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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,

Maria Gullo

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Ms. Ref. No.: PRBI-D-14-00238R1

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

Process Biochemistry

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

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

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

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27 **Abstract**

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

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

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

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

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

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76 1. Introduction

77

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

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

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

97 Food-grade vinegar, which is used worldwide as a preservative and condiment for food
98 [6], is a diluted solution of acetic acid and is produced through a microbial oxidation carried
99 out by AAB [7]. In addition, vinegar has been demonstrated to possess healthful properties
100 [8].

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

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

115 Major studies have also been conducted to examine the prevalent microflora, in order to
116 determine the role of AAB in vinegar fermentation [16-24]. Differences in the species
117 detected correlate with the selective pressure exerted by the acetic acid concentration of
118 collection sites. In particular, highly acidic vinegar environments (acetic acid > 6% (w/v))
119 favor the prevalence of *Gluconacetobacter* species, whose ADH shows a higher stability in
120 high acetic acid content; in low acidity vinegars (acetic acid concentration \leq 6% (w/v))
121 *Acetobacter* species are dominant, although *Gluconacetobacter* has also been found [25-
122 27]. Although the aforementioned studies provided a good understanding of the
123 ecophysiology of AAB in acidic niches, very little literature is available on the functionality
124 of AAB in submerged processes relating to process parameters. The reasons for this lack
125 of information can be mainly attributed to the difficulty of handling of AAB, resulting often in

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

127 In addition, it is well known that a large fraction of microorganisms present in both
128 natural and industrial environments are uncultivable under standard laboratory conditions.
129 Environments in which viable but not cultivable microorganisms have been found include
130 soil [28], activated-sludge process for waste-water treatment [29], clinical samples
131 exhibiting mixed communities of biofilm-forming bacteria [30], vinegars [31] and paper mill
132 [32]. The uncultivability phenomenon limits the understanding of species richness and
133 diversity of these environments and consequently a broad-spectrum strategy to select
134 efficient strains as starter culture is affected.

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

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

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

145

146 **2. Aerobic submerged fermentation**

147

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

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

153 The acetic acid fermentation proceeds according to the basic stoichiometric equation
154 $C_2H_5OH + O_2 \rightarrow CH_3COOH + H_2O$; ($\Delta G^\circ = -455$ kJ/mol).

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

162 The quotient of the total vinegar concentration produced over the total mash concentration
163 indicates the yield (%) and expresses the relation between the input total concentration
164 and the output total concentration of the mash [10]. Theoretically, the total concentration
165 should remain constant throughout the process. Because ethanol is also a carbon source
166 for the synthesis of cellular constituents, it can be depleted at the cytoplasmic level and it
167 can be partially lost by evaporation, so the actual yield is lower than the theoretical one
168 [38].

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

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

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

181 The oxygen transfer is influenced by a high number of parameters including physical
182 properties of gas and liquid, operational conditions and geometrical parameters of the
183 bioreactor. Therefore, the oxygen transfer rate depends on stirring since it facilitates the
184 disruption of large bubbles into smaller ones, on the surface tension of the solution, on the
185 viscosity and on the fraction of gas retained in the bioreactor. A detailed description of the
186 oxygen transfer rate and parameters that affect its efficiency in aerobic SF can be found in
187 [41,42].

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

194 The heat generation during SF is unavoidable because acetic acid fermentation is an
195 exothermic reaction, producing approximately 8.4 MJ for every liter of oxidized ethanol
196 [39]. Moreover, during charge of substrate and discharge of product, the temperature can
197 vary greatly. Fermentation breakdown due to temperature variation is generally avoided by
198 heating and cooling systems.

199

200 *2.1 Fermentation mode*

201

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

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

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

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

220 The simplest semi-continuous operation mode is performed by two bioreactors arranged in
221 series. The first bioreactor contains the inoculum derived from a previous cycle, to which
222 wine or other alcoholic liquids (12-15% (v/v) ethanol, 1-2% (w/v) acetic acid) are added.
223 When the acetifying mass reaches an ethanol content of approximately 2-3% (v/v), it is
224 pumped into the second bioreactor where it will remain until the ethanol is depleted (0.2-
225 0.3% (v/v)) and the required acetic acid content is achieved [10].

226 The duration of a fermentation cycle is between 18 and 30 hours. The length mainly
227 depends on the initial concentration of ethanol, the efficiency of the aeration system and
228 the duration of the bacterial lag phase. For wine, intervals of 24 hours for each cycle were
229 optimal to obtain high acetic acid concentrations; shorter intervals provided higher
230 acetification rates but significantly lower acetic acid concentrations. Intervals of more than
231 30 hours between each loading step correlate with instability of cycles [44].

232

233 **3. Acetic acid bacteria in submerged fermentation**

234

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

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

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

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

251 co-workers [18], who identified *Ga. europaeus* (now *Komagataeibacter europaeus*) [45] as
252 the main component of the microflora in industrial bioreactors. Some unique growth
253 conditions for this species are: ability to grow at pH 2.5, an acetic acid concentration of 10-
254 14% (w/v), a constant oxygen supply and the requirement of acetic acid. However, *Ga.*
255 *europaeus* has also frequently been isolated from low acidity vinegars, during processing
256 steps with constant acetic acid content [27,31].

257 *A. pasteurianus* and *A. aceti*, whose strains have a strong oxidative activity against
258 ethanol, are mostly found as indigenous organisms in low acidity vinegars (~6%)
259 [9,22,31,46]. In contrast, strains of *Ga. xylinus* species that are able to produce suitable
260 amounts of acetic acid can have an opposing role in vinegar production due to their ability
261 to synthesize cellulose, thus potentially causing drawbacks. However, in surface
262 fermentation *Ga. xylinus* is reported to have a high acetic acid productivity; this is most
263 likely because the structure of the cellulose membrane network supports cells close to the
264 air-liquid interface, facilitating oxygen uptake. Conversely, in shake conditions, they
265 exhibited slowed growth and lower substrate consumption [47].

266

267 **4. Oxygen availability in submerged fermentation**

268

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

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

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

285 In submerged conditions of mixed AAB culture, it was noted that at a total concentration of
286 5%, an interruption of aeration for 2-8 minutes had the same effect as an interruption for
287 15-60 seconds when the total concentration was 10-12% [10], whereas in SF of a culture
288 of *A. acetii*, an interruption of aeration for 10 s at 6% (w/v) acetic acid caused a total
289 inhibition of acetic acid production [51].

290 Oxygen deprivation can be harmful not only during SF but also during the transfer of
291 cultures from the precultivation flasks to the acetators and during any transfer from one
292 acetator to another. To reduce bacteria death and to maintain a high respiration activity
293 throughout the whole fermentation process, a so-called RAMOS (respiration activity
294 monitoring system) device, that ensures a constant oxygen supply, was recently proposed
295 [52]. With this method, cultures drained off from bioreactors can be transferred into an
296 aerated bubble column and transported without an interruption of the oxygen supply.
297 When comparing bacteria death and respiration activity, a higher number of living cells
298 were present in cultures transferred by the RAMOS device than those managed with
299 traditional procedures.

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

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

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

310 Similar results (2 mg/L of dissolved oxygen) have been found in semi-continuous mode
311 using *A. aceti*, whereas the same culture in batch mode required less oxygen (0.7 mg/L of
312 dissolved oxygen) [49,57]. Recent studies on a *Ga. xylinus* strain from rice vinegar
313 confirmed the same behavior, with a greater concentration of dissolved oxygen present
314 (5,76 mg/L) in shaking flask fermentation than in surface fermentation (0.3 mg/L) [47].

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

326 an increase in the oxygen partial pressure should improve the oxygen transfer rate and
327 hence the productivity [40]. However, a high dissolved oxygen content can inhibit AAB
328 growth by contributing to oxidative stress and protein damage in cells [59]. Moreover,
329 during SF a correlation has been observed between exponential increases in
330 acetaldehyde concentration with ALDH inhibition when the oxygen content is higher than
331 40% [58].

332

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

334

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

344 During ethanol oxidation and stationary phases AAB accumulated acetic acid in the
345 environment without utilizing it, while during overoxidation phase (ethanol depleted) they
346 oxidize acetic acid to CO₂ and H₂O.

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

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

359

360 *5.1 Acetic acid resistance under different conditions*

361

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

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

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

391

392 *5.2 Acetic acid resistance and species*

393

394 According to the literature, the highest resistance against acetic acid that has been
395 observed is described for the following species: *Ga. europaeus*, *Ga. intermedius*, *Ga.*
396 *oboediens*, and *Ga. entanii* [18,19,25,73,74,26]. Trček and co-workers [26] detected higher
397 ADH activity in *Ga. europaeus* and *Ga. intermedius* than in *A. pasteurianus*, indicating that
398 the expression level of ADH in these species differs. Higher ADH activity might result in a
399 bigger energy pool available for membrane-associated processes, such as the
400 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).

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

430

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

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

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

444 Genomic analysis of this strain revealed more than 280 transposons and five genes with
445 hyper-mutable tandem repeats in the genome. The genome consists of a 2.9-Mb
446 chromosome and six plasmids, which are recognized as characteristics contributing to the
447 hyper-mutability [81]. In contrast, other studies report more phenotypic stability of *A.*
448 *pasteurianus* in terms of acetic acid production for strains maintained with a suitable long-
449 term preservation method. Instead, phenotypic changes can arise due to mutation in
450 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

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

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

509

510 **7. Conclusion**

511

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

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

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

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

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

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

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

536

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540

541 **References**

542

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785

786 **Figure legends**

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

788

789 ¹at concentrations specified by legislation

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

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

794

795 **Fig. 2.** Submerged fermentation in semi-continuous mode for vinegar production.

796 Desirable achievements as a basic platform for processes optimization and product

797 innovation

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

799 ²Slow 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 | |
| <i>A. pasteurianus</i> | 8% | SF ^b | SF ^b | - | Surface fermentation for TBV ^c ; SSF ^d for cereal vinegar | [9,26,23,27] |
| <i>A. aceti</i> | 7% | - | SF ^b , Surface fermentation | - | - | [20,49] |
| ^e <i>Ga. europaeus</i> | 8-15% | SF ^b | SF ^b | SF ^b SF ^b | Surface fermentation for TBV ^c | [18,23,27] |
| <i>Ga. entanii</i> | 7 and 11% | - | - | SF ^b | - | [25] |
| ^e <i>Ga. hansenii</i> | - | SF ^b | Surface fermentation | - | - | [20,23] |
| ^e <i>Ga. intermedius</i> | 6% ^f | SF ^b | - | SF ^b | Kombucha tea ^g | [19] |
| ^e <i>Ga. oboediens</i> | ≤11% ^f | - | SF ^b | - | - | [74] |
| ^e <i>Ga. xylinus</i> | 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 <i>Ga. europaeus</i> (DES11-DSM 6160) | 90-95 ^c | High acetic acid tolerance; absolute requirement of acetic acid | [18] |
| ^b <i>Ga. europaeus</i> V3 and JK2 | 90-95 ^c | High acetic acid tolerance (10-8% wt/v) in broth | [26] |
| ^b <i>Ga. intermedius</i> JK3 | 90-95 ^c | Acetic acid tolerance: 6% (wt/v) | [26] |
| <i>Ga. entanii</i> (LTH 4560 ¹) | 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] |
| <i>A. pasteurianus</i> SKU1108 | 30 | Thermotolerant (37 °C) | [85] |
| <i>A. pasteurianus</i> TI and TH-3 (thermo-adapted strain SKU1108) | 30 | Thermotolerant (40 °C) | [85] |
| <i>A. pasteurianus</i> CICIM B7003-2 | 90 | Physical mutation under acidic stress (60 g/L acetic acid) | [80] |
| <i>A. pasteurianus</i> CWBI-B419 | 20 | Thermotolerant (38 °C) coupled to acidoresistant character | [84] |
| <i>Acetobacter</i> species | 20-30 | Thermotolerant (38-40 °C) (up to 4% (wt/v) acetic acid and 8% (v/v) ethanol) | [83] |
| <i>A. tropicalis</i> CWBI-B418 | 20 | Thermotolerant (35 °C) coupled to acidoresistant character | [84] |
| <i>A. aceti</i> subs. <i>xylinum</i> NBI1002 | 80 | Spheroplast fusion Acetic acid tolerance (50g/L) at 30 °C | [78] |
| <i>A. aceti</i> subs. <i>aceti</i> 1023 | 30 | Spheroplast fusion Thermotolerant (37 °C) | [78] |
| <i>A. aceti</i> No. 116 | 60 | Spheroplast fusion Thermotolerant (37 °C) | [78] |
| <i>A. aceti</i> subsp. <i>xylinum</i> NBI2099 (pMV24) | 68.4 | Plasmid vector developed for <i>Acetobacter</i> | [79] |
| <i>A. aceti</i> subsp. <i>xylinum</i> NBI2099 (pAL25) | 96.6 | Cloning of the 75 kDa subunit of the ALDH complex of <i>A. polyoxogenes</i> into pMV24 | [79] |
| <i>A. aceti</i> 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]

^cData obtained from yield reached in high acidity submerged fermentation

^dAE broth containing 4% acetic acid, 3% ethanol, 2% glucose

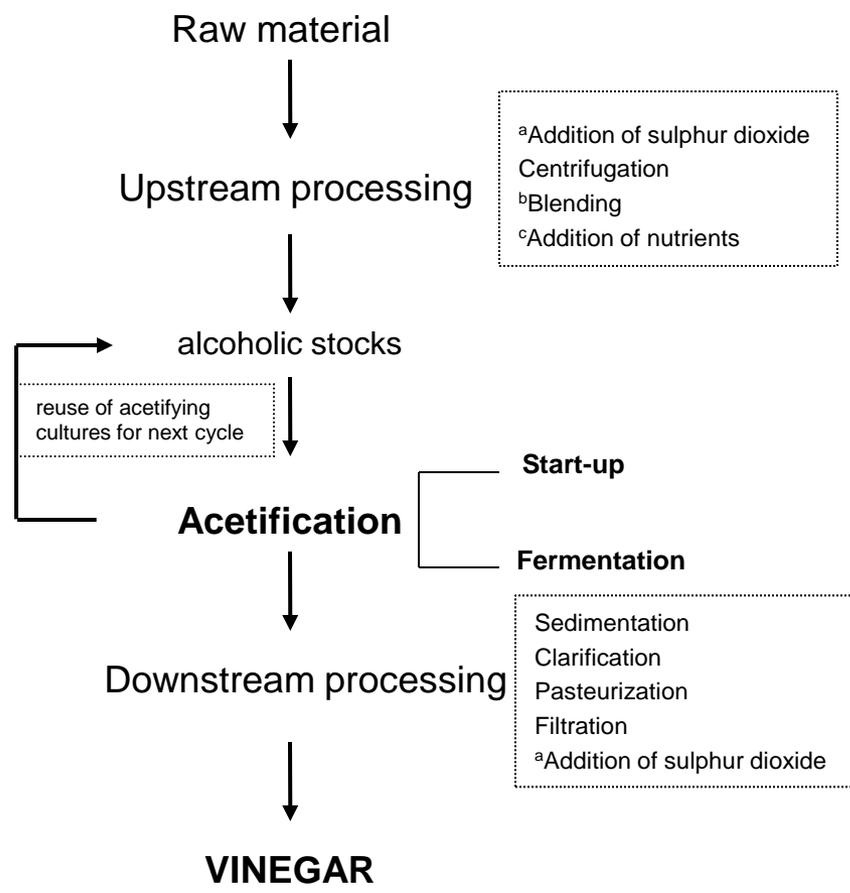


Figure 1. Schematic representation of vinegar production in submerged system

^aat concentrations specified by legislation

^bblending with high acidity vinegar, to block undesired alcoholic fermentation

^cnutrients 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].

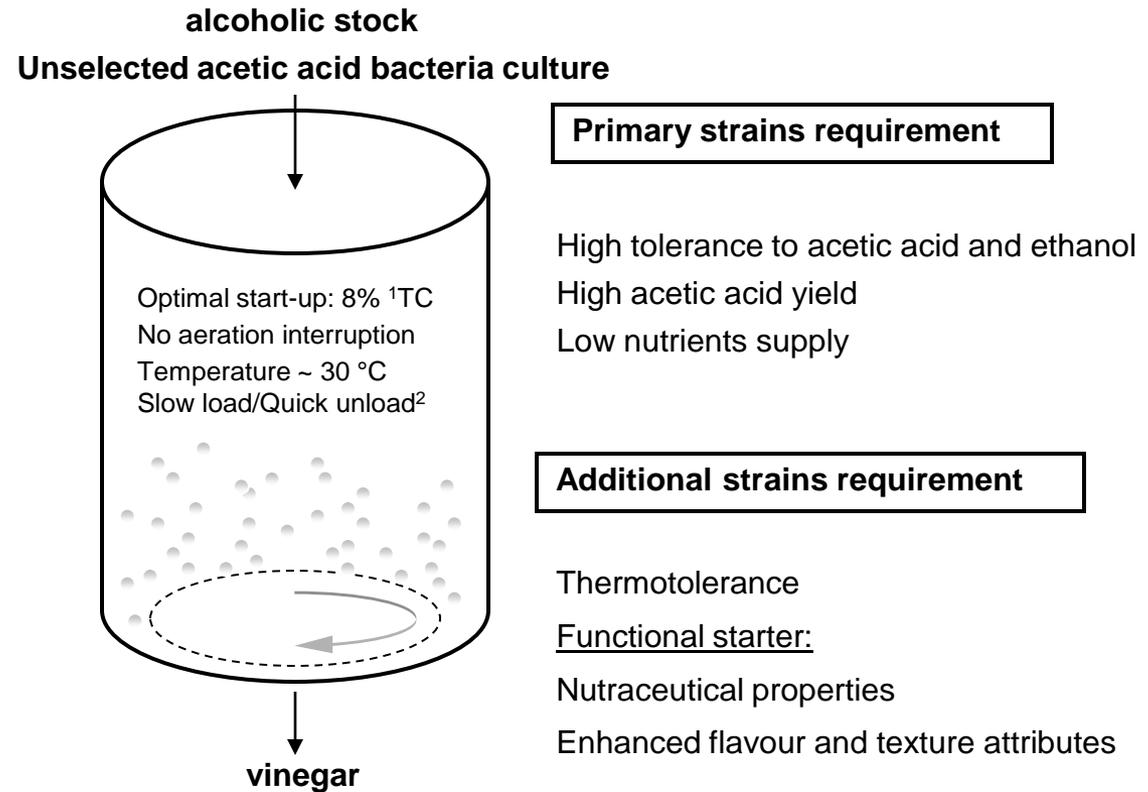


Figure 2. Submerged fermentation in semi-continuous mode for vinegar production. Desirable achievements as a basic platform for processes optimization and product innovation

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

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