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

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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 15.00–27.00 (% 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.

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1. Introduction

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

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

Previous studies have highlighted the occurrence of *Komagataeibacter europaeus* in submerged fermentations for the production of high-acidity vinegar (10–15%) and *Acetobacter pasteurianus* in vinegars that reach acetic acid contents of 6–7%. The fermentative attributes of these species are well studied with respect to acetic acid production in conventional vinegars [8,4,9,10–12]. What is less studied is the behaviour of AAB in the presence of multiple carbon sources (ethanol and glucose) in industrial conditions and how to avoid cellulose formation.

Before scaling up a bioprocess for vinegar production, basic knowledge must be gathered concerning the technological traits of AAB and the fitness of these strains over the course of cultivation [13,3,14,15]. Moreover, exploring the feasibility of fermentation parameter optimization in different culture broths will be necessary to obtain successful fermentations [16,17,18,19–24]. Finally, process scalability is a bottleneck due to the transfer of optimized fermentation conditions from small batches to large fermentations because the different operation modes interfere with microbial

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activity. In particular, the transition from laboratory to industrial scale is affected by the loss of the ability to oxidise ethanol and the loss of acetic acid resistance. These deficiencies have been observed frequently with AAB. Although little is known about the genetic background governing the instability of physiological properties such as ethanol oxidation, acetic acid resistance and cellulose formation, phenotypic modifications by transposon insertion have been previously reported in AAB [25–30].

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

2. Materials and methods

2.1. General experimental plan

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

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

2.2. Bacterial strains, culture media and phenotypic assays

Bacterial strains used in this study were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and UMCC (Unimore Microbial Culture Collection) culture collections. *A. pasteurianus* AB0220 (DSMZ 25273 = UMCC 1754) was previously isolated from vinegar [13], *A. pasteurianus* (DSMZ 3509^T) and *K. xylinus* (DSMZ 2004) were used as type and reference strains, respectively. Subcultures were recovered from preserved aliquots (−80 °C) and cultivated on GY broth (10.0% glucose and 1.0% yeast extract dissolved in deionized water, pH not adjusted). One millilitre of culture was inoculated into tubes containing 5 mL of GY. The cultures were incubated at 28 °C for 5–7 days. Cultivation on solid medium (GYC) was performed on GY supplemented with calcium carbonate (2.0%) and agar (8.0%) at 28 °C for 5–7 days. Frateur medium (30 mL/L ethanol, 1.0% yeast extract, 2.0% calcium carbonate and 2.0% agar) was used to assay acetic acid production from ethanol and over-oxidation. Filter-sterilized ethanol was added to the sterile basal medium after cooling to 50 °C. Cell shape, KOH tests and catalase production were assayed as previously reported [31]. The cellulose production test was performed by collecting the pellicles and boiling them in 4 mL of 5.0% NaOH for 2 h, according to the previously reported method [32]. The presence of cellulose was confirmed when the pellicle did not dissolve after boiling. *K. xylinus* (DSM 2004) was used as a positive control.

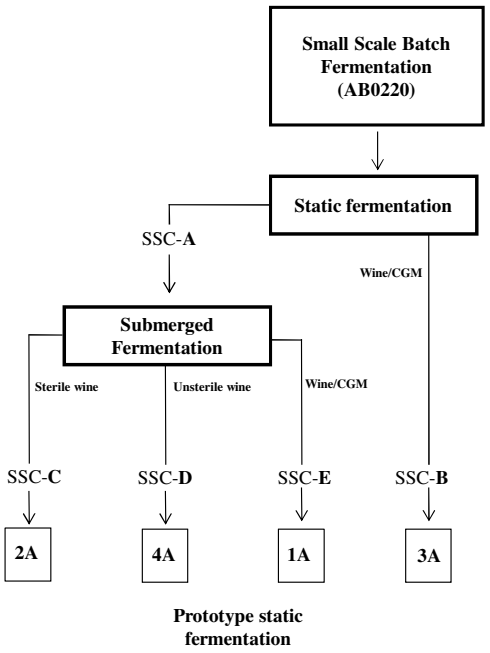

Fig. 1. Schematic of the experimental plan.

2.3. Fermentation substrates

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

Table 1
Substrates for SSC production at laboratory and prototype scales.

Substrate	pH	Titratable 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

(–) not detected.

Table 2
Substrate, fermentation mode and final parameters of the SSCs produced at the laboratory scale.

Name	Substrate	Fermentation mode	Titratable 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

(–) not detected.

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

[a] Yeast extract.
[b] Laboratory scale.
[c] Prototype scale.

substrates were stored at 4 °C until use. To produce SSC-E, a special mash composed of unfiltered wine and CGM was used. The mixture was prepared appropriately to limit the sugar concentration to a range of 20–30%.

2.4. Analytical methods

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

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

2.5. Small-scale batch cultivations and static fermentations

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

2.6. Submerged fermentation

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

2.7. Prototype-scale fermentation

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

2.8. Genomic DNA extraction and typing

Genomic DNA (gDNA) from the strain cultures was extracted using a sodium dodecyl sulfate (SDS) proteinase-cetyltrimethyl ammonium bromide (CTAB) treatment as previously reported [13]. Samples from the prototype system were collected in triplicate from three different points on the tank's surface, streaked on GYC and incubated at 28 °C for 3 days. gDNA was extracted from colonies recovered on plates as previously described. gDNA was visualized by electrophoresis on agarose (Fisher Molecular Biology) gels (1% in 0.5 × TBE buffer) stained with ethidium bromide (0.1 μg/mL) under UV light. Quantification was performed with a spectrophotometer (NanoDrop ND-1000). A 260/280 nm absorption ratio between 1.7 and 2.0 was used to assess the purity of the gDNA. (GTG)5-PCR fingerprinting was performed according to [35] with some modifications. (GTG)5-PCR reproducibility was tested by amplifying gDNA from randomly chosen strains several times. In addition, each PCR mixture was controlled for reproducibility by the inclusion of *A. pasteurianus* 3509^T gDNA. Genomic DNA was titrated to optimize the PCR amplification for a given reaction. No mineral oil was added to the PCRs. Each PCR run contained a negative control (water instead of gDNA). The PCRs were performed in a BioRad thermocycler (My-Thermal Cycler). The GeneRuler 100 bp DNA Ladder Plus molecular marker (Thermo Scientific, Carlsbad, CA, USA) was used to deduce the size of the templates. Digital images were generated in a BioDocAnalyze system (BDA; Germany).

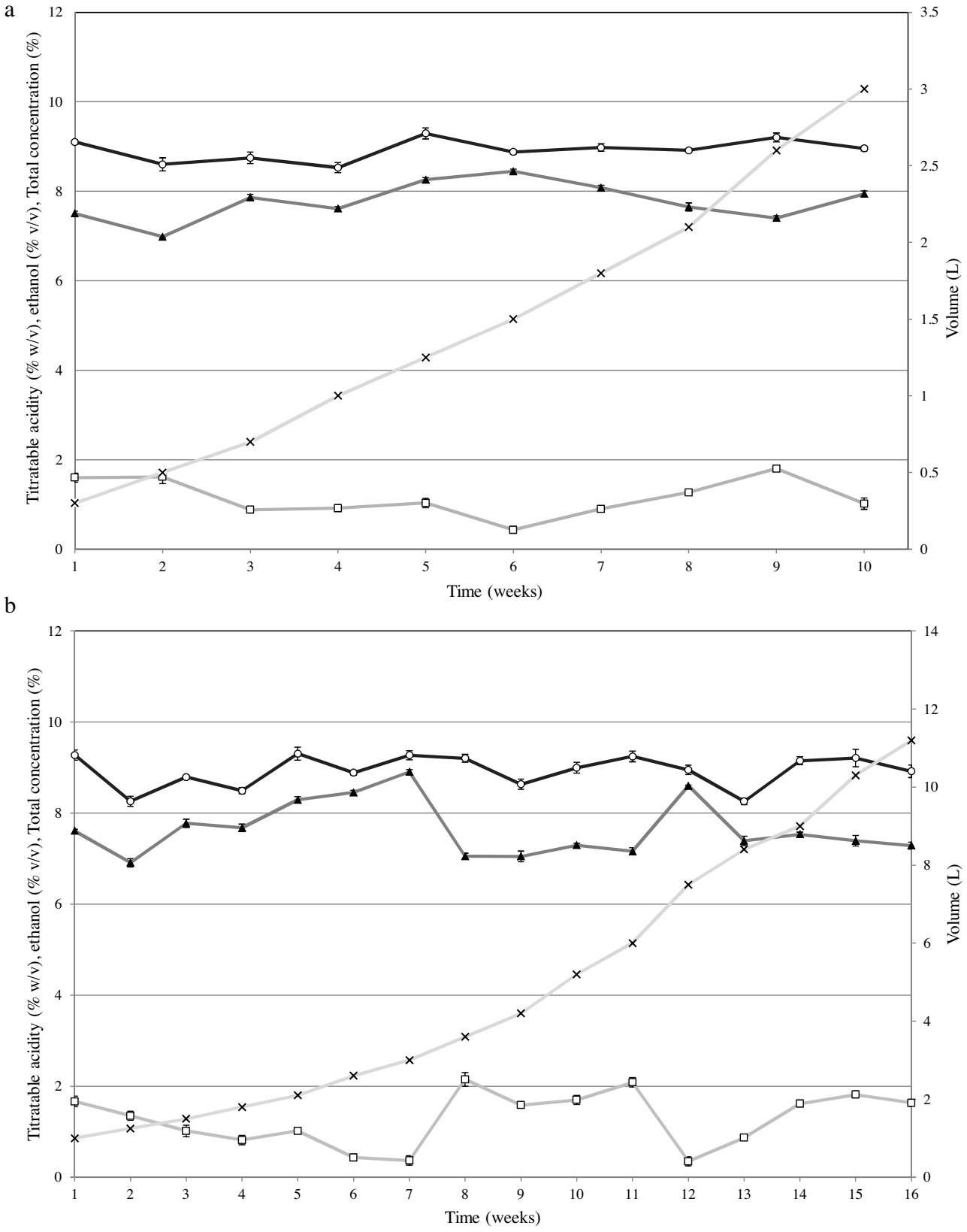

Fig. 2. Trends in analytical parameters during static fermentation (a, b: Selected starter culture-A and Selected starter culture-B). Symbols: (□) ethanol; (▲) titratable acidity; (O) total concentration; (×) volume. Each value is the mean of three parallel replicates ± standard deviation.

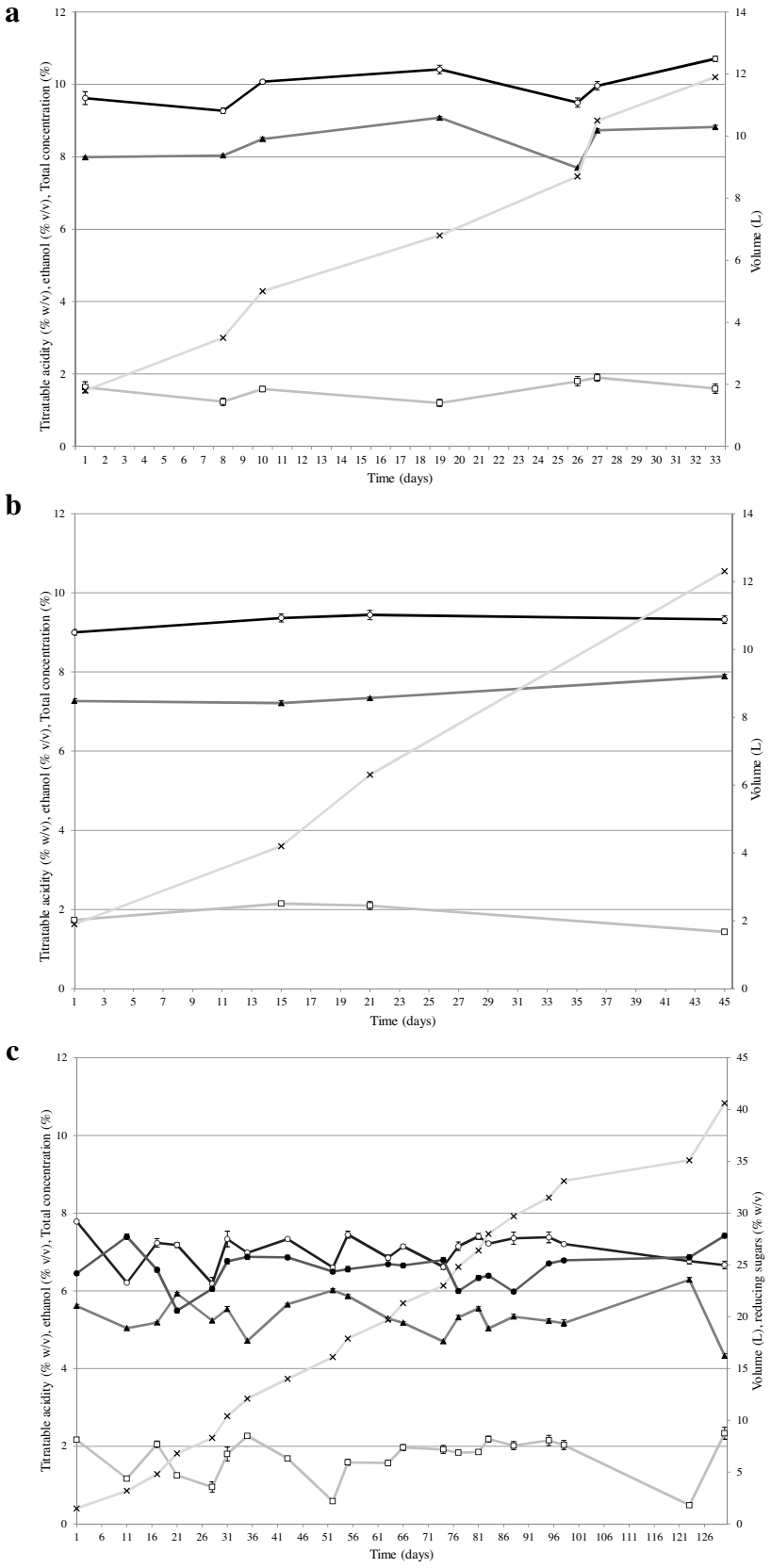

Fig. 3. Semi-continuous fermentation in the submerged system. Trend in analytical parameters during culture scale-ups with different acetification substrates (a–c: Selected starter culture-C, Selected starter culture-D and Selected starter culture-E). Symbols: (□) ethanol; (▲) titratable acidity; (O) total concentration; (×) discharged volume; reducing sugars (●). Each value is the mean of three parallel replicates ± standard deviation.

Table 4
Substrates and final parameters of the prototype-scale batches.

Batch	SSC	Substrate	Titratable 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

(–) not detected.

Table 5
Experimental data for the semi-batch cycles in the submerged mode.

Cycle	Substrate/SSC	Experiment time (h)	Titratable acidity (%w/v)		Ethanol (%v/v)		Acetification Rate (%day^{-1})	Stoichiometric yield (%)
			Initial	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

3. Results and discussion

3.1. Selected starter cultures produced in static fermentation mode

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

To produce a set of vinegars with the parameters detailed in the general experimental plan, first, two SSCs (SSC-A and SSC-B) were produced via small-scale batch cultivation and static fermentation under batch conditions at 28 °C and using the acetification substrates reported in Table 1. SSC-A was started from the strain re-cultivated on GY and then on wine. In contrast, SSC-B was developed from an aliquot of SSC-A after recursive cultivation on wine as the substrate. The final SSC parameters are reported in Table 2.

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

3.2. Selected starter cultures produced in submerged fermentation mode

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

During the first 7 days (start-up), titratable acidity remained stable, whereas the concentration of ethanol declined slight, mainly due to evaporation. The fermentation phase started on the 8th day, and in less than 24 h, almost all of the remaining ethanol was oxidized to acetic acid. According to the trials performed in this study, the start-up was set at 4.6% (v/v) ethanol and 6.0% (w/v) acetic acid. The temperature was kept constant at 30 °C, and the airflow was set to 40 L/h.

To evaluate culture performance in a submerged system, three fermentative cycles of SSC-C were studied in detail. Each cycle was started at 6.0% (w/v) titratable acidity and 4.4% (v/v) ethanol and were considered finished when the titratable acidity reached at least 8.50% (w/v). The efficiency of the fermentation process, expressed as the vinegar stoichiometric yield, was in the range of 93–95% (Table 5). Consistent with previous studies [36], ethanol loss by evaporation was observed because the process was performed without a volatile compound recovery system.

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

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

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

Four fermentation batches (1A, 2A, 3A, and 4A) were developed from the SSCs (B, C, D and E) and were transferred to the prototype scale for obtaining vinegars with different compositions (Table 4).

When the batches reached the maximum volume (approximately 220 L), the discharged vinegars were transferred to four additional vessels prior to successive refilling with the substrate. Start-up required approximately one week for all batches. The respective substrates were added weekly to support acetic acid fermentation.

A total of more than 200 Litres of vinegar per fermenter were produced and were subjected to downstream processing to obtain final products with different acetic acid, sugar and residual ethanol contents. The final analytical values of the cultures are shown in Table 4 (the data refer to the last filling step), whereas the scale-up trends

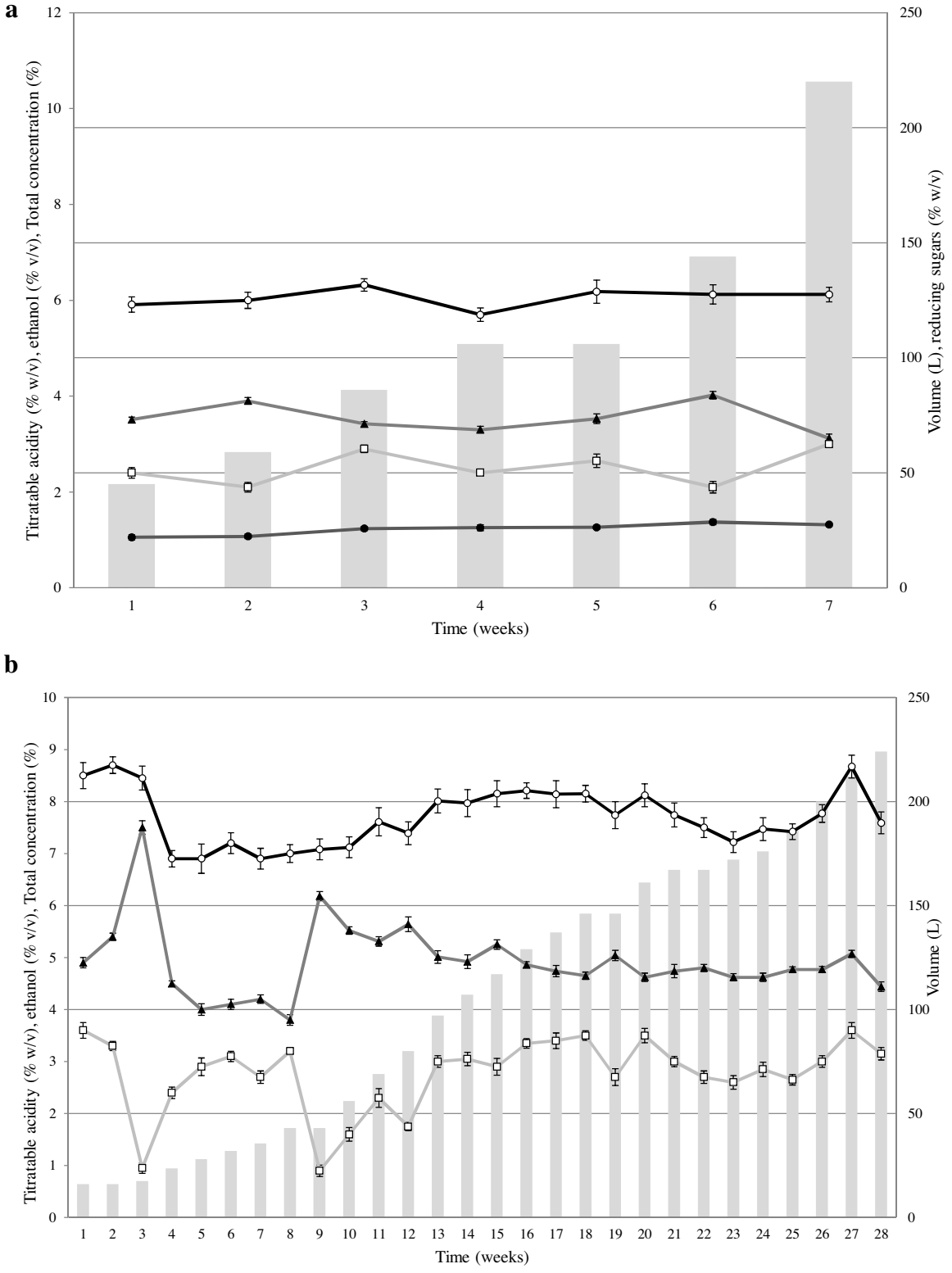

Fig. 4. Prototype-scale fermentation in the static system. Trends in analytical parameters during culture scale-ups with different acetification substrates (a–d: 1A, 2A, 3A and 4A). Symbols: (□) ethanol; (▲) titratable acidity; (O) total concentration; (×) volume; reducing sugars (●). Each value is the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

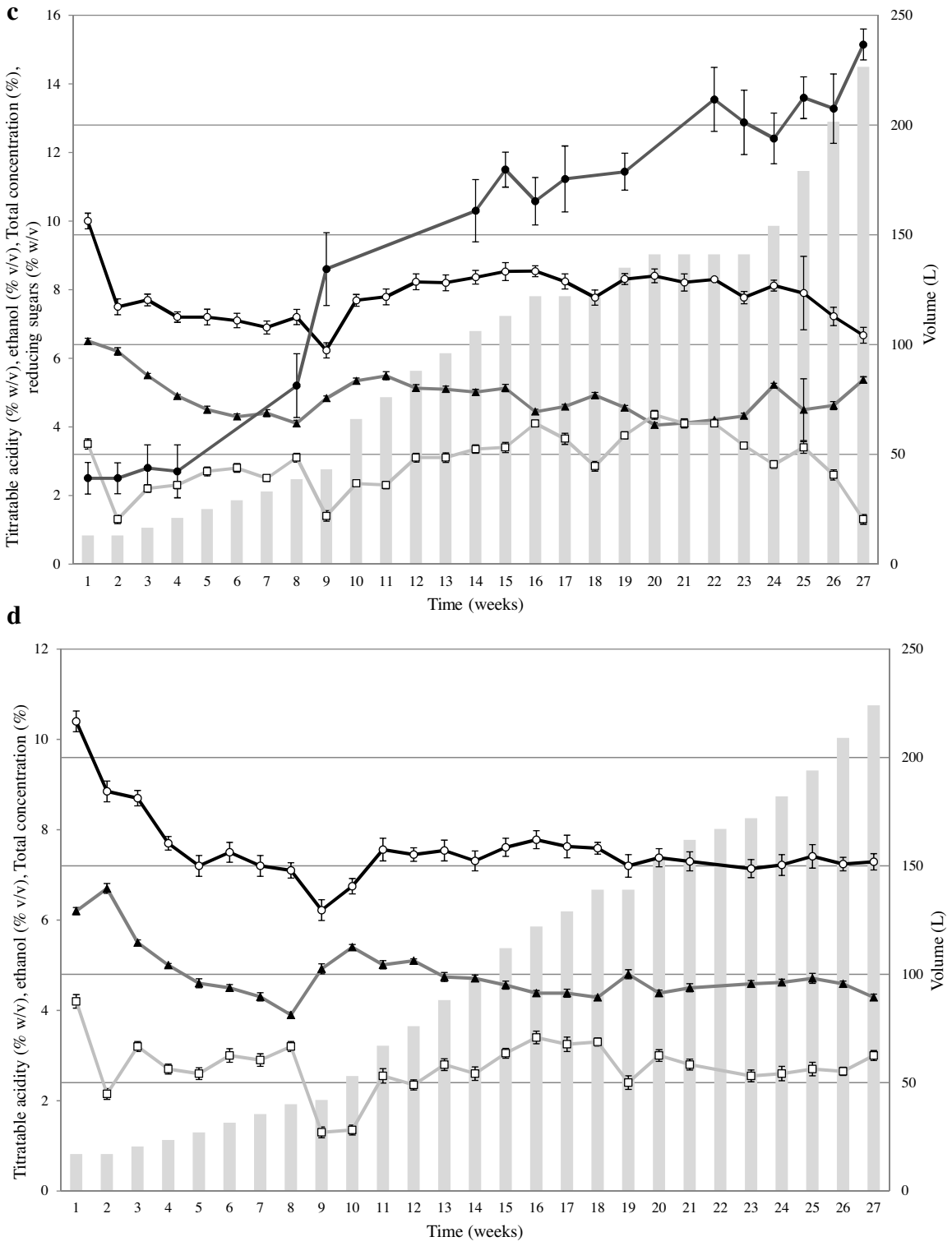

Fig. 4. (*Continued*)

are shown in Figs. 4a–d. The entire process lasted approximately seven months for batches 2A, 3A and 4A and approximately two months for batch 1A. It must be noted that SSC-E, used to start batch 1A, was obtained in submerged operation mode using CGM as the substrate (over 20.0% w/v reducing sugar). Thus, when the culture was transferred to the prototype scale, it was already adapted to a high-sugar-content substrate. Moreover, batch 1A was considerably larger (45 L) compared with the other batches (13, 16 and 17 L, respectively). Considering batches 1A and 3A, produced to obtain vinegars having residual sugars higher than conventional vinegars, the batch 1A provided the best result (more than 27% w/v of residual sugars). It must be taken into account that, differently from conventional vinegars for which the main quality criterion is the amount of acetic acid produced, in the case of vinegars rich in sugars, the main parameter is the residual sugar content. Accordingly batch 1A allowed to obtain high sugar content, acetic acid and ethanol

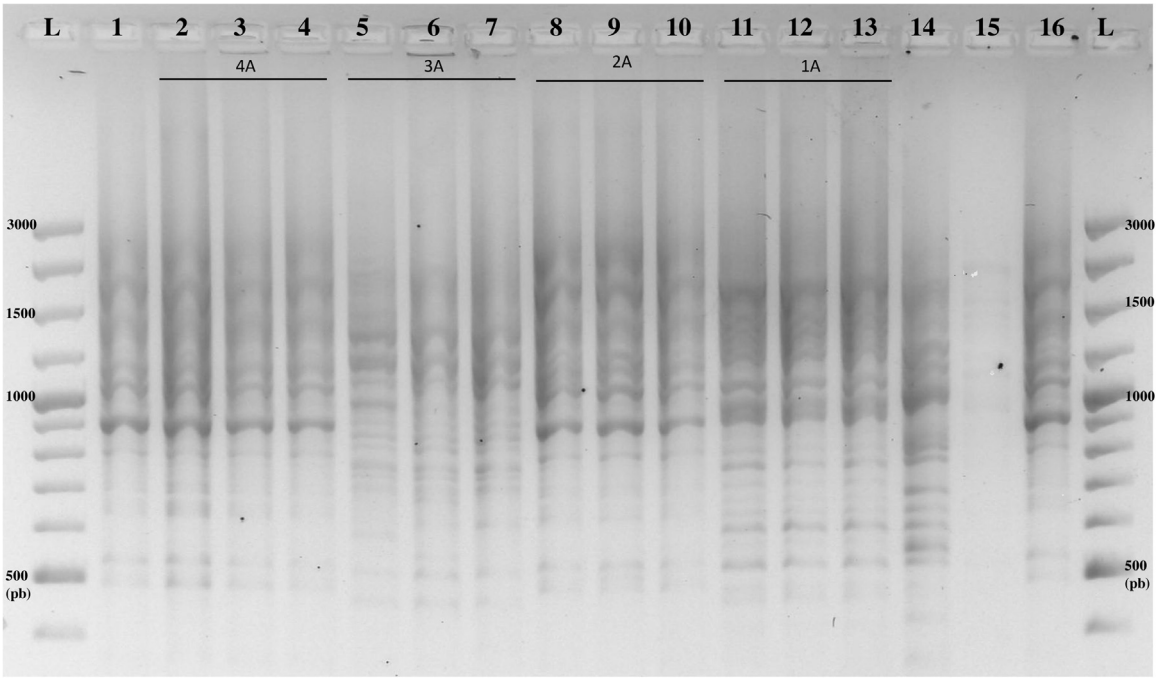

Fig. 5. (GTG)5-PCR fingerprinting patterns. L: 100 bp Plus DNA Ladder (Thermo Scientific, Carlsbad, CA, USA); 1: AB0220 (culture strain); 2–4: (triplicates of sample 4A); 5–7: (triplicates of sample 3A); 8–10: (triplicates of sample 2A); 11–13: (triplicates of sample 1A); 14: AB0220; 15: negative control;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

content suitable to prevent spoiling by other organisms (Table 4). Therefore the procedure to obtain batch 1A was the best one for the production of vinegars rich in sugar.

3.4. Dominance of the microbial culture over fermentation time

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

Assays were conducted on biofilms recovered from GYC plates. The cells were rod-shaped, KOH-positive and catalase-positive. Cultivation on GYC medium showed vigorous growth mainly as a biofilm spread across the plate's surface. The oxidation of ethanol to acetic acid and acetate assimilation were shown on Frateur medium by a clear halo around bacterial growth, followed by the reappearance of opacity on the bottom of the plates due to acetate oxidation. No cellulose production was observed in samples except for batch 3A, for which the analysis of the exopolysaccharide-containing pellicle confirmed the presence of cellulose.

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

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

As observed in a previous study [34], our hypothesis is that an indigenous strain became dominant at the prototype scale as a consequence of supplying a mixture of wine and CGM as the substrate at ethanol values below 2.0% (v/v). It is interesting to note that the strain used as SSC was active in batch 1A, which derived from a submerged fermentation containing 27.0% (w/v) of reducing sugars. Instead, a loss of dominance was observed in batch 3A, which had been produced in static conditions at a lower sugar concentration (15.15% (w/v)). Moreover, the length of the process was shorter for batch 1A (2 months) than for batch 3A (6 months). The operation mode to obtain each batch was also different; the main variation concerned the step at which the CGM was added. In particular, batch 1A was developed from SSC-E; thus the culture was adapted to a high-sugar environment during growth in the submerged system. Batch 3A was obtained from SSC-B cultured in static conditions and using wine as the substrate. At the prototype-scale, batch 3A was scaled up using wine as the substrate for the first 5 months, and from month 6 onwards, a mash containing wine and CGM was added, reaching 15% reducing sugars, 5.5% (w/v) acetic acid and approximately 1.0% (v/v) ethanol.

Ethanol depletion (< 2.0% (v/v)), corresponded to the addition of glucose- and fructose-containing CGM, which may have induced

cellulose formation from glucose. Although a cellulose layer was detected in batch 3A, the final product reached the desired acetic acid content (> 5.0% (w/v)). On the basis of these observations, the indigenous strain that conducted the last phases of fermentation was able to produce both acetic acid and cellulose, as in the case of *K. xylinus*.

Indeed, strains of this species are able to produce both acetic acid and cellulose when growing in vinegar environments. In this species, acetic acid produced periplasmically can accumulate in the surrounding liquid or enter the cell. The acetate in the cell is fed by extracellular acetic acid, and it can be excreted or phosphorylated into acyl-phosphate, which is transformed into acetyl-coenzyme A to feed the tricarboxylic acid cycle [41]. Oxaloacetate produced by the glyoxylate shunt is decarboxylated into pyruvate, forming glucose 6-phosphate (via the gluconeogenesis pathway). Then, pyruvate is used by the gluconeogenesis pathway to produce glucose, that is the building block of cellulose [42]. Glucose 6-phosphate and fructose 6-phosphate, which are freely interconverted by a phosphohexose isomerase, feed a glucan-synthase enzyme complex that permits cellulose biosynthesis from glucose 6-phosphate [43]. Therefore, with different energy balances, both acetic acid and cellulose can be produced from the diauxic consumption of ethanol and glucose as carbon sources.

4. Conclusions

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

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

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