* and *Gluconacetobacter* three genes in the *aar* gene cluster are required for acetic acid resistance: *aarA* encodes a citrate synthase, *aarB* encodes a functionally unknown protein,

and *aarC* encodes a protein involved in acetic acid assimilation [61,62]. Additionally, the *pmt* gene encoding phoshatidylethanolamine N-metyltransferase [63] and the *aatA* gene encoding an ATP-binding cassette transporter [64] are involved in the mechanism of acetic acid resistance. An efflux pump in the cytoplasmic membrane specific for acetic acid has also been reported as an additional machinery in the mechanism of acetic acid resistance in AAB. When cells are in the presence of high concentrations of acetic acid the efflux pump, which is driven by a proton motive force, pumps acetic acid from the cytoplasm to outside the cell [65].

5.1 Acetic acid resistance under different conditions

Under industrial conditions, acetic acid concentration is a major physiological stressor of cells. Undissociated acetic acid can penetrate the cell membrane, disrupting membrane transport processes, and then dissociate inside the cell, resulting in toxic levels of the anion and an associated increase in acidity [66]. Although AAB are tolerant to acetic acid at concentrations that are detrimental to the majority of microorganisms, significant variation among AAB species exists. Moreover, during the different fermentation phases of SF, strains exhibit different degrees of resistance to acetic acid. This resistance is affected by the number and the modality of recursive cultivations in acetic acid media, and the time between strain isolation and industrial application [67-70]. Therefore the effect of acetic acid on AAB growth is a function of the concentrations of substrate (ethanol) and product (acetic acid) and of the growth conditions. For instance in shaking ethanol cultures of *Acetobacter* strains, it was found that 10 g/L of acetic acid has an activating effect on growth, and lower concentrations resulted in a significant decrease in the logarithmic growth phase [71].

Activation and inhibition effects on AAB growth as a function of the total concentration have been observed during the start-up phase in SF. In these conditions a total concentration of approximately 8% (ethanol between 35.5 and 47 g/L and acetic acid between 30 and 45 g/L) was determined to be optimal for a wine vinegar start-up, using an AAB culture of the prevailing *A. aceti* cells [48].

Conversely, during the fermentation step AAB are able to grow at higher concentrations of acetate. The level of acetate resistance depends on the physiological adaptation under selective pressure due to acetic acid content, with significant variation according to species, evolved or wild-type strains (Table 2). Strains of *Ga. europaeus* isolated from industrial vinegar bioreactors tolerate acetic acid concentrations up to 100 g/L [72]. *A. aceti* in continuous cultivation with ethanol as substrate grows at concentrations of acetate exceeding 70 g/L [56]. Moreover, cultivation of *A. aceti* at increasing acetate concentrations for long periods of time (corresponding to 240 generations) produced acetate-resistant cultures that had acquired the ability to grow at more than 50 g/L of acetic acid [68].

5.2 Acetic acid resistance and species

According to the literature, the highest resistance against acetic acid that has been observed is described for the following species: *Ga. europaeus*, *Ga. intermedius*, *Ga. oboediens*, and *Ga. entanii* [18,19,25,73,74,26]. Trček and co-workers [26] detected higher ADH activity in *Ga. europaeus* and *Ga. intermedius* than in *A. pasteurianus*, indicating that the expression level of ADH in these species differs. Higher ADH activity might result in a bigger energy pool available for membrane-associated processes, such as the acetate/acetic acid export system, which may be involved in the resistance mechanism of

401 *Ga. europaeus* to high acetic acid concentrations.

402 *Ga. intermedius* exhibits a shorter lag phase than *Ga*. *europaeus* in ethanol media, but its
403 maximal acetic acid resistance is 40% lower than that of *Ga*. *europaeus* [26]. These
404 results suggest a different adaptation mechanism against acetic acid between the two
405 species. The isolation source of the AAB might partially explain the different behavior
406 toward high concentrations of acetic acid. For instance, the tested strains of *Ga*.
407 *intermedius* reported above were isolated from cider vinegar, where the sugar content of
408 the apples usually limits the final acidity of vinegar to 6%. In contrast, strains of *Ga*.
409 *europaeus* originated from 10% wine vinegar. Although the *A. pasteurianus* tested in this
410 comparative study were also derived from industrial vinegar reactors, (with acetic acid
411 concentration higher than 6% (w/v)), its tolerance to acetic acid was lower. These results
412 are supported by the fact that the majority of studies report *A. pasteurianus* as a common
413 species in low-acidity vinegars, and there is no evidence for a role as a stable component
414 of high-acidity vinegar microflora. *A. pasteurianus* was previously found as the main
415 microbial component in SSF for cereal vinegars (approximately 6% (w/v) acetic acid
416 concentration) [75,9]; other studies reported the suitability of strains of *A. pasteurianus* as
417 starter cultures during the scale-up of static fermentations with acetic acid concentration
418 maintained in a range between 1.7 and 5% [27].

419 Over the past few years, there have been many studies attempting to understand the
420 mechanisms of acetic acid resistance, and also for strain development for high acidity
421 vinegar production [76-78] (Table 2). Fukaya and co-workers [78] developed a spheroplast
422 fusion between *A. aceti subsp. aceti* and *A. aceti subsp. xylinum,* which showed enhanced
423 acetic acid production at higher temperatures (37 °C). Further improved production of
424 acetic acid (1.4-fold increase respect to wild strain) was later achieved by cloning the
425 ALDH gene of *A. polyoxogenes* into *A. aceti* [79] (Table 2).

Recently, UV mutagenesis under acidic stress was used to screen for a thermo-adapted *A. pasteurianus* mutant with a higher fermentation ability (103 g/L within 160 hours) than the wild type. The mutant also showed phenotypic stability over repeated cycles of semi-continuous fermentation [80].

From an industrial point of view, ethanol oxidation and acetic acid resistance are necessary phenotypic traits for strains to be effective. However, one of the limitations of using selected AAB strains in vinegar production is that strains can lose these important phenotypic traits over multiple cultivation cycles.

Previous works have reported that the characteristics of AAB are strongly affected by the "history" of strains [67]. For instance, the tolerance to ethanol and acetic acid decreases when isolates have been used as inocula and then have been kept for a long time in short-term preservation; a high tolerance was observed for strains used immediately after the isolation. This inconsistency is likely due to the genetic instability of strains.

Recursive cultivation of A. *pasteurianus* (NBRC 3283) produced a high rate of ethanol oxidation-deficient mutants. The formation of a multiphenotype cell complex with different textures (rough and smooth) of colony surfaces, as a result of high number of cultivation cycles, was also observed.

Genomic analysis of this strain revealed more than 280 transposons and five genes with hyper-mutable tandem repeats in the genome. The genome consists of a 2.9-Mb chromosome and six plasmids, which are recognized as characteristics contributing to the hyper-mutability [81]. In contrast, other studies report more phenotypic stability of *A. pasteurianus* in terms of acetic acid production for strains maintained with a suitable long-term preservation method. Instead, phenotypic changes can arise due to mutation in subcultures preserved by short-term preservation [70]. These are still standing issues for

451 establishing stable industrial strains, and especially those used in high-acidity vinegar
452 production.
453
454 **6. Growth temperature**
455
456 For industrial submerged conditions the optimal working temperature is approximately
457 30 °C [66]. Temperatures increase above the optimal value occurs during SF because
458 acetic acid fermentation is a thermodynamically favorable aerobic process. A temperature
459 increase induces the denaturation of nucleic acids and proteins and causes cell damage.
460 These reactions, together with the dispersion of cellular compounds due to membrane
461 damage, can irreversibly reduce the metabolic functions of the cells [57]. Additionally, the
462 toxic effects of acetic acid concentration in the medium increase the sensitivity of AAB to
463 high temperatures.
464 De Ory and co-workers [57] found that in wine vinegar production the optimal temperature
465 to maximize the specific growth rate of *A. aceti* is 30.9 °C. The maximum temperature
466 beyond which bacterial growth was totally inhibited was 35 °C, while the minimum
467 temperature was 8 °C (less than 1% of the specific growth rate).
468 The effect of temperature on acetic acid fermentation in the semi-continuous process for
469 wine vinegar production was also studied, utilizing both isothermal and a gradient-
470 temperature approach [82]. The results showed that the overall productivity improved
471 (approximately 15-20%) when using a 32-30 °C decreasing temperature-gradient
472 condition, with a concomitant reduction in the process time from 29 to 24.5 h, compared to
473 isothermal conditions at 30 °C. In this experiment, an initial temperature of 32 °C was
474 maintained until an acetic concentration of 95 g/L (starting from 70 g/L) was reached, at
475 which point the temperature was gradually reduced to 30 °C and maintained until the end

476 of the process. The use of a temperature gradient is an appealing prospect for both
477 improving the process productivity and reducing cooling expenses.
478 The availability of strains that are able to produce acetic acid at temperatures other than
479 the optimal values for mesophilic AAB have been considered by several authors (Table 2).
480 Thermotolerant *Acetobacter* strains that are able to acetify at 38 to 40 °C with higher
481 fermentation rates at higher temperatures were isolated from fruits [83]. Strains of the
482 species *A. tropicalis* and *A. pasteurianus* were isolated from different products in sub-
483 Saharan Africa and selected for their capacity to produce high acetic acid content at 35
484 and 38 °C, respectively [84].
485 Additionally, a number of attempts to obtain thermotolerant mutants have been made. A
486 genetically modified *A. aceti* strain was isolated that produced suitable acetic acid
487 concentrations at 37 °C in continuous acetic acid fermentation with 1-2% (v/v) ethanol [78]
488 (Table 2).
489 Recently, thermo-adapted strains (which stably perform acetic acid fermentation at 40 °C)
490 were obtained by recursive cultivation cycles using *A. pasteurianus* (SKU1108) [85].
491 It is well known that thermotolerant AAB can accumulated a large number of mutations
492 during the adaptation to high temperatures niches and evolve defense mechanisms
493 against thermal stress. These mechanisms contribute to an increase in genetic diversity,
494 and induce the expression of a wide variety of stress-response genes and alternative
495 metabolic pathways. Recently some studies have elucidated the role of a number of genes
496 involved in AAB thermotolerance. Three genes play a crucial role in thermotolerance and
497 fermentation at high temperature by AAB: the amino acid transporter (APT 1698), the
498 transcriptional regulator MarR (APT 2081) and the C4-dicarboxylate transporter (APT
499 2237) [85]. An analysis of genes involved in the thermotolerance mechanism of *A.*
500 *tropicalis* SKU1100 revealed a complex of 24 genes responsible for thermotolerance. In

addition to genes involved in heat shock or stress response, other genes were identified, including those required for cell cycle and cell division, which may be related to DNA replication errors and damage at high temperature, and also those involved in cell wall or cell membrane biosynthesis which play important roles as the first line of defense against environmental stress [86].

A deeper understanding of the molecular mechanisms regulating heat-stress adaptation could lead to improvements in SF, including an innovative high-temperature fermentation system.

7. Conclusion

SF is the main method used to produce vinegar at the industrial scale. Although acetic acid in vinegar can be derived from synthetic pathways, the bioconversion of ethanol into acetic acid is used worldwide to produce food-grade vinegar.

The availability of oxygen, acetic acid, ethanol and the process temperature are key factors for successful fermentation processes. Historically, the development of the vinegar production techniques has formed two principal technological fronts: optimizing process control and the evaluation and development of optimized AAB strains. However, these efforts have not met the industrial demand for stable and robust strains.

Although many studies report the successful genetic improvement of AAB strains, which are mainly selected for acetic acid resistance and thermotolerance, it must be emphasized that all of the attempts have been conducted on *Acetobacter* species, and no studies have been done on *Ga. europaeus* or other more suitable species for SF.

Based on current knowledge, it is clear that AAB species show significant variability in the technological characteristics that are important for vinegar production. Therefore, the

selection of optimal AAB strains is a very important means of increasing the productive capacity of this sector, and so far has not been explored.

In light of this, the fastest progress may come from the selection and validation of AAB for pure technological features, such as acetic acid tolerance and thermotolerance. This advancement could then serve as basic platform for the search of functional starters in order to increase nutritional and quality benefits.

Finally, many AAB metabolites are able to inhibit the growth of undesirable microorganisms, display nutraceutical properties, and contribute to flavour and texture properties. The study of specific metabolites produced by AAB is a promising field for future research that is of industrial interest.

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Figure legends

Fig. 1. Schematic representation of vinegar production in submerged system

[1]at concentrations specified by legislation

[2]blending with high acidity vinegar, to block undesired alcoholic fermentation

[3]nutrients containing carbon and nitrogen sources, vitamins and minerals are supplemented especially to produce high acidity vinegar (>12% of acetic acid) from alcoholic stocks containing no carbon sources except for ethanol [10].

Fig. 2. Submerged fermentation in semi-continuous mode for vinegar production. Desirable achievements as a basic platform for processes optimization and product innovation

[1] TC:Total concentration (sum of ethanol and acetic acid concentrations)

[2] Slow load of raw material/quick unload of final product

Table 1 Main acetic acid bacteria species in vinegars produced by different systems and their resistance to ethanol and acetic acid (expressed as total concentration)

Species[a]	Total concentration	Vinegar				References
		Cider	Wine	Spirit	Other	
A. pasteurianus	8%	SF[b]	SF[b]	-	Surface fermentation for TBV[C]; SSF[d] for cereal vinegar	[9,26,23,27]
A. aceti	7%	-	SF[b], Surface fermentation	-	-	[20,49]
[e]*Ga. europaeus*	8-15%	SF[b]	SF[b]	SF[b] SF[b]	Surface fermentation for TBV[c]	[18,23,27]
Ga. entanii	7 and 11%	-	-	SF[b]	-	[25]
[e]*Ga. hansenii*	-	SF[b]	Surface fermentation	-	-	[20,23]
[e]*Ga. intermedius*	6%[f]	SF[b]	-	SF[b]	Kombucha tea[g]	[19]
[e]*Ga. oboediens*	≤11%[f]	-	SF[b]	-	-	[74]
[e]*Ga. xylinus*	8%	SF[b]	-	-	Surface fermentation for TBV[C]; SSF[d] for cereal vinegar	[22,23,47]

(–) not detected
[a] *A*: *Acetobacter* genus; *Ga*: *Gluconacetobacter* genus
[b] SF: submerged fermentation
[C] TBV: traditional balsamic vinegar
[d] SSF: solid state fermentation
[e] *Komagataeibacter* according to Yamada and coworkers [45]
[f] Value obtained with experiments in tube test
[g] low acidity beverage obtained by alcoholic and acetic fermentation in static conditions

Table 2 Characteristics and/or growth condition for wild and engineered acetic acid bacteria used for ethanol oxidation

Organism[a]	Yield (g/L acetic acid)	Characteristic/Condition	Reference
[b]*Ga. europaeus* (DES11-DSM 6160)	90-95[c]	High acetic acid tolerance; absolute requirement of acetic acid	[18]
[b]*Ga. europaeus* V3 and JK2	90-95[c]	High acetic acid tolerance (10-8% wt/v)) in broth	[26]
[b]*Ga. intermedius JK3*	90-95[c]	Acetic acid tolerance: 6% (wt/v)	[26]
Ga. entanii (LTH 4560^T)	90-95[c]	Cultivation in an atmosphere at relative humidity > 95% at total concentration > 6%; does not over-oxidize acetic acid in AE broth[d]	[25]
A. pasteurianus SKU1108	30	Thermotolerant (37 °C)	[85]
A. pasteurianus TI and TH-3 (thermo-adapted strain SKU1108)	30	Thermotolerant (40 °C)	[85]
A. pasteurianus CICIM B7003-2	90	Physical mutation under acidic stress (60 g/L acetic acid)	[80]
A. pasteurianus CWBI-B419	20	Thermotolerant (38 °C) coupled to acidoresistant character	[84]
Acetobacter species	20-30	Thermotolerant (38-40 °C) (up to 4% (wt/v) acetic acid and 8% (v/v) ethanol)	[83]
A. tropicalis CWBI-B418	20	Thermotolerant (35 °C) coupled to acidoresistant character	[84]
A. aceti subs. *xylinum* NBI1002	80	Spheroplast fusion Acetic acid tolerance (50g/L) at 30 °C	[78]
A. aceti subs. aceti 1023	30	Spheroplast fusion Thermotolerant (37 °C)	[78]
A. aceti No. 116	60	Spheroplast fusion Thermotolerant (37 °C)	[78]
A. aceti subsp. *xylinum* NBI2099 (pMV24)	68.4	Plasmid vector developed for *Acetobacter*	[79]
A. aceti subsp. *xylinum* NBI2099 (pAL25)	96.6	Cloning of the 75 kDa subunit of the ALDH complex of *A. polyoxogenes* into pMV24	[79]
A. aceti M23	50	Continuous culture with 45 g/L acetic acid	[56]

[a]*Ga*: *Gluconacetobacter* genus; *A*: *Acetobacter* genus
[b]*Komagataeibacter* according to Yamada and coworkers [45]
[c]Data obtained from yield reached in high acidity submerged fermentation
[d]AE broth containing 4% acetic acid, 3% ethanol, 2% glucose

figure1

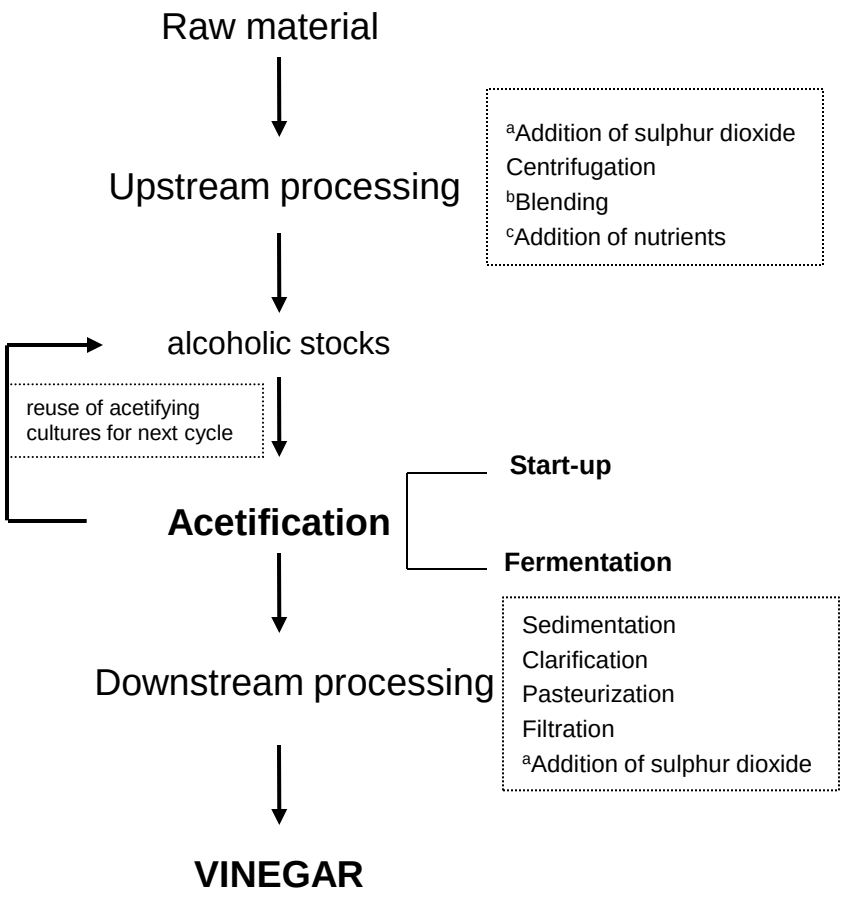

Figure 1. Schematic representation of vinegar production in submerged system

[a]at concentrations specified by legislation
[b]blending with high acidity vinegar, to block undesired alcoholic fermentation
[c]nutrients containing carbon and nitrogen sources, vitamins and minerals are supplemented especially to produce high acidity vinegar (>12% (wt/v) of acetic acid) from alcoholic stocks containing no carbon sources except for ethanol [10].

figure2

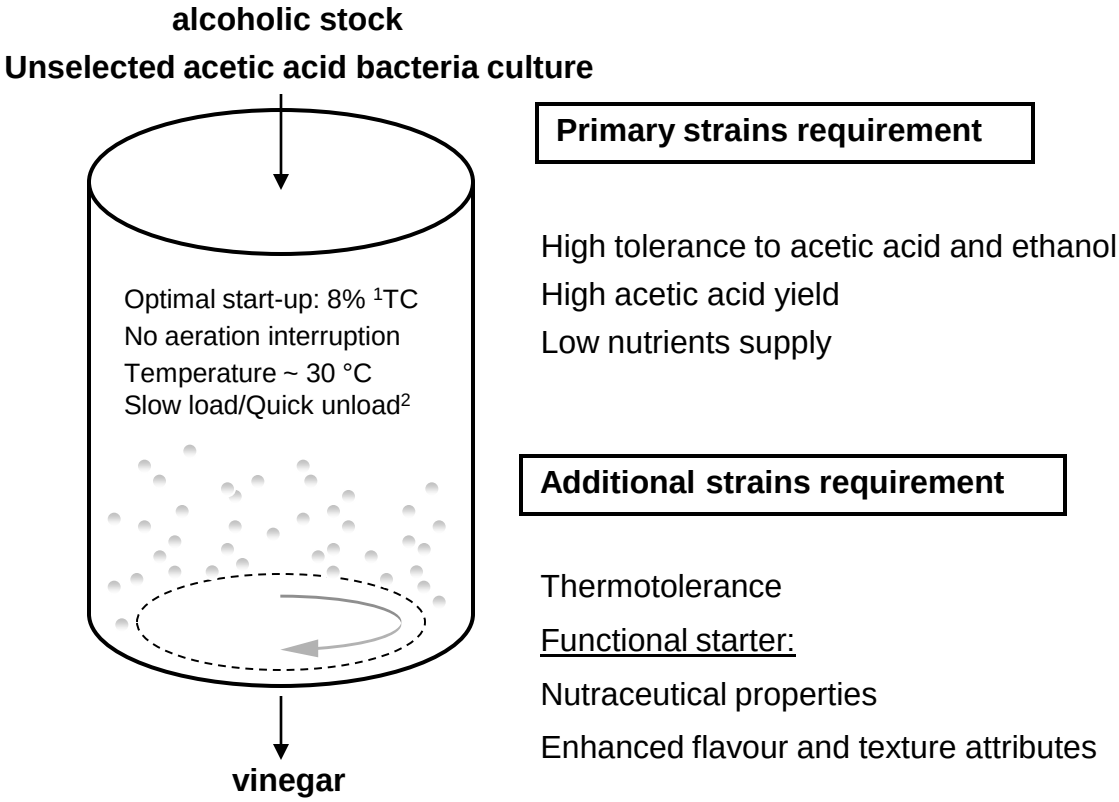

Figure 2. Submerged fermentation in semi-continuous mode for vinegar production. Desirable achievements as a basic platform for processes optimization and product innovation

[1] TC:Total concentration (sum of ethanol and acetic acid concentrations)

[2] Slow load of raw material/quick unload of final product