

Saccharomyces cerevisiae Biodiversity During the Brewing Process of an Artisanal Beer: A Preliminary Study

Luca Cocolin*, Simona Campolongo, Roberta Gorra, Luca Rolle
and Kalliopi Rantsiou

ABSTRACT

J. Inst. Brew. 117(3), 352–358, 2011

In this study we investigated the biodiversity of *Saccharomyces cerevisiae* during the brewing of an artisanal beer, as well as during its storage in the bottle for 107 days at 20°C. After inoculation with an active dried yeast (ADY), the yeast counts were followed during fermentation and after bottling. Yeast loads remained stable at 10⁶–10⁷ colony forming units (cfu)/mL, and only after day 21, were they reduced to 10⁴ cfu/mL. After three months in the bottle they spanned 10²–10⁵ cfu/mL. Almost all isolated yeasts were identified as *S. cerevisiae* and after molecular characterization, unexpected results were obtained. The ADY did not take over the fermentation process and only after 21 days did isolates from the beer share similarities with the inoculated strain. During storage, a high diversity was found, underlining that each bottle developed its own micro-ecosystem. This study highlighted the necessity for better investigations of *S. cerevisiae* population dynamics during artisanal brewing. Even when the chemical parameters measured confirmed a correct fermentation process, the inoculated strain was not the main yeast involved in the fermentation and consequently, the final product may have different sensory characteristics from the ones expected by the producers.

Key words: beer, biodiversity, *Saccharomyces cerevisiae*, SAU-PCR.

INTRODUCTION

Beer production is an ancient fermentation process in which the ingredients, such as malt (mainly from barley, but also other cereals malted and unmalted), hops, water and yeast, are managed through the malting, mashing, and fermentation steps. After steeping, germination and kilning¹⁰, the malt is subjected to boiling followed by an inoculation with fermenting yeasts⁷.

Undoubtedly, the *Saccharomyces sensu stricto* species complex²², containing some of the most relevant species and strains for the fermentation industry, represents the main group to refer to for the selection of yeasts to be

used in beer production. Specifically, *S. cerevisiae* and *S. pastorianus* (synonym *S. carlsbergensis*, a strain obtained from a natural interspecific fusion-cross between *S. cerevisiae* and *S. bayanus*), are the two species that most often are used as starter cultures in breweries¹⁴. Generally, a distinction between top-fermenting yeasts (mainly belonging to *S. cerevisiae*) producing ale beers, and bottom-fermenting yeasts (mainly belonging to *S. carlsbergensis*) responsible for the fermentation of lager beers, is made, and this differentiation is also correlated to the temperature variations used during the fermentation process¹¹.

Based on the afore-mentioned parameters, the choice of the yeasts to inoculate is crucial in beer production, as they possess important characteristics that affect beer flavour and demand different technological adjustments. Overall, carbohydrate fermentation must be prompt and rapid, the yeast should possess appropriate flocculation and sedimentation characteristics, genetic stability, osmotolerance, ethanol tolerance and the ability to produce esters, higher alcohols and flavours. Moreover, high cell viability for repeated pitching as well as temperature tolerance must also be evaluated⁷.

The capability of a yeast strain to dominate the fermentation process is of primary importance for the production of a beer with desired and standardized sensory and stability characteristics. Because of this, a study of the microbial ecology and dynamics during brewing represents a focal point for quality assurance. Nowadays, it is becoming more and more relevant to be able to assess the dominance of the inoculated yeast strain. In the past, this aspect was monitored by using traditional microbiological techniques, such as the plate count, and it was assumed that if the yeast cell number increased promptly from the beginning of the fermentation, then the inoculated strain was able to predominate. With the application of molecular biology techniques in the field of the food and beverage fermentations, a number of new methods have become available, and these can be used in order to comprehend the strain dynamics during fermentation. This approach is becoming common practice in other fermentations, such as wine (with application of mitochondrial DNA restriction fragment length polymorphisms³, randomly amplified polymorphic DNA (RAPD)-PCR²⁴ and minisatellite¹⁷). To our knowledge, no study focusing on the monitoring of the inoculated strain during artisanal brewing has been published.

DIVAPRA, Agricultural Microbiology and Food Technology Sector, Faculty of Agriculture, University of Turin, Italy.

* Corresponding author. E-mail: lucasimone.cocolin@unito.it

Publication no. G-2011-0930-1146

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The aim of this study was i) to investigate fermentation dynamics and pitching yeast performances in a craft beer produced by a micro-brewery in the Northwest of Italy and ii) to assess the starter culture dominance, during fermentation and after the bottling, by molecular characterization of isolated yeasts throughout the process using SAU-PCR.

MATERIALS AND METHODS

Fermentation process and sampling procedures

One batch of 44 hL of malted barley wort, for the production of an ale-style beer, was employed. After mashing and boiling, the temperature was reduced to 24°C and a commercial active dried yeast (*S. cerevisiae*) (ADY), routinely used by the micro-brewery, was inoculated to reach an estimated cell count of 10⁷ colony forming units (cfu)/mL. Primary fermentation was conducted for 8 days at 24°C and the secondary fermentation (maturation) was carried out for 14 days at 4°C. Both processes took place in a stainless steel fermentor, subjected to a clean-in-place (CIP) process before bottle filling. Bottling took place immediately after the secondary fermentation process, in bottles of 0.75 L, after filtration through a 10 µm filter, and the addition of 6.5 g/L fructose. The bottles were left to rest at 20°C in a controlled temperature room, and they were analyzed over a period of three months. Usually beer is sold by the micro-brewery after 30 days (after the bottling); however in this work the bottles were kept longer in order to define yeast viability during storage.

Sampling, in triplicate, was performed immediately after the boiling process, after 3 h from the inoculation of the ADY, and at days 1, 4, 7 and 8 (primary fermentation), and after 14 days of maturation (secondary fermentation). After bottling, 3 bottles were sampled at 0 (immediately after filling), 2, 4, 7, 9, 15, 48, 78 and 107 days of storage at 20°C.

The wort before inoculation was at pH 5.6 and 18.3°P.

Microbiological counts

Yeast counts were determined by plating the appropriate beer sample dilutions, prepared in Ringer solution (Oxoid, Milan, Italy), on WLN medium (Oxoid, Milan, Italy), incubated at 30°C for 3–5 days. After this period, colonies with a morphology and colour resembling *S. cerevisiae*²⁴ were counted, and means and standard deviations were calculated. From each sample considered in this study, 10 yeast colonies, with *S. cerevisiae* colony morphology, were randomly selected, streaked onto YPD agar (yeast extract 1% [w/v], bacteriological peptone 2% [w/v], dextrose 1% [w/v], and agar 1.5% [w/v], all from Oxoid) and stored at –20°C in YPD broth containing 30% (v/v) glycerol (Sigma, Milan, Italy) before being subjected to molecular identification and characterization. A total of 170 colonies were isolated: 80 from the fermentation tank (primary and secondary fermentation) and 90 from the bottles during storage. Lastly, 15 colonies were also isolated from the ADY, after its rehydration and plating on WLN medium as described above.

Molecular identification and characterization of the isolates

Isolates coming from the ADY, the fermentation (primary and secondary) and the bottles during storage were

subjected to molecular identification through the use of Denaturing Gradient Gel Electrophoresis (DGGE), species specific PCR and 26S rRNA gene sequencing. DNA was extracted by using a mechanical treatment in a beat-beater machine (Fast-Prep®-24 Instrument, MP Biomedicals, Illkirch, France) following the protocol developed by Cocolin et al.⁵. After precipitation, the DNA was resuspended in 50 µL of DNase/RNase free water (Sigma) and quantified by using the NanoDrop instrument (Celbio, Milan, Italy). DNA from the isolates was standardized at 100 ng/µL and stored at –20°C for further applications. First, isolates were amplified by using the primers NL1GC (5'-CGC CCG CCG CCG CGC GGC GGC GGC GCG GGG GCA CGG GGG CCA TAT CAA TAA GCG GAG GAA AAG-3') and LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') and used for DGGE analysis in order to group isolates with identical electrophoretic profile. The protocols used were as described by Cocolin et al.⁵. Strains migrating as *S. cerevisiae* DBVPG 6173 (used as migration control) were confirmed in the identification through the amplification of the Intergenic Spacer Regions (ITS) by using the species specific primers Schaf (5'-GTA GTG AGT GAT ACT CTT-3') and Schar (5'-AGA ACA TGT TGC CTA GAC-3') as reported by Manzano et al.¹⁵. Lastly, isolates that did not migrate as *S. cerevisiae* and were not amplified by the specific primers, were subjected to the sequencing of the D1-D2 loop of the 26S rRNA encoding gene after its amplification with the primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') as previously described¹³.

Isolates belonging to the *S. cerevisiae* species were molecularly characterized by SAU-PCR following the procedure described by Cocolin et al.⁶, with the exception of the primer used being SCA2 (5'-CCG CCG CGA TCG T-3')⁸ instead of SAG1. SAU-PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gels in 0.5X TBE at 120 V for 4 h. Gels were stained in 0.5X TBE buffer containing 0.5 µg/mL of ethidium bromide (Sigma) for 20 to 30 min. Pictures of the gels were digitally captured by BioImaging System GeneGenius (SynGene), and BioNumerics software (Applied Maths, Kortrijk, Belgium) was used for pattern analysis. The calculation of similarities in the profiles of bands was based on the Pearson product-moment correlation coefficient. Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm²³. A coefficient of correlation of 70% was arbitrarily selected to distinguish the clusters. Yeast isolates were subjected to SAU-PCR analysis at least twice.

Chemical analysis

Principal chemical compounds, maltose, glucose, fructose, glycerol and ethanol were determined by high performance liquid chromatography (HPLC) as described by Castellari et al.⁴. Samples were centrifuged for 5 min (7,000 rpm) and the supernatant was filtered through a 0.20 µm disposable syringe membrane filter (Sartorius AG, Göttingen, Germany). Analysis was carried out using a chromatograph consisting of a P100 pump, an AS3000 auto-sampler (Spectra Physics Analytical, Inc, San Jose, CA, USA), a refractive index detector (RI-150) and a

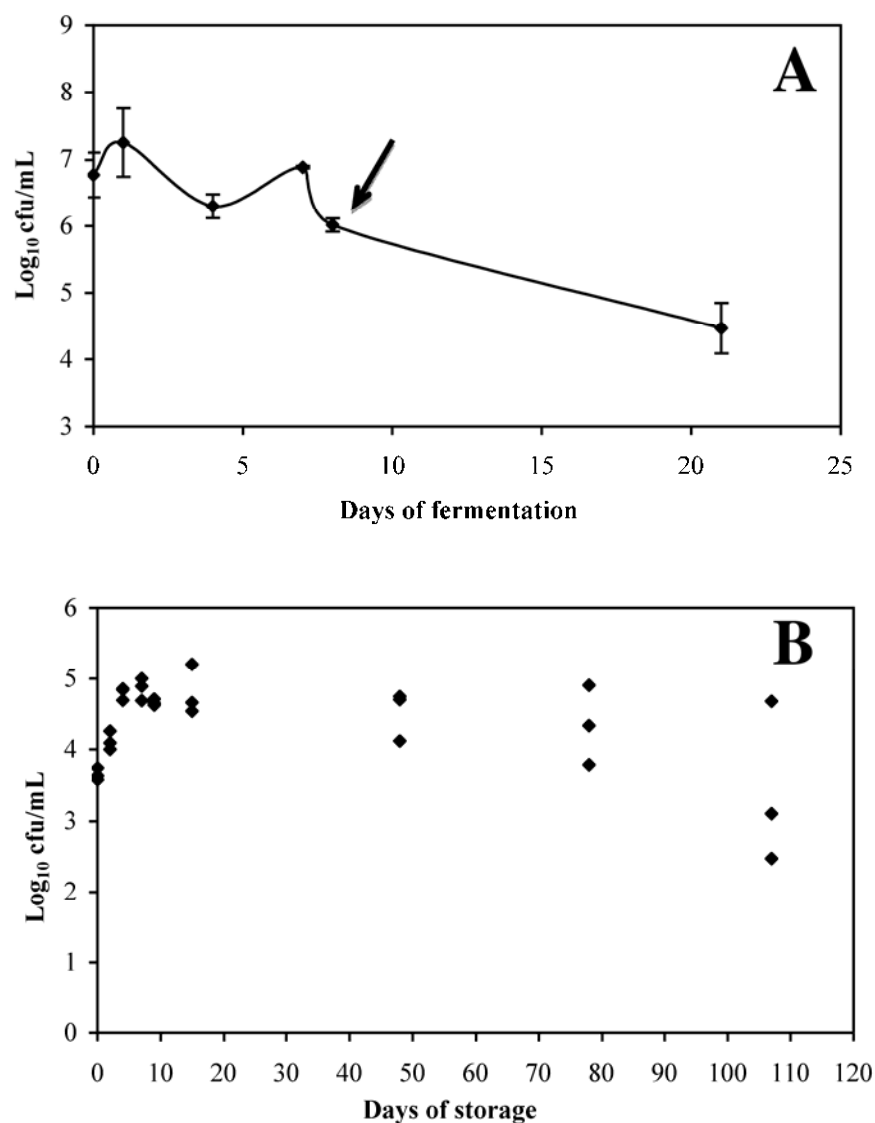


Fig. 1. (A) Yeast counts as determined on WLN media during the fermentation and maturation process. The arrow indicates the beginning of the maturation period when the temperature was reduced to 4°C. (B) Yeast counts during storage of the beer after bottling. At each sample point, three bottles were analyzed.

Reodyne injection valve equipped with a 20 µL sample loop. Chromatographic separations were performed on an Aminex HPX-87H column 300 × 7.8 mm i.d. cation exchange column equipped with a Cation H⁺ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA).

Isocratic elution at a flow rate of 0.5 mL/min and 45°C was carried out using a mixture of 0.0045N sulphuric acid and acetonitrile (6%, v/v). Peak detection was made using the cells of the RI detector kept at 40°C. Solvents were filtered through a 0.20 µm membrane filter and degassed under vacuum. The injection volume was 20 µL. Data treatment was carried out using the ChromQuest™ chromatography data system (ThermoQuest, Inc, San Jose, CA, USA). Peak identification was carried out using pure standards. HPLC-grade solvents and all other chemicals were purchased from Sigma. Solutions were prepared in

deionised water produced by a Purelab Classic system (Elga Labwater, Marlow, United Kingdom).

RESULTS AND DISCUSSION

Beer fermentation is an ancient microbial process and its production pre-dates to the Sumarians in 1800 BC¹². Handling and management of starter cultures is essential for the completion of the fermentation and in order to reach specific sensory characteristics in the final product¹⁴. Based on these considerations, the study of *S. cerevisiae* dynamics and diversity during beer production should be a requisite in order to assess yeast performance. In the past traditional microbiological techniques used could not completely answer the question of which strains were conducting the fermentation and, at best, biochemi-

Table I. Results of the chemical analysis carried out during the fermentation and maturation of the beer followed in this study.

| Sampling days | Maltose (g/L) | Glucose (g/L) | Fructose (g/L) | Glycerol (g/L) | Ethanol (mL/L) |
|----------------|---------------|---------------|-----------------|----------------|----------------|
| 0 ^a | 76.58 ± 0.89 | 21.50 ± 0.22 | 14.07 ± 0.18 | 0.24 ± 0.07 | 1.71 ± 0.32 |
| 1 | 53.78 ± 0.55 | 2.57 ± 0.09 | 5.67 ± 0.10 | 2.24 ± 0.09 | 32.10 ± 0.24 |
| 4 | 1.25 ± 0.11 | 0.10 ± 0.03 | nd ^b | 3.61 ± 0.12 | 68.33 ± 0.26 |
| 7 | 0.69 ± 0.15 | 0.09 ± 0.02 | nd | 3.54 ± 0.10 | 69.21 ± 0.18 |
| 8 | 0.76 ± 0.10 | 0.07 ± 0.01 | nd | 3.37 ± 0.08 | 69.34 ± 0.17 |
| 22 | 0.81 ± 0.12 | 0.10 ± 0.01 | nd | 3.33 ± 0.08 | 70.52 ± 0.15 |

^a 3 h after yeast inoculation, average ± standard deviation (n = 3).

^b Not determined.

cal characterization would identify the species of *Saccharomyces* involved. Currently, advancements in molecular biology have allowed for several methods to become available that permit a more detailed description of yeast dynamics during a fermentation. Molecular methods have been used to identify contaminating yeasts in breweries^{1,19}, to delimitate the brewing yeast strains²⁰ and to attempt to differentiate *S. cerevisiae* strains^{9,16}. It is surprising that there are few publications regarding *S. cerevisiae* strain dynamics and biodiversity during the brewing process. Müller and Lösche¹⁸ investigated the performance of a commercial strain of *S. cerevisiae* during three successive cycles of a brewing yeast by the means of flow cytometry and reported the formation of various subpopulations, characterized by changes in structural and functional parameters.

In this study, craft beer production in the Northwest part of Italy was followed from a microbiological and chemical point of view. Moreover, strains of *S. cerevisiae*, as well as the active dry yeast (the ADY inoculated), were monitored throughout the process and characterized molecularly by the use of the SAU-PCR technique. This technique, first developed for prokaryotes⁸, was recently applied for the study of the biodiversity of yeasts, such as *S. cerevisiae*⁶ and *Brettanomyces bruxellensis*².

The yeast counts during the 8 days of fermentation, after maturation at 4°C, as well as during storage in bottles for 107 days, are shown in Fig. 1A and 1B, respectively. Wort after boiling and before inoculation of the ADY had a yeast count of about 100 cfu/mL (data not shown) and ADY was inoculated to reach a count of 10⁷ cfu/mL. ADY presented an initial count of about 5 × 10¹⁰ cfu/g and was inoculated to reach a load of 10⁷ cfu/mL. Counts remained stable throughout the process and at the end of the primary fermentation they were 10⁶ cfu/mL. After this period, the temperature was decreased to 4°C and the beer was matured until day 21, when it was filtered and bottled. After maturation, the counts were ~10⁴ cfu/mL and filtration did not affect the final number of yeasts that the beer contained. Immediately before bottling, the beer was supplemented with fructose in order to start a mild, “in bottle” fermentation, and the bottles kept at 20°C. As shown in Fig. 1B, yeast increased in the first days after bottling and reached a count of about 10⁵ cfu/mL, and the count remained stable for about 80 days. After 107 days (last sampling point), the bottles had counts ranging from 10²–10⁵ cfu/mL, suggesting that the yeast dynamics were specific to each bottle. To this respect, the sugar consumption rate could be different in each bottle explaining the different death rates during storage.

The results of the chemical analysis are presented in Table I. They reflect the yeast counts described above, with the sugars maltose, glucose and fructose completely consumed in the first 4 days of fermentation. Also, the major end products of the alcoholic fermentation, namely ethanol and glycerol, showed a constant increase up to day 4 and then stabilized. The beer at the end of the primary fermentation contained an ethanol concentration of about 70 mL/L, a small residual amount of maltose (<1 g/L) and possessed a glycerol content of about 3.3 g/L. These last parameters also remained constant during cold maturation and storage of the bottles, while an increase in ethanol content (about 4 mL/L) was measured in the final product due to the addition of fructose. The added fructose was consumed completely within the first 15 days of beer storage (data not shown).

A total of 185 colonies of presumptive *S. cerevisiae* were isolated during the fermentation stage, bottle storage and from the ADY. After DGGE analysis, 5 different profiles could be observed, with the majority of the isolates belonging to one single electrophoretic pattern (data not shown). After species-specific PCR for *S. cerevisiae* isolates, three out of the five migration groups did not get amplified, highlighting their appurtenance to another species. This was confirmed by 26S rRNA gene sequencing. Twelve isolates not amplified were identified as *Candida* sp., and all came from the first day of the fermentation, while the other 173 were confirmed to be *S. cerevisiae* (data not shown).

Seventy-one and eighty-seven isolates, respectively from the transformation process (primary fermentation and maturation) and the storage period, plus 15 *S. cerevisiae* from the ADY were subjected to SAU-PCR for their molecular characterization. Unexpected results were obtained when the isolates from the fermentation and the ADY were compared by the way of the BioNumerics program. As reported in Fig. 2, by using an arbitrary coefficient of similarity of 70%, a total of 9 clusters and 3 single-strain clusters could be differentiated. Seven clusters (I to V, VII and IX) were formed by strains isolated throughout the process; however they were all different from the isolates of the ADY. Interestingly, their composition correlated well with the day of isolation, allowing one to speculate that there was a succession of strains during the fermentation. Only cluster IX grouped *S. cerevisiae* was isolated throughout the period. Two clusters (VI and VIII) contained yeasts obtained from both the fermentation and the starter culture. It is important to observe that only 13 (18.3%) of 71 isolates shared similarities with the starter culture, and these were all isolated at

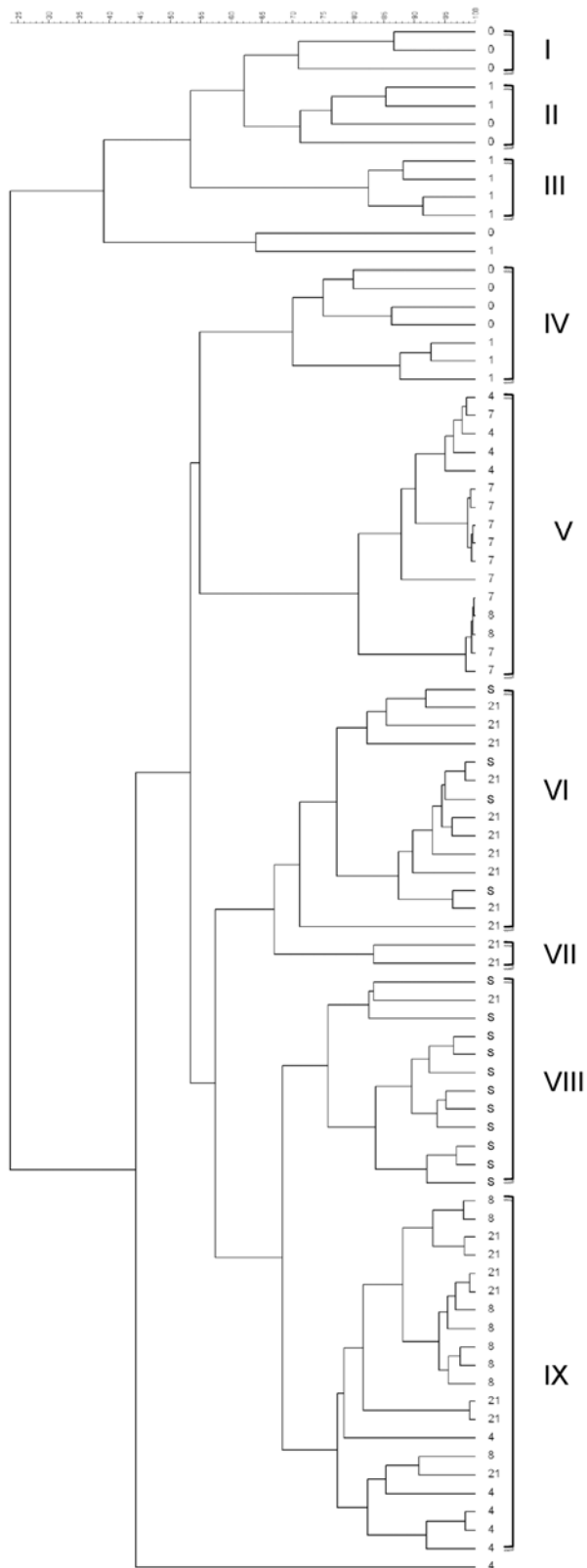


Fig. 2. Dendrogram of *S. cerevisiae* isolated during the fermentation (from days 0 to 8) and maturation process (day 21) subjected to SAU-PCR analysis. The numbers indicate the day of isolation, while the S stands for isolates from the starter culture added. The clusters resulting by using a similarity coefficient of 70% are identified by roman numerals.

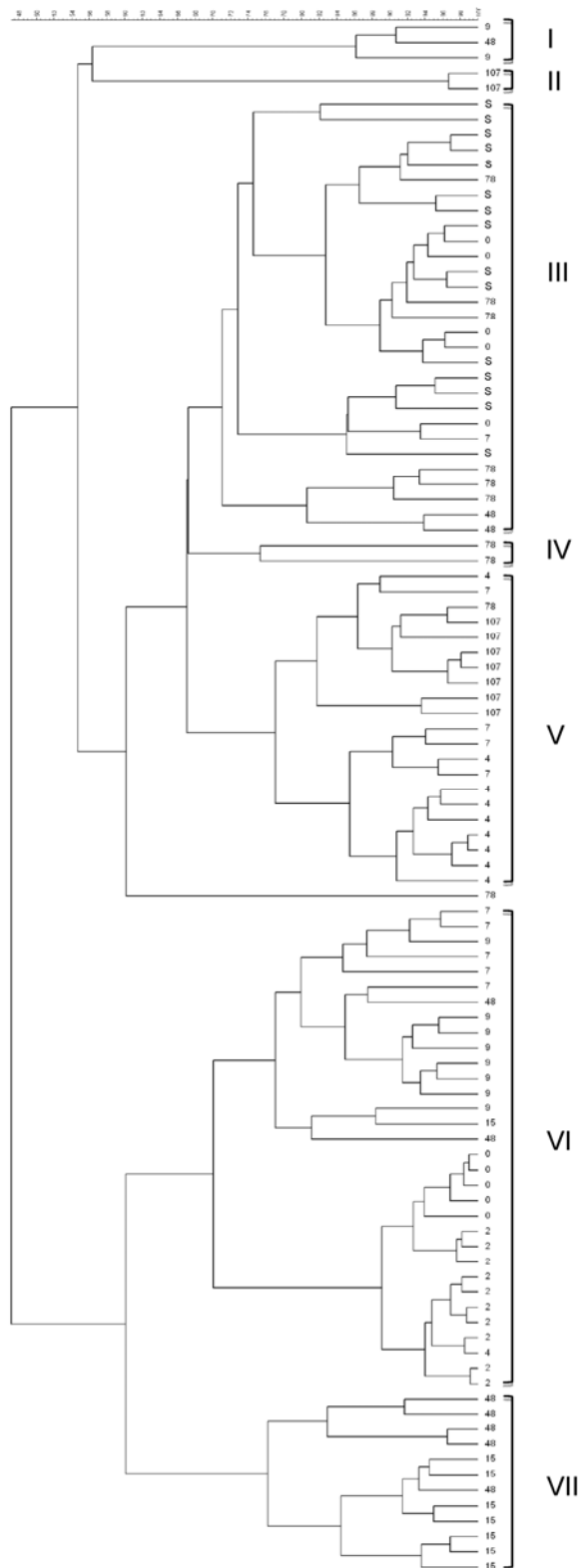


Fig. 3. Dendrogram of *S. cerevisiae* isolated from the bottles of the beers during their storage at 20°C and subjected to SAU-PCR analysis. The numbers indicate the day of isolation, while the S stands for isolates from the starter culture added. The clusters resulting by using a similarity coefficient of 70% are identified by roman numerals.

the end of the maturation process (21 days). This evidence emphasises that the inoculated yeast was not able to dominate until the end of the fermentation. In Fig. 3, the dendrogram obtained from the analysis of the yeasts isolated from the bottles during storage is presented. The results correlated well with the previously presented picture of *S. cerevisiae* biodiversity during fermentation. By using a 70% coefficient of similarity, a total of 7 clusters could be differentiated plus 1 single-strain cluster. All of them were constituted by isolates coming from different sampling points, and only cluster III contained ADY. Clusters V and VI, containing respectively 21 and 32 isolates, were the most numerous. These outcomes suggest that every bottle had developed its own microbial ecosystem, which could be connected with the type of *S. cerevisiae* contained in the beer before the bottling.

CONCLUSIONS

Based on this evidence, it can be speculated that organoleptic differences may occur between bottles. In this study, the inability of the ADY inoculated into the wort to dominate the fermentation has been highlighted. The chemical parameters followed suggested that the transformation process was proceeding without any potential problem, reaching almost complete consumption of the sugars (residual 4.5°P) (wort maltotriose was not measured) and the maximum level of glycerol and ethanol were achieved after only 4 days of fermentation. However, the study of *S. cerevisiae* biodiversity highlights that the populations conducting the alcoholic fermentation were not associated with the starter culture. These results were most likely associated with inappropriate handling of the ADY and more specifically of its rehydration process. This step is crucial to fully re-establish metabolic activity of the ADY, a pre-requisite for its dominance. Recently, gene expression studies have highlighted that the rehydration process plays a fundamental role in the sugar assimilation machinery re-establishment and that based on the media in which rehydration was performed (water or water plus sucrose) a different induction of the hexose transporters is observed²¹. The origin of the *S. cerevisiae* strains that co-conducted the fermentation is still unknown. It is unlikely that there would be residual yeast in the boiled wort; the aspect of the colonies on WLN could not be associated with *S. cerevisiae* (data not shown) or environmental contamination. The micro-brewery employed in this study was producing different kinds of craft beers, for which different ADYs were used. Although during this experimentation, dedicated equipment was devoted to the studied fermentation, it cannot be excluded that cross contaminations had not occurred. This study focused on a single brewing batch, which highlights the need for a better investigation of the dynamics and biodiversity of *S. cerevisiae* during artisanal beer fermentation. This will allow a better comprehension of the performances of the starter culture, possibly identifying incorrect handling and management of the inoculum, which results in its inability to take over the fermentation under the conditions of this study.

ACKNOWLEDGEMENTS

Authors express their gratitude to the technical staff of the brewery that provided the beer samples studied.

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(Manuscript accepted for publication July 2011)