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Title: Feasible acetic acid fermentations of alcoholic and sugary substrates in a combined operation mode

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Keywords: *Acetobacter pasteurianus*, acetic acid fermentation, submerged fermentation, static fermentation

Abstract: Starting from small-scale batch cultivations, acetic acid fermentations in static and submerged systems have been performed by a single acetic acid bacterial strain. To provide user-friendly selected starter cultures for industry, the versatility of these cultures in using different oxidation substrates under different conditions was assessed. In all cases, vinegars with the desired acetic acid, residual ethanol and reducing sugar contents were obtained.

An appropriate small-scale batch cultivation subjected to strict process control was pivotal for obtaining the desired acetic acid concentrations and an active culture for submerged fermentation. This achievement enabled the generation of selected starter cultures for submerged vinegar production, which reached an acetic acid content of 8.00–9.00% (w/v), as well as prototype-scale vinegar production. The production of vinegars with reducing sugars in the range of 15.00 to 27.00 (% w/v) was achieved, and cellulose production was avoided. The dominance of the microbial culture in this process was shown via (GTG)5-PCR. These results are valuable for introducing the use of selected acetic acid bacteria cultures in industrial vinegar production.

Highlights

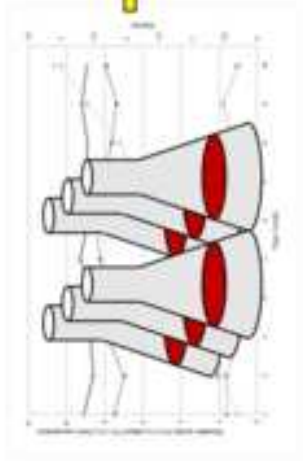
Acetic acid fermentations of alcoholic and sugary media

Transition from batch cultivation to a prototype scale

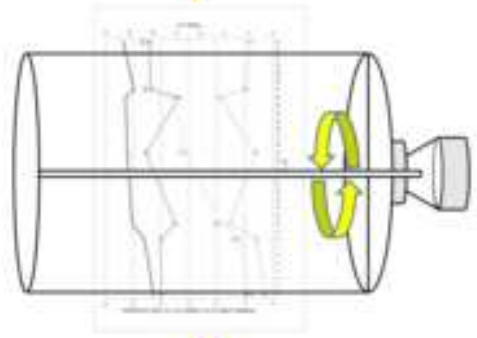
Selected AAB cultures for industrial applications

Static and submerged fermentations via a single AAB strain

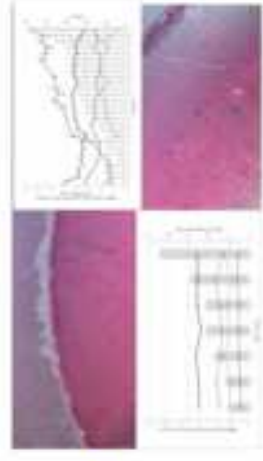
**Microscale cultivation
in static system**



**Submerged
fermentation**



**Prototypal scale
fermentation**



1 **Feasible acetic acid fermentations of alcoholic and sugary substrates in combined operation**
2 **mode**

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9 **Abstract**

10 Starting from small-scale batch cultivations, acetic acid fermentations in static and submerged
11 systems have been performed by a single acetic acid bacterial strain. To provide user-friendly
12 selected starter cultures for industry, the versatility of these cultures in using different oxidation
13 substrates under different conditions was assessed. In all cases, vinegars with the desired acetic
14 acid, residual ethanol and reducing sugar contents were obtained.

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19 vinegar production. The production of vinegars with reducing sugars in the range of 15.00 to 27.00
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21 culture in this process was shown via (GTG)5-PCR. These results are valuable for introducing the
22 use of selected acetic acid bacteria cultures in industrial vinegar production.

23

24 **Keywords:** *Acetobacter pasteurianus, acetic acid fermentation, submerged fermentation, static*
25 *fermentation*

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33 **Introduction**

34 Acetic acid bacteria (AAB) fermentations are oxidative fermentations performed by AAB growing
35 on carbon substrates under aerobic conditions [1]. Vinegar production is the most common example
36 of an AAB fermentation and occurs without the use of selected starter cultures (SSCs), both at small
37 and large industrial scales [2-5].

38 The low cost of vinegar is one of the reasons why the industry does not use SSCs for vinegar
39 fermentation. Indeed, the use of indigenous AAB cultures, propagated by a back-slopping
40 procedure, satisfies the main needs of the industry: low production costs, high performance and no
41 specialized expertise required to perform the fermentation because the back-slopping procedure is
42 easily customized. In the industrial production of some vinegars (for example, spirit vinegar), the
43 acidity reaches 20% (expressed as acetic acid (% w/v)), whereas for wine vinegar, the acetic acid
44 content is approximately 10-12%, which derives from approximately 95-98% of the ethanol content
45 of the original wine [6]. In these productions, the substrate to be oxidized is ethanol, and the main
46 goal is to reach the highest conversion yield of acetic acid. In contrast, for some high-priced
47 vinegars, such as sherry, traditional balsamic, and some eastern cereal vinegars, the final high
48 acidity and acetic acid yield are not the main attributes. Moreover, consumer demand is strongly
49 oriented towards sweet vinegars, which are generally obtained by blending sugars and vinegar,
50 rarely by fermenting liquid media containing both sugar and ethanol [7]. For these vinegars, new
51 fermentations performed by AAB with specific traits, such as the ability to grow in high-sugar
52 environments without depleting the sugar and to produce cellulose from glucose, are required.

53 Previous studies have highlighted the occurrence of *Komagataeibacter europaeus* in submerged
54 fermentations for the production of high-acidity vinegar (10-15%) and *Acetobacter pasteurianus* in
55 vinegars that reach acetic acid contents of 6-7%. The fermentative attributes of these species are
56 well studied with respect to acetic acid production in conventional vinegars [8, 4, 9,10, 11, 12].

57 What is less studied is the behaviour of AAB in the presence of multiple carbon sources (ethanol
58 and glucose) in industrial conditions and how to avoid cellulose formation.

59 Before scaling up a bioprocess for vinegar production, basic knowledge must be gathered
60 concerning the technological traits of AAB and the fitness of these strains over the course of
61 cultivation [13, 3, 14, 15]. Moreover, exploring the feasibility of fermentation parameter
62 optimization in different culture broths will be necessary to obtain successful fermentations [16-
63 17,18-19,20,21,22,23,24]. Finally, process scalability is a bottleneck due to the transfer of
64 optimized fermentation conditions from small batches to large fermentations because the different
65 operation modes interfere with microbial activity. In particular, the transition from laboratory to
66 industrial scale is affected by the loss of the ability to oxidize ethanol and the loss of acetic acid
67 resistance. These deficiencies have been observed frequently with AAB. Although little is known
68 about the genetic background governing the instability of physiological properties such as ethanol
69 oxidation, acetic acid resistance and cellulose formation, phenotypic modifications by transposon
70 insertion have been previously reported in AAB [25-26-27-28-29,30].

71 In this work, an AAB strain selected for its particular technological traits, including the inability to
72 produce cellulose while growing on ethanol and glucose-rich media, was used to develop SSCs via
73 static and submerged fermentations of wine and fermented grape musts rich in **glucose**. Specific
74 operation modes were established to maintain culture functionality during the use of different
75 fermentation methods at both the laboratory and industrial prototype scales.

76

77 **2. Materials and Methods**

78 2.1 General experimental plan

79 Figure 1 summarizes the general experimental design to obtain a set of vinegars with reducing sugar
80 contents in the range of 7.00 to 25.00% (w/v), an acetic acid content of 5.5-7.0% (w/v) and residual
81 ethanol less than 1.0% (v/v).

82 The culture produced in the small-scale fermentation system was scaled up in static conditions and
83 used to produce the SSC for the submerged and prototype static fermentations. A total of five SSCs
84 were produced, two in static fermentation systems and three in submerged fermentations. These
85 SSCs were used to develop the static fermentation at the prototype scale.

86

87 2.2 Bacterial strains, culture media and phenotypic assays

88 *A. pasteurianus* AB0220 (DSM 25273/UMCC 1754), previously isolated from vinegar [13], *A.*
89 *pasteurianus* (DSM 3509^T) and *K. xylinus* (DSM 2004) were used in this study. Subcultures were
90 recovered from preserved aliquots (-80 °C) and cultivated on GY broth (10.0% glucose and 1.0%
91 yeast extract dissolved in deionized water, pH not adjusted). One millilitre of culture was inoculated
92 into tubes containing 5 ml of GY. The cultures were incubated at 28 °C for 5-7 days. Cultivation on
93 solid medium (GYC) was performed on GY supplemented with calcium carbonate (2.0%) and agar
94 (8.0%) at 28 °C for 5-7 days. Frateur medium (30 m/L ethanol, 1.0% yeast extract, 2.0% calcium
95 carbonate and 2.0% agar) was used to assay acetic acid production from ethanol and over-oxidation.
96 Filter-sterilized ethanol was added to the sterile basal medium after cooling to 50 °C. Cell shape,
97 KOH tests and catalase production were assayed as previously reported [31]. The cellulose
98 production test was performed by collecting the pellicles and boiling them in 4 ml of 5.0% NaOH
99 for 2 hours, according to the previously reported method [32]. The presence of cellulose was
100 confirmed when the pellicle did not dissolve after boiling. *K. xylinus* (DSM 2004) was used as a
101 positive control.

102

103 2.3 Fermentation substrates

104 Wine (sterile and unsterile) and concentrated grape must (CGM) rich in glucose were used as
105 acetification substrates to produce the five SSCs (Tables 1 and 2). Wine sterilization was performed
106 by filtration (0.22-µm Millipore Express[®] PLUS membranes). All substrates were stored at 4 °C

107 until use. To produce SSC-E, a special mash composed of unfiltered wine and CGM was used. The
108 mixture was prepared appropriately to limit the sugar concentration to a range of 20 to 30%.

109 2.4 Analytical methods

110 pH and titratable acidity were measured using an automatic titrator (TitroLine[®] EASY) equipped
111 with an SI Analytics electrode. Samples were neutralized with NaOH (0.1 N) at pH 7.2. It was
112 assumed that all sample acidity was due to acetic acid. Reducing sugars were determined by the
113 standard Fehling method [33]. Ethanol % (v/v) was measured as follows: the hydroalcoholic
114 solutions were analysed directly with a Malligand ebulliometer. CGM was first subjected to
115 distillation (distiller Enochimico Gibertini[®]) and then analysed with a Malligand ebulliometer. All
116 experiments were performed in triplicate.

117 The “total concentration” parameter was calculated by adding ethanol (mL per 100 mL) and acetic
118 acid (g per 100 mL) concentrations. This parameter expresses the maximal concentration of acetic
119 acid that can be obtained in a complete fermentation. The vinegar stoichiometric yield was
120 calculated as the percentage of ethanol in the liquid medium converted into acetic acid. In contrast,
121 the acetification rate was expressed as the ratio between acidity produced and time (hours) [6].

122

123 2.5 Small-scale batch cultivations and static fermentations

124 Small-scale batch cultivations were performed in 250-mL and 2-L Erlenmeyer flasks. First, 5 ml of
125 revitalized culture were transferred into a 250-mL flask containing 50 mL of GY broth enriched
126 with 2.0% ethanol. New alcoholic broth was added after the ethanol concentration dropped below
127 1.0% (v/v). To conduct the static fermentations, the refilling procedure was performed by fixing
128 1.0% and 3.0% as the upper and lower limits for ethanol content, respectively, and 3.0% and 8.0%
129 (w/v) as the lower and maximum limits for acetic acid content, respectively. After a sufficient
130 volume was achieved (1 L), the culture was transferred to a 2-L Erlenmeyer flask and scaled-up as
131 previously described [34].

132 2.6 Submerged fermentation

133 Submerged fermentations were performed in an 8.0-L fermenter (CETOTEC® GmbH, Germany).
134 The operating conditions during the start-up phase were as follow: volume of the starting mash, 4L;
135 aeration, 40 l/h; and temperature, 30°C. The starting mash (6.0% (w/v) titratable acidity and 4.6%
136 (v/v) ethanol) was composed of three litres of SSC-A produced in the static system fermentation
137 and one litre of wine. In the fermentation phase, the volume was gradually increased to 6 L and the
138 aeration raised to 80 l/h. The bioreactor was operated in semi-batch mode. Approximately 1/3 of the
139 fermentation liquid was discharged when the residual ethanol concentration reached 1.2-2.0% (v/v)
140 and was then replaced with fresh mash. At the beginning of a new cycle, the ethanol and titratable
141 acidity concentrations were approximately 4.0-4.5% (v/v) and 6.0-6.5% (w/v), respectively, for
142 both SSC-C and SSC-D, versus 2.5-3.0% (v/v) and 3.5-4.0% (w/v), respectively, for SSC-E.

143 2.7 Prototype-scale fermentation

144 To develop the fermentation at a prototype scale, four custom-made stainless steel 200-L fermenters
145 equipped with a bubbler, a thermostat and sampling devices were used. Analytic parameters were
146 measured weekly using the procedures described above. Two batches (1A and 3A) were developed
147 starting from SSC-E and SSC-B, respectively, and were refilled with CGM. Batches 2A and 4A
148 were started from SSC-C and SSC-D, respectively, adding unfiltered wine. The amount of sugar in
149 batch 1A was kept constant (approximately 25 % (w/v)), whereas in batch 3A, it was gradually
150 increased to 15% (w/v).

151 152 2.8 Genomic DNA extraction and typing

153 Genomic DNA (gDNA) from the strain cultures was extracted using a sodium dodecyl sulfate
154 (SDS) proteinase-cetyltrimethyl ammonium bromide (CTAB) treatment as previously reported [13].
155 Samples from the prototype system were collected in triplicate from three different points on the
156 tank's surface, streaked on GYC and incubated at 28 °C for 3 days. gDNA was extracted from

157 colonies recovered on plates as previously described. gDNA was visualized by electrophoresis on
158 agarose (Fisher Molecular Biology) gels (1% in 0.5 X TBE buffer) stained with ethidium bromide
159 (0.1 µg/mL) under UV light. Quantification was performed with a spectrophotometer (NanoDrop
160 ND-1000). A 260/280 nm absorption ratio between 1.7 and 2.0 was used to assess the purity of the
161 gDNA. (GTG)₅-PCR fingerprinting was performed according to [35] with some modifications.
162 (GTG)₅-PCR reproducibility was tested by amplifying gDNA from randomly chosen strains several
163 times. In addition, each PCR mixture was controlled for reproducibility by the inclusion of *A.*
164 *pasteurianus* 3509^T gDNA. Genomic DNA was titrated to optimize the PCR amplification for a
165 given reaction. No mineral oil was added to the PCRs. Each PCR run contained a negative control
166 (water instead of gDNA). The PCRs were performed in a BioRad thermocycler (My-Thermal
167 Cycler). The GeneRuler 100 bp DNA Ladder Plus molecular marker (Thermo Scientific, Carlsbad,
168 CA, USA) was used to deduce the size of the templates. Digital images were generated in a
169 BioDocAnalyze system (BDA; Germany).

170

171 **3. Results and Discussion**

172 3.1. Selected starter cultures produced in static fermentation mode

173 In this study, strain AB0220 was chosen as a microbial culture because of its versatility in
174 performing acetic acid fermentations under different conditions (Table 3). Previously, this strain
175 was successfully used at the industrial scale to produce vinegar [34], and the phenotypic stability of
176 its subcultures after long storage times has been proven [20]. The observation that this strain could
177 be used to generate starter cultures provided the basis for developing SSCs suitable for use under
178 different fermentation conditions.

179 To produce a set of vinegars with the parameters detailed in the general experimental plan, first, two
180 SSCs (SSC-A and SSC-B) were produced via small-scale batch cultivation and static fermentation
181 under batch conditions at 28 °C and using the acetification substrates reported in Table 1. SSC-A

182 was started from the strain re-cultivated on GY and then on wine. In contrast, SSC-B was developed
183 from an aliquot of SSC-A after recursive cultivation on wine as the substrate. The final SSC
184 parameters are reported in Table 2.

185 The higher acetification speed during the scale-up of SSC-B (0.5 L per week) compared with that of
186 SSC-A (0.25 L per week) (Figs. 2a and b) was expected because it has been shown that AAB cells
187 maintained with acetate as a selective pressure acquire resistance and preserve physiological traits
188 such as acetic acid resistance and the ability to oxidise ethanol [14,24,15]. From an industrial
189 perspective, this observation is one reason why vinegar processes are conducted with AAB cultures
190 recovered from previous fermentations, which are cyclically propagated in the fermentation broth.

192 3.2. Selected starter cultures produced in submerged fermentation mode

193
194 A total of three different starter cultures, SSC-C, SSC-D and SSC-E, were developed in submerged
195 systems using SSC-A as the inoculum (Table 2). SSC-C and SSC-D were produced under the same
196 conditions except the substrate (wine) was sterile for SSC-C and unsterile for SSC-D. Unsterile
197 wine was used to evaluate the dominance of the microbial culture over the extant microflora in
198 commercial wine and later in industrial conditions, in which wine is not sterilized and
199 contaminations from the environment cannot be excluded. No significant differences were observed
200 with respect to start-up time and fermentation parameters between SSC-C and SSC-D, suggesting
201 that the microbial culture is also effective in unsterile fermentation broths.

202 Figure 3 shows the start-up phase (Fig. 3a) and fermentation phase (Fig. 3b) for SSC-C. During the
203 first 7 days (start-up), titratable acidity remained stable, whereas the concentration of ethanol
204 declined slight, mainly due to evaporation (Fig. 3a). The fermentation phase started on the 8th day,
205 and in less than 24 hours, almost all of the remaining ethanol was oxidized to acetic acid (Fig. 3b).

206 According to the trials performed in this study, the start-up was set at 4.6% (v/v) ethanol and 6.0%
207 (w/v) acetic acid. The temperature was kept constant at 30 °C, and the airflow was set to 40 L/h.
208 To evaluate culture performance in a submerged system, three fermentative cycles of SSC-C were
209 studied in detail. Each cycle was started at 6.0% (w/v) titratable acidity and 4.4% (v/v) ethanol and
210 were considered finished when the titratable acidity reached at least 8.50% (w/v). The efficiency of
211 the fermentation process, expressed as the vinegar stoichiometric yield, was in the range of 93 to
212 95% (Table 5). Consistent with previous studies [36], ethanol loss by evaporation was observed
213 because the process was performed without a volatile compound recovery system.

214 SSC-E, produced using a mixture of wine and CGM (see Table 1), was developed to evaluate the
215 ability of the microbial culture to ferment acetic acid in the presence of multiple carbon sources
216 (ethanol and glucose).

217 As shown in Table 3, SSC-E achieved lower values of maximum titratable acidity ($6.15 \pm 0.06\%$)
218 compared to SSC-C ($9.08 \pm 0.09\%$). This result is mainly due to the lower total concentration of the
219 CGM/wine mixture used. Cycles were started at approximately 4.0% (w/v) titratable acidity and
220 3.0% (v/v) ethanol. Fermentation was considered finished when the titratable acidity reached at
221 least 5.0% (w/v) (Table 5). No decrease in sugars was observed during this process. The average
222 length of the cycles for SSC-E was approximately 76 h, noticeably higher than that of SSC-C and
223 SSC-D (both approximately 24 h); as a consequence, a lower acetification rate and stoichiometric
224 yield were observed. However, the stoichiometric yield increased (13.0%) with subsequent cycles
225 due to the extended cultivation of the culture with wine/CGM as the substrate (Table 5). The SSC-
226 C, SSC-D and SSC-E scale-up trends are reported in Figs. 4A-c.

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231 3.3. Prototype-scale fermentation start-up and products development

232

233 Four fermentation batches (1A, 2A, 3A, and 4A) were developed from the SSCs (B, C, D and E)
234 and were transferred to the prototype scale for obtaining vinegars with different compositions
235 (Table 4). When the batches reached the maximum volume (approximately 220 L), the discharged
236 vinegars were transferred to four additional vessels prior to successive refilling with the substrate.
237 Start-up required approximately one week for all batches. The respective substrates were added
238 weekly to support acetic acid fermentation. A total of more than 200 litres of vinegar per fermenter
239 were produced and were subjected to downstream processing to obtain final products with different
240 acetic acid, sugar and residual ethanol contents. The final analytical values of the cultures are
241 shown in Table 4 (the data refer to the last filling step), whereas the scale-up trends are shown in
242 Figs. 5a-d. The entire process lasted approximately seven months for batches 2A, 3A and 4A and
243 approximately two months for batch 1A. It must be noted that SSC-E, used to start batch 1A, was
244 obtained in submerged operation mode using CGM as the substrate (over 20.0% w/v reducing
245 sugar). Thus, when the culture was transferred to the prototype scale, it was already adapted to a
246 high-sugar-content substrate. Moreover, batch 1A was considerably larger (45 L) compared with the
247 other batches (13, 16 and 17 L, respectively).

248

249 3.4. Dominance of the microbial culture over fermentation time

250 The dominance of the microbial culture from the laboratory to the prototype scale was proven with
251 phenotypic and molecular assays, using the working culture maintained at laboratory scale as a
252 purity control. Both phenotypic and molecular tests were performed on samples recovered from the
253 prototype scale after 6 months (batches 2A, 3A and 4A) or two months (batch 1A) of fermentation.

254 Assays were conducted on biofilms recovered from GYC plates. The cells were rod-shaped, KOH-
255 positive and catalase-positive. Cultivation on GYC medium showed vigorous growth mainly as a

256 biofilm spread across the plate's surface. The oxidation of ethanol to acetic acid and acetate
257 assimilation were shown on Frateur medium by a clear halo around bacterial growth, followed by
258 the reappearance of opacity on the bottom of the plates due to acetate oxidation. No cellulose
259 production was observed in samples except for batch 3A, for which the analysis of the
260 exopolysaccharide-containing pellicle confirmed the presence of cellulose.

261 High-resolution fingerprinting patterns were obtained via (GTG)5-PCR and allowed the persistence
262 of the strain to be mapped and the detection of a single loss-of-dominance event during the
263 fermentation process (Fig. 6, lanes 5, 6, and 7). The size of the DNA fragments obtained after
264 amplification ranged from 300 to 3000 bp (Fig. 5). (GTG)5-PCR reproducibility was monitored by
265 including the control strain (DSM 3509^T) in each reaction. The PCRs and electrophoresis were
266 performed in triplicate from the same DNA stock and the same reagents; no qualitative differences
267 in the banding patterns were observed. Consistent with previous data [37], repeated fingerprintings
268 were obtained, confirming that the banding patterns of samples 1A, 2A and 4B were identical to
269 each other and to that of the laboratory working culture. In contrast, a different pattern was obtained
270 from sample 3A.

271 (GTG)5-PCR analysis is able to reveal that a given species is represented by different strains within
272 the same sample or even to detect the dominance of a single strain throughout a process. Recently,
273 many studies have focused on the high-throughput identification and typing of a broad range of
274 AAB using (GTG)5-PCR fingerprinting with a single primer [38,39,40]. Moreover, (GTG)5-PCR
275 enabled the detection of indigenous AAB belonging to *A. pasteurianus* species and strains of the
276 *Komagataeibacter* genus in vinegars produced by a selected *A. pasteurianus* strain as a result of
277 dominance loss [4]. In our study, (GTG)5-PCR analysis suggested that in batch 3A, two strains
278 appeared (the inoculated strain and a contaminant strain) or that the dominant indigenous strain was
279 able to produce both acetic acid and cellulose. This result is in agreement with the phenotypic
280 assays, in which a cellulosic pellicle was identified in sample 3A.

281 As observed in a previous study [34], our hypothesis is that an indigenous strain became dominant
282 at the prototype scale as a consequence of supplying a mixture of wine and CGM as the substrate at
283 ethanol values below 2.0% (v/v). It is interesting to note that the strain was active in batch 1A,
284 which derived from a submerged system containing 27.0% (w/v) reducing sugars. Instead, a loss of
285 dominance was observed in batch 3A, which had been produced in static conditions at a lower sugar
286 concentration (15.15% (w/v)). Moreover, the length of the process was shorter for batch 1A (2
287 months) than for batch 3A (6 months). The operation mode to obtain each batch was also different;
288 the main variation concerned the step at which the CGM was added. In particular, batch 1A was
289 developed from SSC-E; thus the culture was adapted to a high-sugar environment during growth in
290 the submerged system. Batch 3A was obtained from SSC-B cultured in static conditions and using
291 wine as the substrate. At the prototype-scale, batch 3A was scaled up using wine as the substrate for
292 the first 5 months, and from month 6 onwards, a mash containing wine and CGM was added,
293 reaching 15% reducing sugars, 5.5% (w/v) acetic acid and approximately 1.0% (v/v) ethanol.
294 Ethanol depletion (< 2.0% (v/v)), corresponded to the addition of glucose- and fructose-containing
295 CGM, which may have induced cellulose formation from glucose. Although a cellulose layer was
296 detected in batch 3A, the final product reached the desired acetic acid content (>5.40% (w/v)). On
297 the basis of these observations, the indigenous strain that conducted the last phases of fermentation
298 was able to produce both acetic acid and cellulose, as in the case of *K. xylinus*.
299 Indeed, strains of this species are able to produce both acetic acid and cellulose when growing in
300 vinegar environments. Briefly, acetic acid produced periplasmically can accumulate in the
301 surrounding liquid or enter the cell. The acetate in the cell is fed by extracellular acetic acid, and it
302 can be excreted or phosphorylated into acyl-phosphate, which is transformed into acetyl-coenzyme
303 A to feed the tricarboxylic acid cycle [41]. Oxaloacetate produced by the glyoxylate shunt is
304 decarboxylated into pyruvate, forming glucose 6-phosphate (via the gluconeogenesis pathway).
305 Then, pyruvate is used by the gluconeogenesis pathway to produce glucose, the building block of

306 cellulose [42]. Glucose 6-phosphate and fructose 6-phosphate, which are freely interconverted by a
307 phosphohexose isomerase, feed a glucan-synthase enzyme complex that permits cellulose
308 biosynthesis from glucose 6-phosphate [43]. Therefore, with different energy balances, both acetic
309 acid and cellulose can be produced from the diauxic consumption of ethanol and glucose as carbon
310 sources.

311

312 **4. Conclusions**

313 Scalable fermentations require robust strains able to dominate unsterile environments and to
314 maintain their traits throughout the process. Moreover, rational process development requires many
315 considerations to drive the transition from microlitre to industrial scales. Most bioprocesses that use
316 AAB, while technically feasible, are still confined to the laboratory scale due to the difficulty of
317 handling active cultures throughout the process. Consequently, the use of SSCs in the vinegar
318 industry is not a common practice. In this study, a selected AAB strain was scaled up from the
319 laboratory (millilitres) to a prototype scale (hundreds of litres) in a combined fermentation mode
320 (static and submerged systems). The combination of static and submerged system fermentation by
321 *A. pasteurianus* AB0220 has proven to reliably produce viable SSCs at both laboratory and
322 prototype scales. This approach successfully produced small amounts of SSCs in a static system
323 that were able to start prototype-scale fermentations, whereas a submerged system greatly sped up
324 the process.

325 Two sweetened fermented vinegars were developed, and cellulose was not observed in the presence
326 of ethanol, suggesting the robustness of the designed SSC strict process controls. The long-term
327 process stability in the static, submerged and prototype-scale systems confirmed the feasibility of
328 using selected AAB cultures in industrial acetic acid fermentations.

329

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333 the BioloMICSNet Software of UMMC culture collection.

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454 **Figure legends**

455 Fig. 1: Schematic of the experimental plan.

456 Fig. 2. Trends in analytical parameters during static fermentation (a, b: SSC-A and SSC-B).

457 Symbols: (□) ethanol; (▲) titratable acidity; (O) total concentration; (×) volume. Each value is the
458 mean of three parallel replicates ± standard deviation.

459 Fig. 3. Trend in analytical parameters in the main phases of submerged fermentation (a, b: start-up
460 and fermentation). Symbols: (□) ethanol; (▲) titratable acidity; (O) total concentration. Each value
461 is the mean of three parallel replicates ± standard deviation.

462 Fig. 4. Semi-continuous fermentation in the submerged system. Trend in analytical parameters
463 during culture scale-ups with different acetification substrates (a-c: SSC-C, SSC-D and SSC-E).

464 Symbols: (□) ethanol; (▲) titratable acidity; (O) total concentration; (×) discharged volume;
465 reducing sugars (●). Each value is the mean of three parallel replicates ± standard deviation.

466 Fig. 5. Prototype-scale fermentation in the static system. Trends in analytical parameters during
467 culture scale-ups with different acetification substrates (a-d: 1A, 2A, 3A and 4A). Symbols: (□)
468 ethanol; (▲) titratable acidity; (O) total concentration; (×) volume. Each value is the mean of three
469 parallel replicates ± standard deviation.

470 Fig 6. (GTG)₅-PCR fingerprinting patterns. L: 100 bp Plus DNA Ladder (Thermo Scientific,
471 Carlsbad, CA, USA); 1: AB0220 (culture strain); 2-4: (triplicates of sample 4A); 5-7: (triplicates of
472 sample 3A); 8-10: (triplicates of sample 2A); 11-13: (triplicates of sample 1A); 14: AB0220; 15:
473 negative control; 16: DSMZ 3509^T (*A. pasteurianus*).

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476 **Table 1**

477 Substrates for SSC production at laboratory and prototype scales

478

Substrate	pH	Titrateable acidity (% w/v)	Ethanol (% v/v)	Reducing sugars (% w/v)
Sterile wine	3.50 ± 0.05	0.36 ± 0.03	10.00 ± 0.15	-
Unsterile wine	3.50 ± 0.03	0.71 ± 0.06	14.80 ± 0.20	-
Unsterile CGM	2.75 ± 0.04	1.71 ± 0.02	1.20 ± 0.10	80.00 ± 3.00

479 (–) not detected

480

481 **Table 2**

482 Substrate, fermentation mode and final parameters of the SSCs produced at the laboratory scale

483

Name	Substrate	Fermentation mode	Titrateable acidity (% w/v)	Ethanol (% v/v)	Reducing sugar (% w/v)	Volume (L)
SSC-A	Sterile wine	Static	7.98 ± 0.03	1.00 ± 0.09	-	3.00
SSC-B	Sterile wine	Static	7.35 ± 0.08	0.30 ± 0.07	-	11.20
SSC-C	Sterile wine	Submerged	9.10 ± 0.04	0.30 ± 0.15	-	11.90
SSC-D	Unsterile wine	Submerged	8.80 ± 0.03	0.40 ± 0.13	-	12.30
SSC-E	Unsterile wine/CGM	Submerged	5.50 ± 0.06	0.40 ± 0.05	22 ± 2.00	40.60

484 (–) not detected

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Table 3

Maximum titratable acidity of *Acetobacter pasteurianus* (AB0220) under different conditions

Medium	Operation mode	Maximum titratable acidity (w/v)	Reference
Wine/ethanol 12%	^b Static after revitalization	6.80 ± 0.34	[20]
Wine/ethanol 7.5%	^b Static fermentation	3.59 ± 0.71	[34]
Wine + ^a YE (2%)/ethanol 7.5%	^b Static fermentation	5.07 ± 0.71	[34]
CGM/ethanol 7.5%	^b Static fermentation	5.35 ± 0.05	[34]
SSC-B wine/ethanol 10.5%	^b Static fermentation	8.90 ± 0.05	This study
SSC-C wine/ethanol 10.5%	^b Semi-continuous submerged fermentation	9.08 ± 0.09	This study
SSC-E wine-CGM/ethanol 4.60%	^b Semi-continuous submerged fermentation	6.15 ± 0.06	This study
Wine/ethanol 14.80%	^c Static fermentation	7.50 ± 0.13	This study
Wine-CGM/ethanol 7.00%	^c Static fermentation	5.49 ± 0.12	This study

^aYeast extract
^bLaboratory scale
^cPrototype scale

509 **Table 4**

510 Substrates and final parameters of the prototype-scale batches

511

Batch	SSC	Substrate	Titrateable acidity (% w/v)	Ethanol (% v/v)	Reducing sugar (% w/v)	Volume (L)
1A	E	CGM	3.12 ± 0.09	3.00 ± 0.06	27.45 ± 1.02	220
2A	C	Unsterilized wine	4.44 ± 0.04	3.15 ± 0.12	-	224
3A	B	CGM	5.37 ± 0.09	1.30 ± 0.09	15.15 ± 0.45	226
4A	D	Unsterilized wine	4.29 ± 0.05	3.10 ± 0.14	-	224

512 (–) not detected

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Table 5

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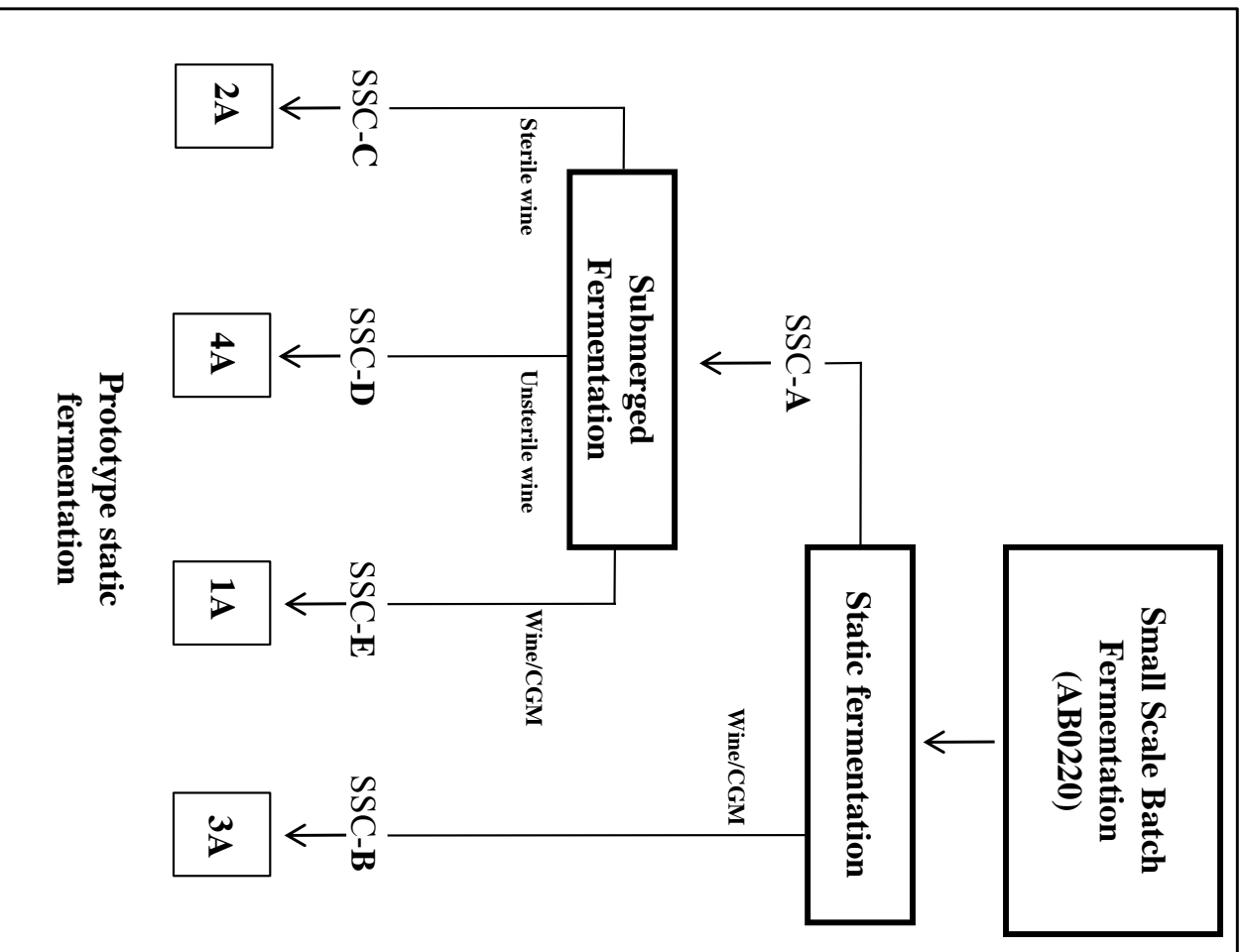
Experimental data for the semi-batch cycles in the submerged mode

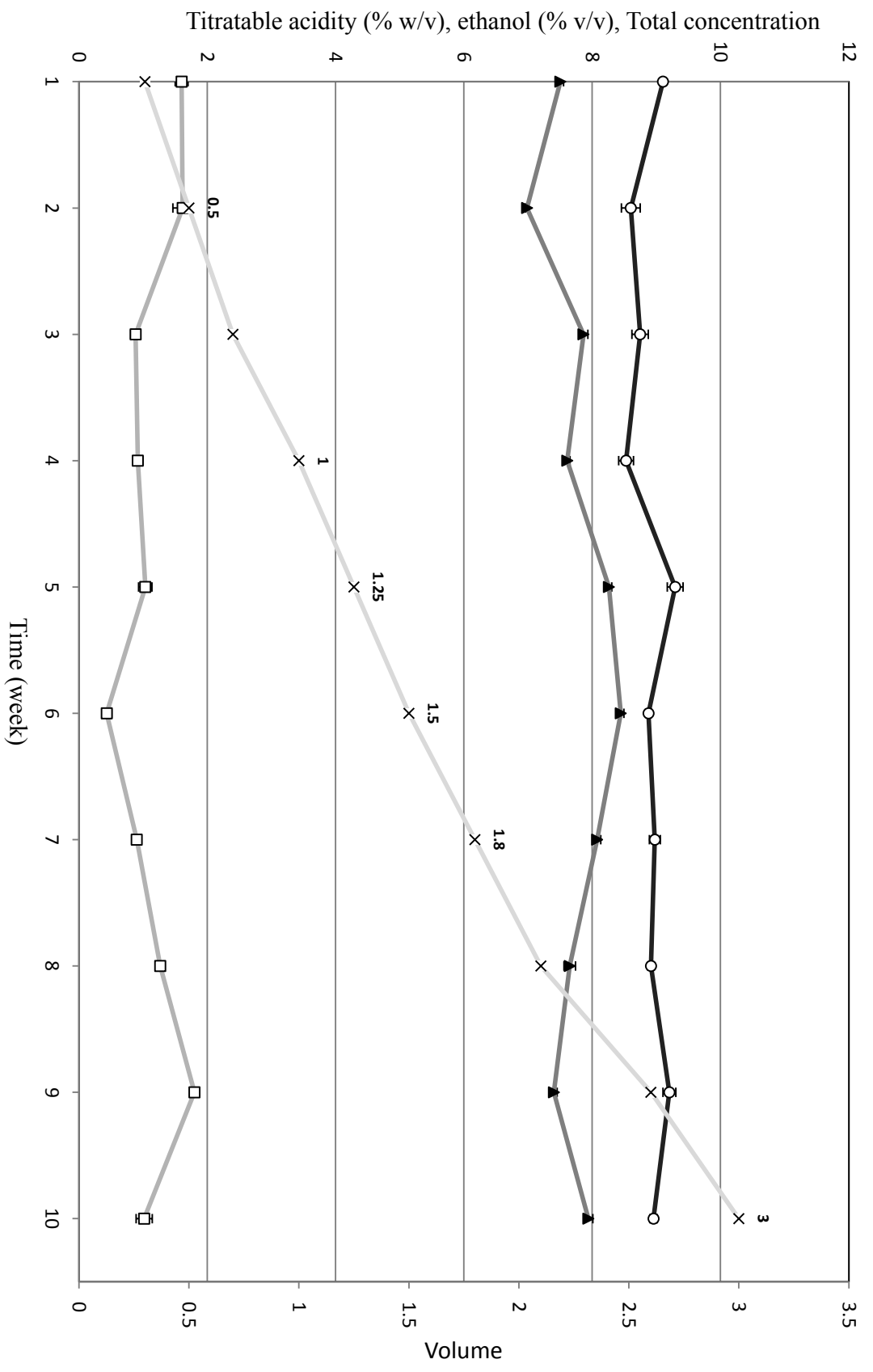
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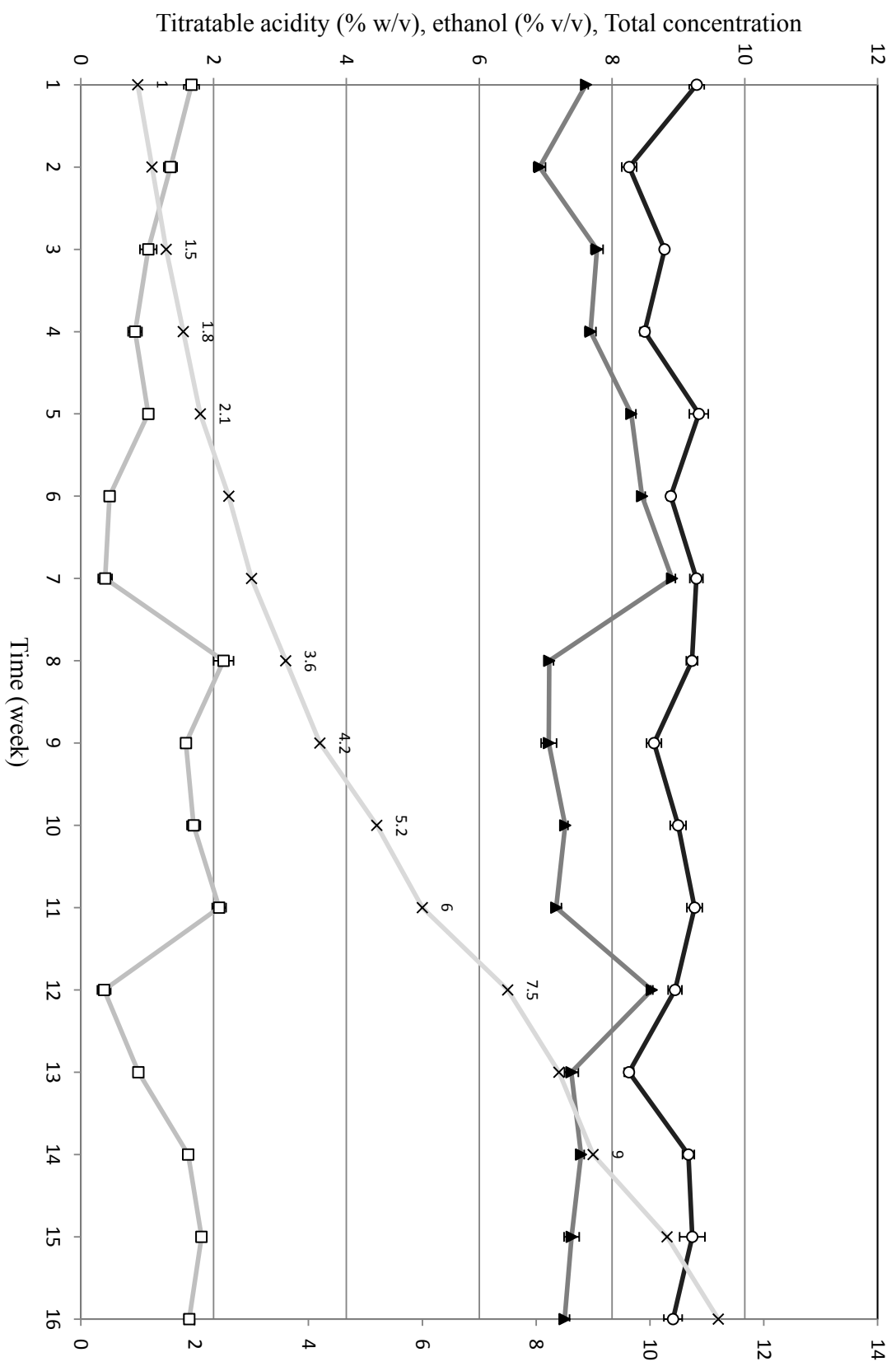
Cycle	Substrate/SSC	Experiment time (h)	Titratable acidity (%)		Ethanol (% v/v)		Acetification Rate (% day ⁻¹)	Stoichiometric yield (%)
			Initial w/v	Final	Initial	Final		
1	Wine/SSC-C	24	6.03 ± 0.08	9.08 ± 0.09	4.33 ± 0.11	1.20 ± 0.07	3.05 ± 0.04	93.41 ± 1.52
2	Wine/SSC-C	24	6.36 ± 0.07	8.73 ± 0.09	4.44 ± 0.15	1.70 ± 0.08	2.37 ± 0.03	94.46 ± 1.74
3	Wine/SSC-C	25	6.25 ± 0.04	8.82 ± 0.08	4.42 ± 0.09	1.60 ± 0.10	2.47 ± 0.01	94.84 ± 1.01
1	CGM/SSC-E	97	3.78 ± 0.01	5.23 ± 0.04	2.67 ± 0.12	0.95 ± 0.05	0.36 ± 0.03	80.81 ± 1.62
2	CGM/SSC-E	74	3.90 ± 0.07	6.01 ± 0.04	2.90 ± 0.14	0.58 ± 0.13	0.68 ± 0.03	92.14 ± 0.84
3	CGM/SSC-E	48	4.10 ± 0.06	6.15 ± 0.06	2.90 ± 0.12	0.48 ± 0.09	1.09 ± 0.05	93.88 ± 0.82

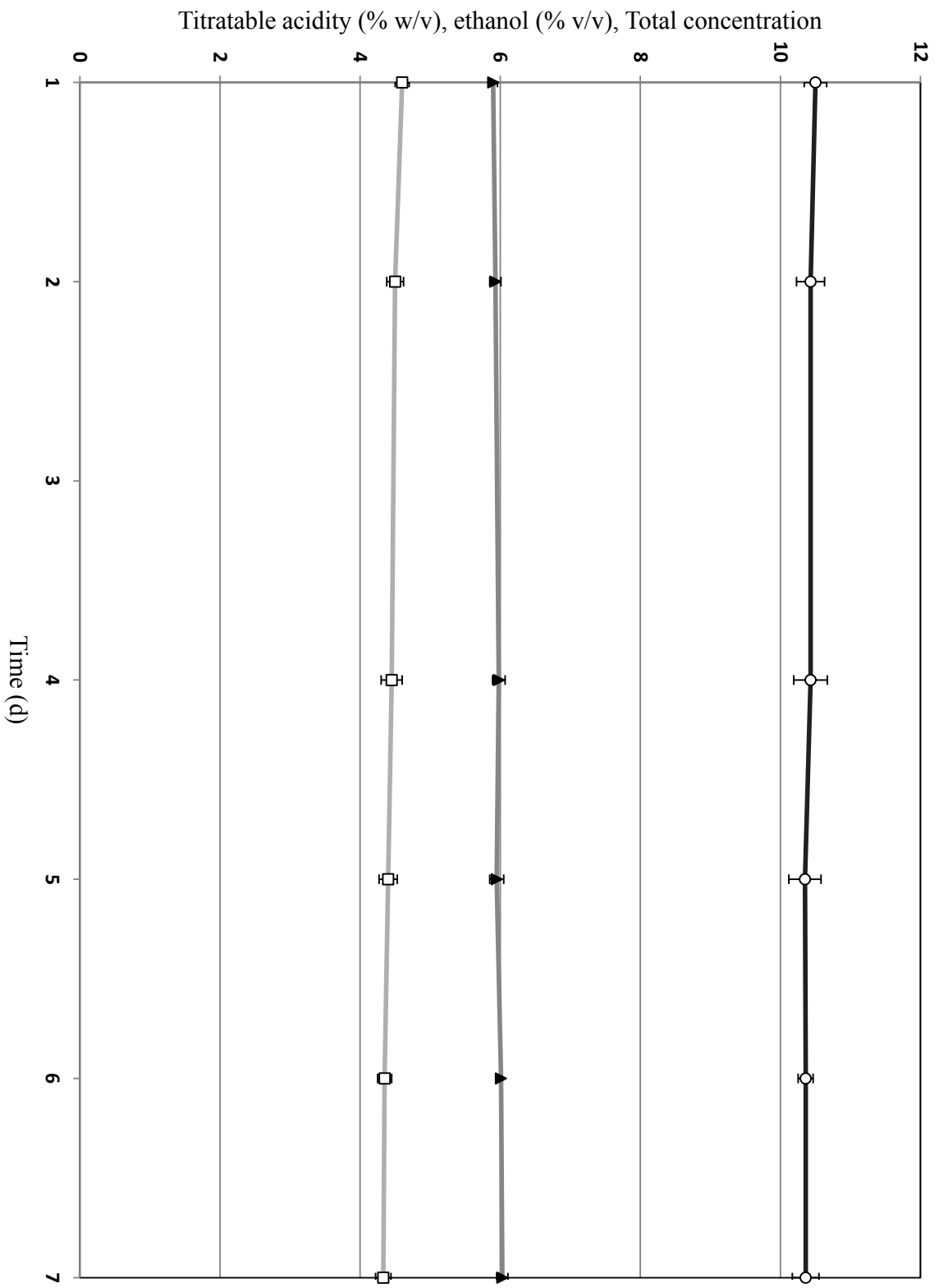
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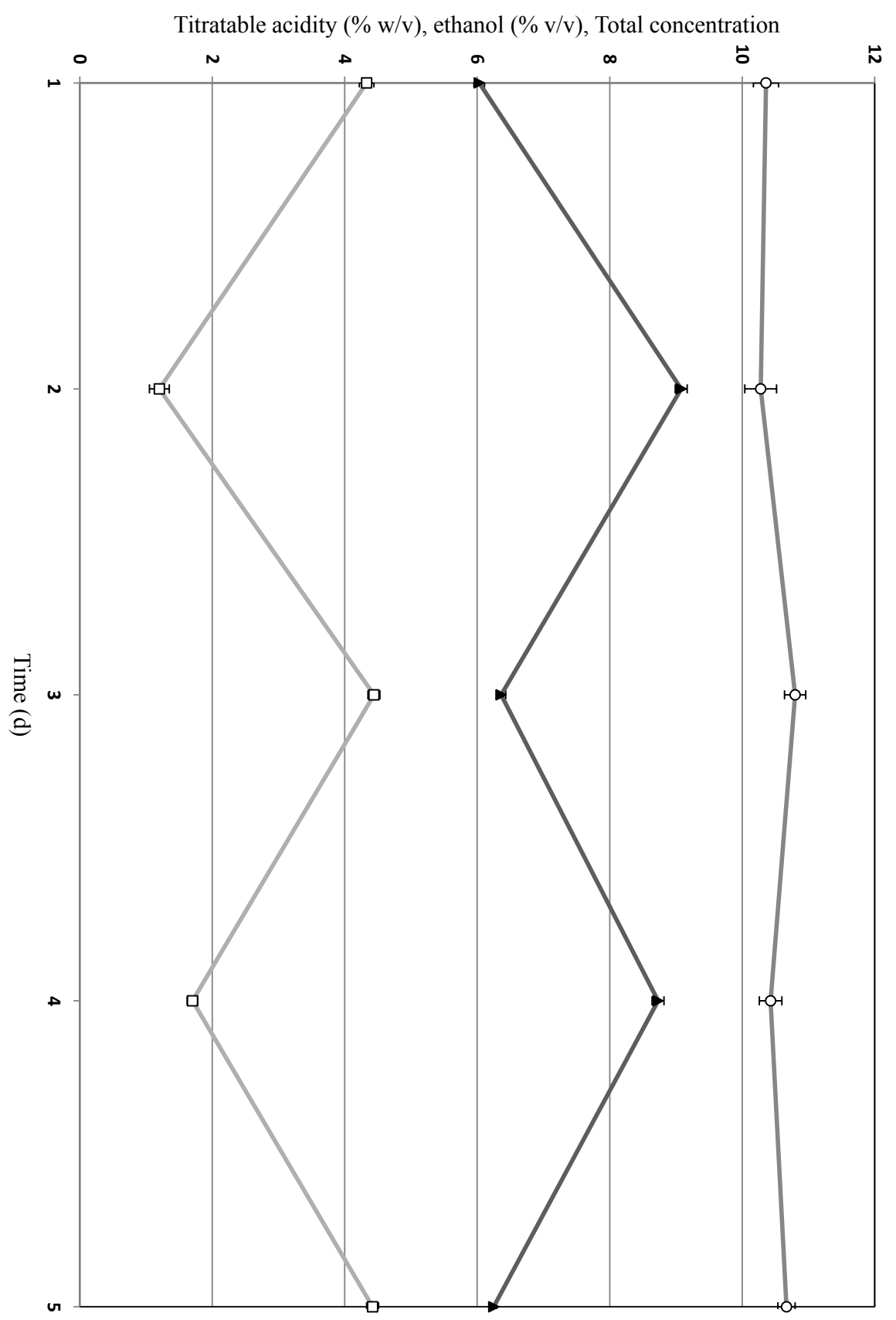
Figure 1

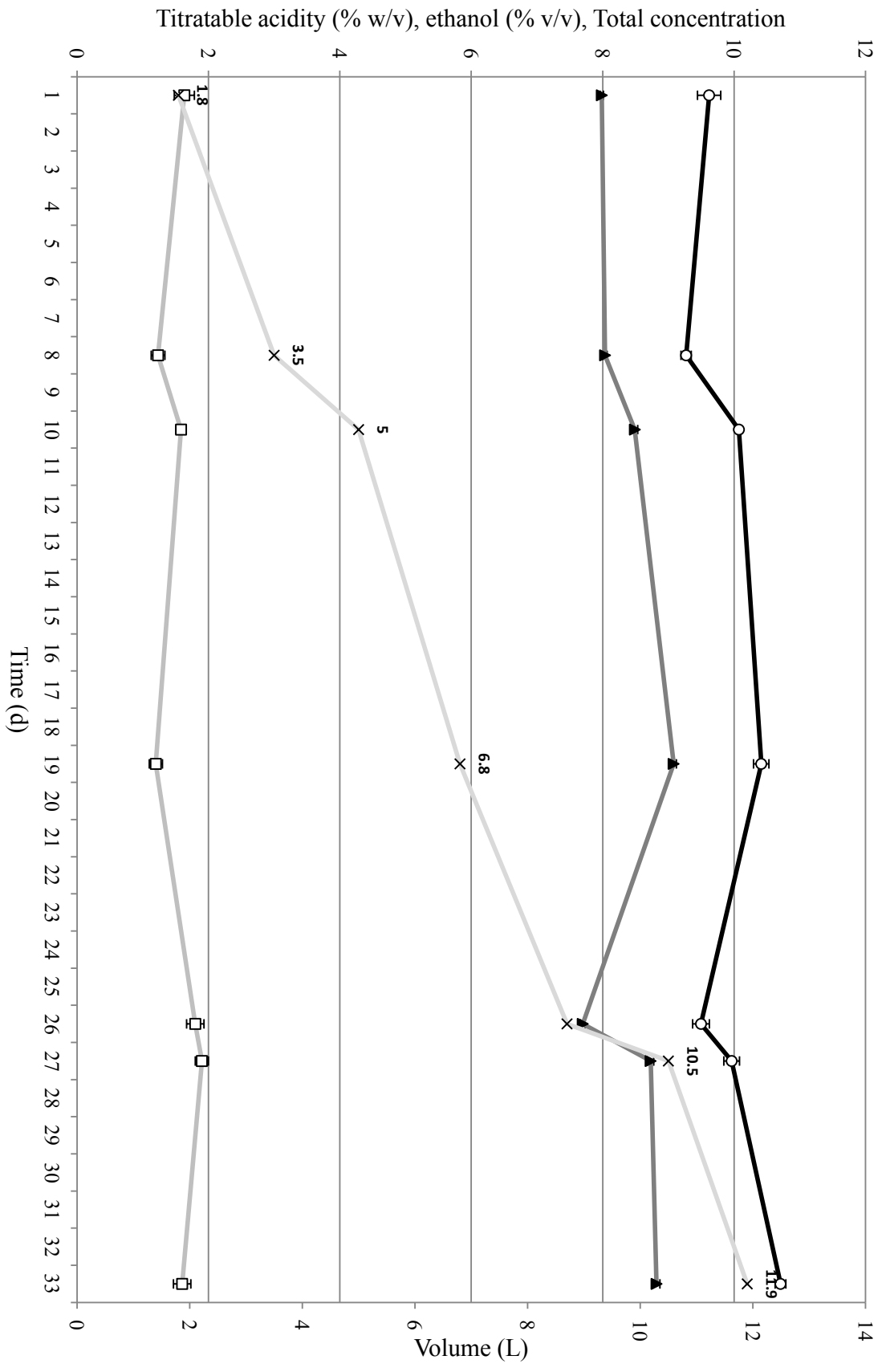


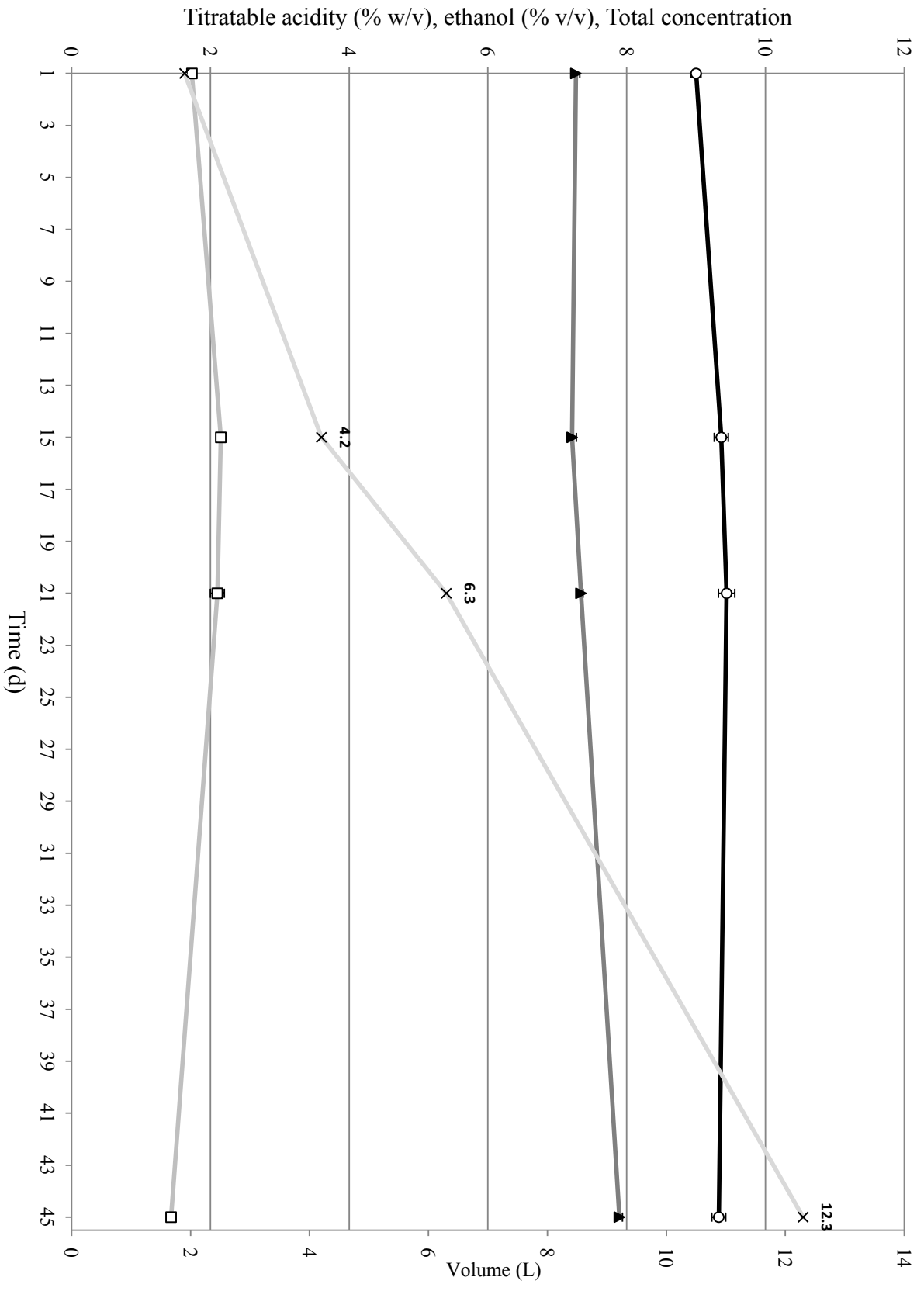


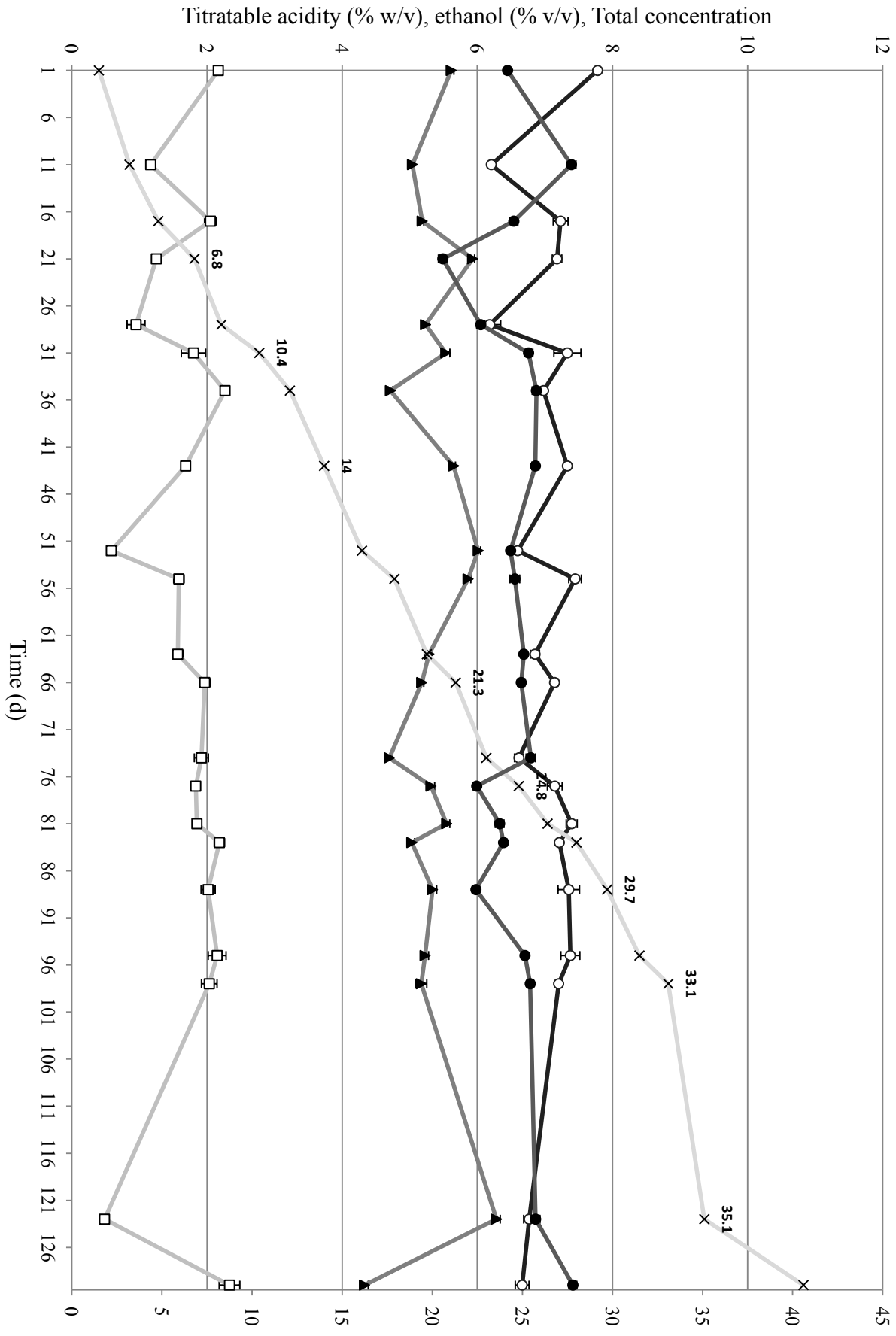


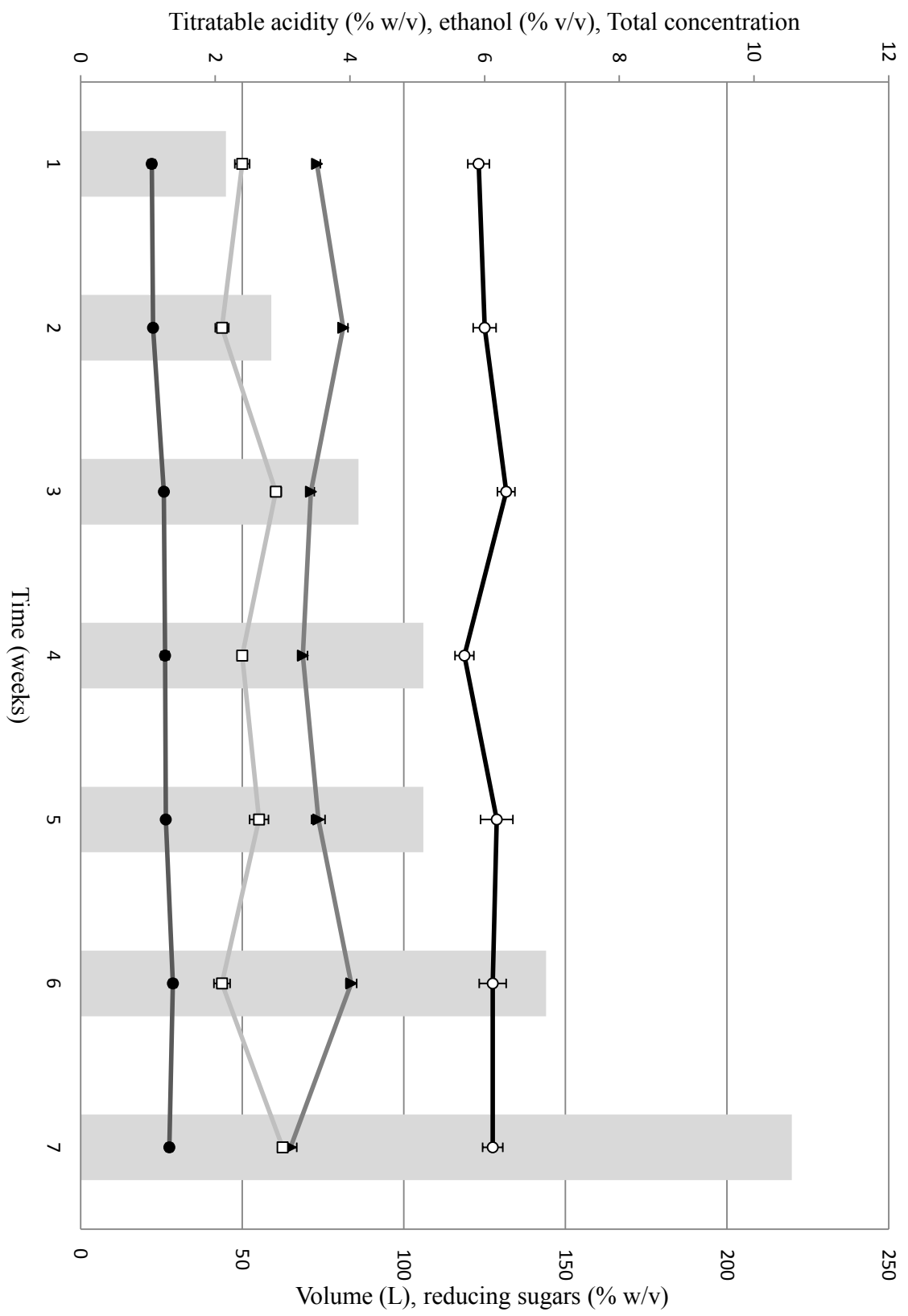


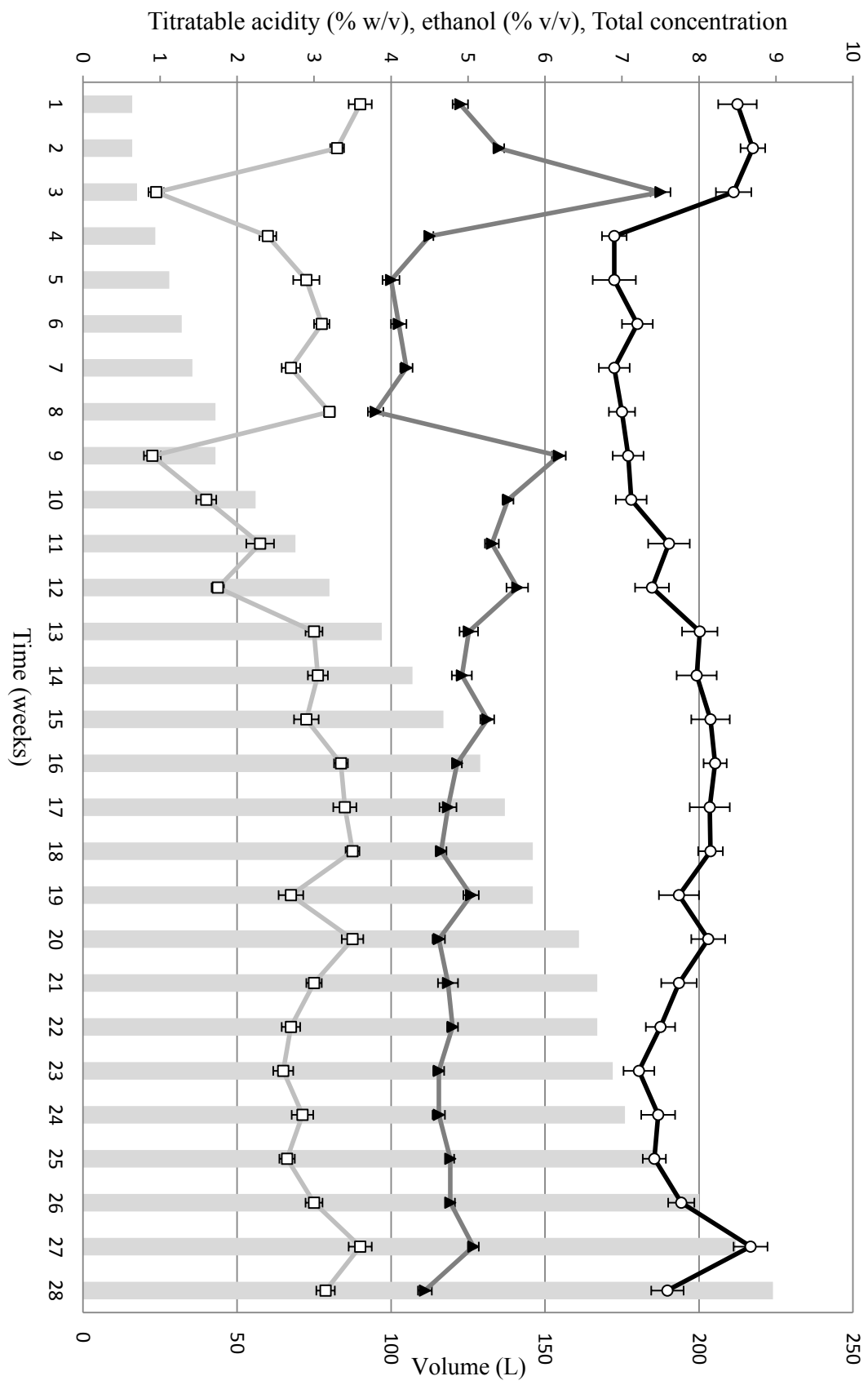


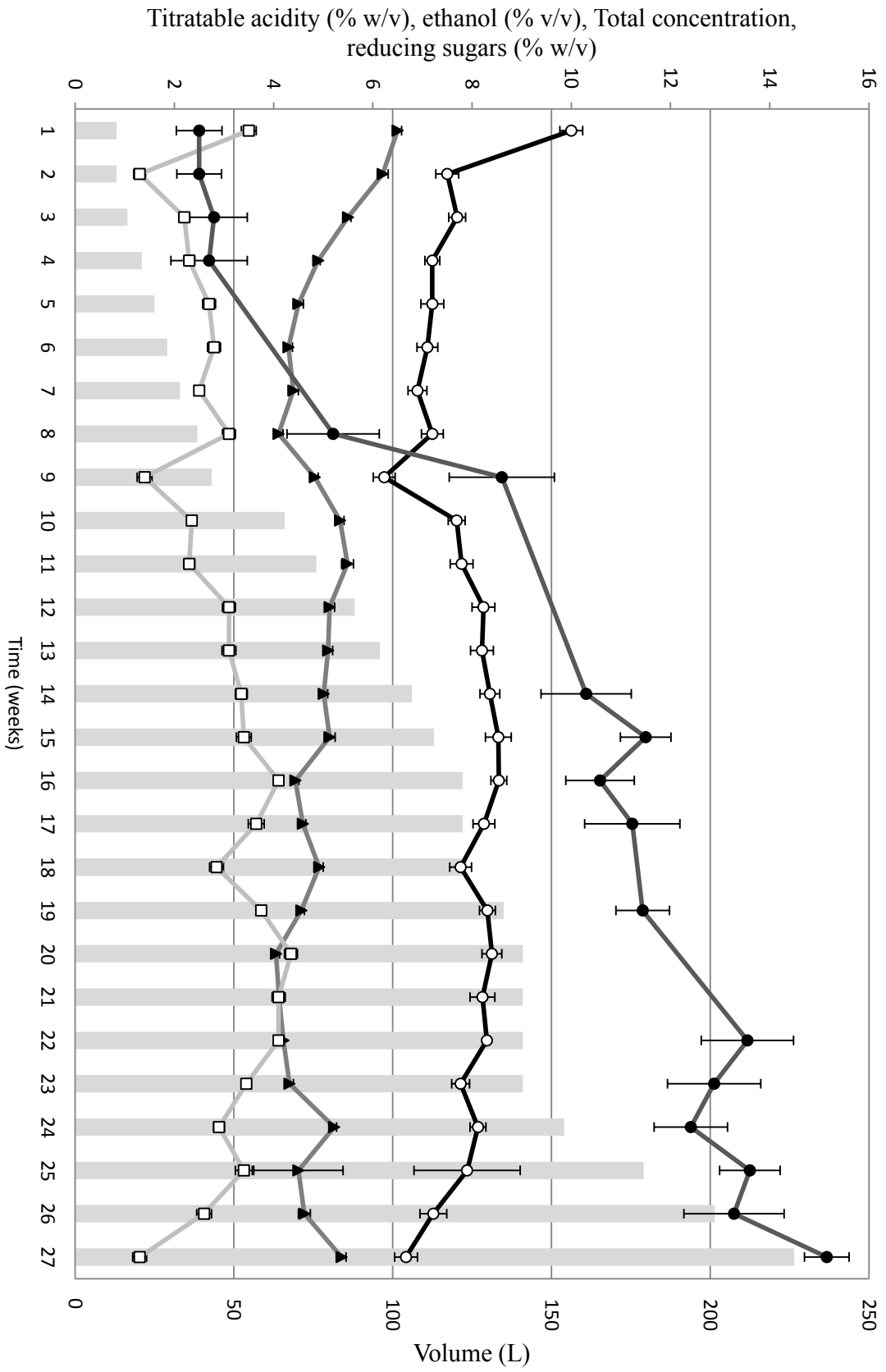












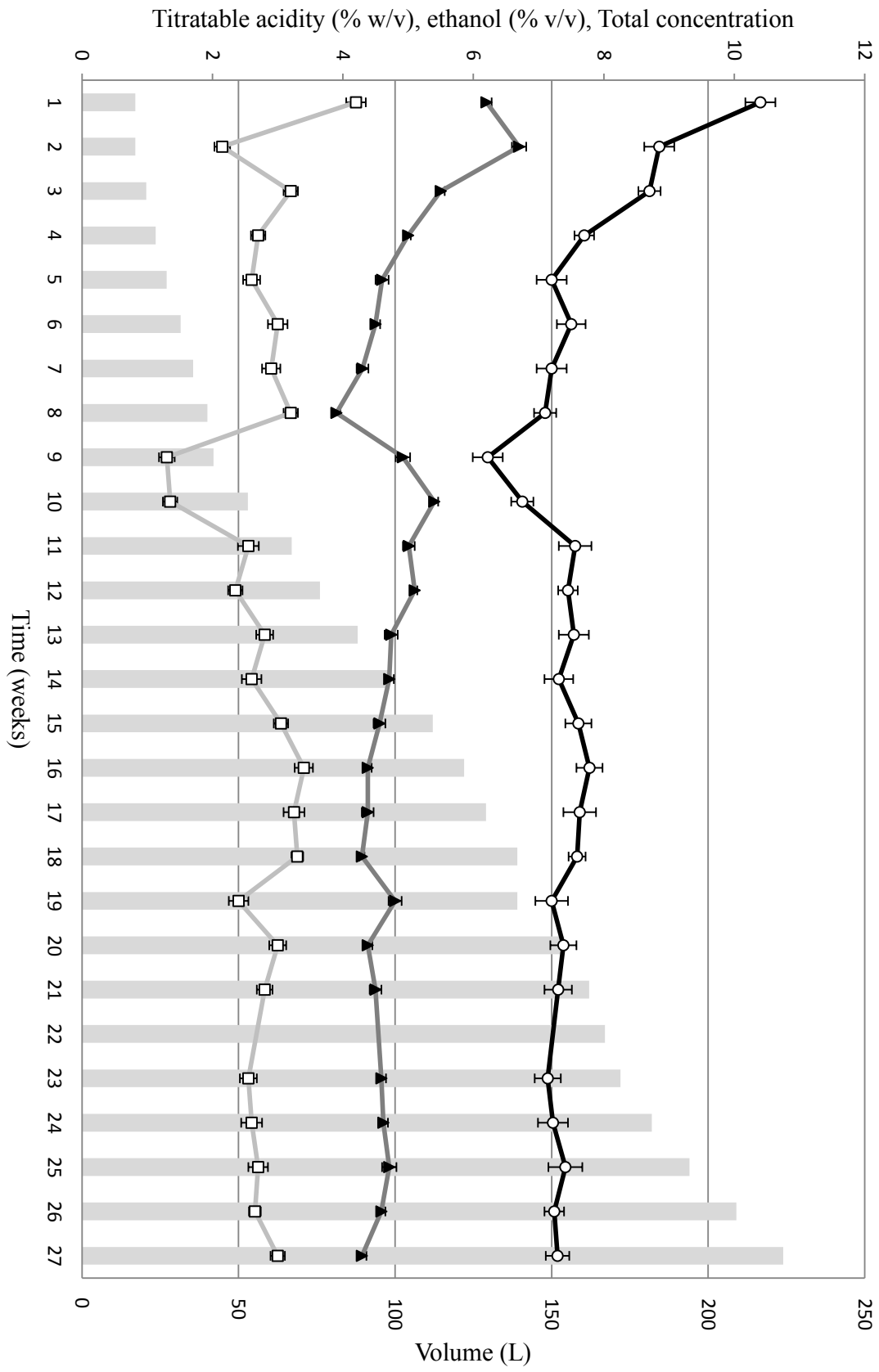


Figure 6

