Fermentative behavior of the Candida stellata yeast under different aeration conditions

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ABSTRACT

The influence of aeration on the fermentative activity of Candida stellata RIVE 3-16-1 was studied. The strain was cultured either in Erlenmeyer flasks or a bioreactor containing sterilized and aroma-removed apple juice. Cultures in Erlenmeyer flasks were done under shaken or static condition, whereas in the bioreactor, a constant air flow regime was kept. Chemical compounds produced during fermentation were determined by GC and HPLC. The agitation of Erlenmeyer flasks increased the production of total higher alcohols as compared to static culture and enhanced dramatically the ethyl acetate production. Meanwhile, the production of acetic acid and glycerol were higher under static culture. Bioreactor fermentation at constant air flow was important to visualize the effect of oxygen on the production of compounds and its impact in the quality of alcoholic beverages. It is reported a specific growth rate of 0.13 h⁻¹. Aeration promoted the cell growth affecting the ethanol yield. At the end of culture, malic acid naturally present in apple juice and the ethanol produced were consumed after sugar depletion. Moreover, the acetic acid produced at the end of the fermentation served as carbon source too. The best results in terms of acceptability of the fermented beverages were obtained when cultivated statically.

Keywords: Candida stellata, alcoholic fermentation, higher alcohols, ethyl acetate

Introduction

Non-Saccharomyces yeasts have been always regarded as contaminants in the production of wines, ciders and beers. For this reason, no attention has been paid to the potential use of these yeasts for fermented beverages production processes. Just few studies have addressed their fermentative behaviour and the factors influencing it, with Kloeckera apiculata, Saccharomyces cerevisiae, Hansenula anomala and Hanseniaspora uvarum as the most relevant non-Saccharomyces yeasts studied in the last years [1-4]. All of them showed that these yeasts are strongly influenced by the presence of oxygen in the fermentation medium and, consequently, it impacts in the production of compounds of sensory importance, such as esters and higher alcohols.

Among the non-Saccharomyces yeasts, Candida spp. are gaining importance from the industrial point of view due to its particular fermentative behavior. For instance, some Candida species are being considered potential candidates for the fermentation of wines and beers respectively [5-7]. Of them, Candida stellata is commonly isolated from grape must and survives during spontaneous wine fermentation for longer periods of time [8, 9]. Studies on the fermentative activity of C. stellata showed that it could positively affect the taste and flavour of alcoholic beverages [10].

How to cite (Vancouver style):

Palabras clave: Candida stellata, fermentación alcohólica, alcoholes superiores, etil acetato

Comportamiento fermentativo de la levadura Candida stellata en diferentes condiciones de aireación. Se evaluó la influencia de la aireación en la actividad fermentativa de Candida stellata RIVE 3-16-1. La cepa se cultivó en frascos de Erlenmeyer y en un bioreactor que contenía jugo de manzana esterilizado y sin aroma. El cultivo en erlenmeyers se realizó en condiciones estáticas o con agitación, mientras que en el bioreactor se mantuvo bajo régimen constante de flujo de aire. Los compuestos químicos producidos durante la fermentación se determinaron por cromatografía de gases y cromatografía líquida de alta presión (HPLC). La agitación de los frascos de Erlenmeyer incrementó la producción de alcoholes superiores en comparación con el cultivo estático y mejoró acentuadamente la producción de etil acetato. Mientras tanto, la producción de ácido acético y glicerol fue mayor en el cultivo estático. El efecto del oxígeno en la producción de compuestos y su impacto en la calidad de bebidas alcohólicas se visualizó en cultivo en bioreactor a flujo de aire constante. Se observó una tasa de crecimiento específico de 0.13 h⁻¹. La aireación promovió el crecimiento celular afectando el rendimiento de etanol. Al final del cultivo, el ácido málico presente naturalmente en el jugo de manzana y el etanol producido fueron consumidos después del agotamiento del azúcar. Adicionalmente, el ácido acético producido al final del proceso sirvió como fuente de carbono. Los mejores resultados en términos de aceptabilidad de las bebidas fermentadas se obtuvieron en cultivo estático.

Palabras clave: Candida stellata, fermentación alcohólica, alcoholes superiores, etil acetato

Moreover, this yeast shows a strong fructophilic and osmophilic character [11] and it is a potent producer of glycerol under aerobic growth conditions and especially in anaerobic ones [12]. Regarding its metabolism, some authors refer to *C. stellata* as Crabtree positive yeast [13], since it shows different metabolic behaviour depending on the oxygen and glucose concentration in the fermentation medium. Under anaerobic or oxygen-limited conditions, this type of yeasts exhibit alcoholic fermentation [14], but in fully aerobic ones, a mixed respiro-fermentative metabolism is observed due to the excess of sugar concentration over a given threshold value. That value tends to be about 1 mM in *Saccharomyces cerevisiae* [15]. Conditions leading to sugar fermentation result in the production of major fermentative compounds in detriment of biomass yield, including ethanol, acetic acid and glycerol, with small amounts of higher alcohols, esters, volatile fatty acids and carbonyl compounds (less than 1% v/v of the utilized sugar). Due to these metabolic losses, the complete fermentation of hexose by yeasts yields 94-96% of the theoretical ethanol yield [16].

While complementary to major fermentative products, those minor chemical compounds are of paramount sensory importance, including esters, higher alcohols, organic acids, aldehydes and others. In fact, esters are the most important flavour compounds in many alcoholic beverages such as beer and wine [17]. Esthers are produced from the reaction between alcohols and fatty acids, which is catalyzed by the enzyme alcohol acetyl transferase (AATase) [18]. Noteworthy, AATase activity is strongly inhibited by trace amounts of oxygen [19]. Regarding higher alcohols, they are produced either catabolically from the degradation of cell-imported amino acids or anaerobically via the biosynthetic route from the carbon source [20]. Oxygen supplementation and temperature increase during fermentation enhance the production of higher alcohols by metabolism activation and, consequently, it promotes the cell growth [21].

In order to elucidate the balance between all these processes, in this work it was studied the effect of aeration on the fermentative metabolism of *Candida stellata* RIVE 3-16-1, and the production of sensory-relevant compounds, using apple juice as fermentation medium. Two culture conditions were considered, attending to their technological importance: fermentation under highly limited oxygen concentration (static culture) or with moderate oxygen (agitated culture). Additionally, a batch culture in a bioreactor containing juice of a specific apple variety under constant air flow was done to evaluate the metabolic fermentative behavior of *C. stellata* RIVE 3-16-1.

**Materials and methods**

**Microorganism and maintenance**

The *Candida stellata* RIVE 3-16-1 yeast strain was acquired from the collection of yeasts at the Research Institute of Viniculture and Enology of Bratislava, Slovak Republic, as used in the experiments as further specified. The strain was maintained on malt extract agar at 7 °C and renewed every three months, until use.

**Propagation of inoculum**

The inoculum was propagated in 100 mL of sterile apple juice at 28 °C for 24 h. Flasks were shaken at 200 min⁻¹ in an orbital shaker. Cell biomass was separated by centrifugation (3000 rpm for 10 min) and washed three times with sterile physiological solution. Fermentation media were inoculated with 1.0 ± 0.1 g of cells based on wet weight [1-4].

**Synthesis of compounds of sensory importance**

Sensory-relevant compounds were produced by fermentation in concentrated, sterilized and aroma-removed apple juice (Severofrukt a.s, Terezin, Czech Republic). It was reconstituted with sterilized distilled water up to a total sugar concentration of 12.8 w/v and pH 3.8 [1-4, 22]. Fermentations were done in triplicate, both static and agitated at 28 °C in 500 mL Erlenmeyer flasks containing 250 mL of medium. Under agitation, flasks were shaken at 200 rpm for 8 d. Static cultures were fermented for 15 d, approximately. Fermentations were regarded as finished when sugar was depleted.

**Batch culture in bioreactor**

Apple juice of Rubin variety containing 13% w/v of total sugars and pH 3.8 was utilized in the experiments. Apples were acquired from CZ-fruit, (Prague-CR, Czech Republic), and the juice was extracted by pressing and then placed in 10-L glass containers. Then, juice was pasteurized in a thermostat at 65-70 °C for 10 h (including the cooling time) to eliminate microflora and the varietal volatile compounds [1-4, 23]. Subsequently, juice was supplemented with 1.2 g/L KH₂PO₄ and 1.2 g/L (NH₄)₂SO₄ as cell growth promoters and [1-4, 24].

Cultures were carried out in a 2-L bioreactor (BIOSTAT, B. Braun International, Germany) containing 1.5 L of apple juice. The equipment was provided with a pH-meter, thermometer, stirrer and an electrode for measurement of dissolved oxygen. The bioreactor was connected to a micro-DCU-300 regulation and measurement unit. The following parameters were kept constant during the entire process: temperature, 18 °C; stirring frequency, 300 rpm; air flow rate, 25 L/h (0.2 mol O₂/h). Cultures were stopped when observing an increase of the dissolved oxygen value to its initial value (100%).

The inoculum was propagated in 80 mL of synthetic medium containing: 100 g/L glucose; 10 g/L peptone, 1.2 g/L KH₂PO₄, 1.2 g/L (NH₄)₂SO₄ and 10 g/L yeast extract; pH was adjusted to 3.8 with a 5% v/v HCl solution. Cells were propagated at 28 °C for 48 h in an orbital shaker at 150 rpm. Subsequently, the cells were separated by centrifugation (3000 rpm for 10 min), washed with sterile physiological solution and finally inoculated into the bioreactor.

**Analytical methods**

Volatile compounds (higher alcohols and esters) produced during fermentation were analyzed by a gas chromatograph (Hewlett-Packard 5990II), equipped with a HP5 column (30 m × 0.32 mm) and FID detector. The working parameters were: injector temperature, 240 °C; oven conditions, 40 °C initial temperature for 5 min, then was raised to 220 °C at 4 °C/min.
and kept at this temperature for 5 min. The samples (three replicates for each experiment) were previously centrifuged and then filtered through 0.45 μm membranes. Volatile compounds were analyzed using the method of dichloromethane micro extraction [25]. Finally, 1 μL of each extract was injected into the column of the equipment.

Acetic, succinic and malic acids, ethanol, glycerol, fructose and glucose were analyzed by a HPLC (Pump LPC 4000), equipped with a re-packing watex 250 × 8 mm column (Ostion LGKS 0800 H+ form) and a RID detector. Malic acid was analyzed only in fermentations carried out with Rubin apple juice. The conditions of analysis were: column temperature 80 °C, mobile phase 5 mM H₂SO₄, 1 mL/min flow rate. Once centrifuged at 10 000 rpm and filtered, samples were diluted with demineralized water (1:3) before injecting to the equipment [1-4, 24].

Cell biomass was determined by gravimetry. Cells were separated by centrifugation (3000 rpm for 10 min), then washed three times with distilled water, dried at 110 °C until constant weight and finally weighed. Additionally, biomass yield and ethanol (Y₀,₅ and Yₐ) and, the specific growth rate (μ) were determined at the end of the experiment.

Sensory and statistical analyses

Sensory assessment of ciders fermented statically and in agitation was performed using an affective and descriptive analysis in the same manner as in previous studies carried by Estela-Escalante et al. [1-4, 24]. Attributes such as taste, aroma and odor were evaluated using a 5-points Hedonic scale (1, dislike very much; 2, dislike somewhat; 3, neither like nor dislike; 4, like somewhat; 5, like very much). Samples were evaluated by a trained panel of 10 judges, (all men, and aged 25-30 years). The sensory evaluation was done according to Meilgaard et al. [26], and sensory evaluation data were presented as means of the score of all the judges. A standard Student’s t test was used to analyze the statistical significance (p < 0.05) of the differences observed between the scores for two fermented beverages (cultivated under agitation or static). Statistical analysis was done using Statistica v.8.0 software.

Results and discussion

Culture under static and agitated conditions

Culture experiments done in shake-flasks are regarded as oxygen-limited [27], this culture condition affecting the fermentative metabolism of yeasts. In fact, high concentration of sugar present in apple juice leads to alcoholic fermentation in Crabtree positive species even when oxygen is present in enough concentration. Culture without agitation (statically) are even more oxygen-limited, since only the surface of the liquid gets into contact with the air and the movement of cells in the liquid is very slow. Oxygen is necessary to improve yeast metabolism and, thus, to successfully complete the fermentation. Results consistent with these observations and derived from the production of chemical compounds during fermentation of apple juice by C. stellata RIVE 3-16-1 in static and agitated cultures are shown in Table 1. Compounds analyzed in ciders fermented with different strains of S. cerevisiae as reported by different authors were included for comparison.

Glycerol production

The production of glycerol under static culture reached 1.9 ± 0.1 g/L, significantly different (p < 0.05; determinations in triplicates) from results in static culture (3.8 ± 0.3 g/L). The decrease of glycerol production under agitated conditions was also observed in previous studies with Kloeckera apiculata, S. cerevisiae, Saccharomyces ludwigii and Hanseniaspora uvarum under similar culture conditions [1, 2, 4, 24]. Aerobic conditions promote cellular respiration and severely decrease glycerol production. Noteworthy, C. stellata is considered as a high producer of glycerol and amounts up to 1.76 g/L have been reported [34]. Despite, the 3.8 ± 0.3 g/L glycerol obtained in this study is low as compared to values reported in the literature, what would suggest that glycerol production by C. stellata could be strain-dependent. From the technological point of view, a higher production of glycerol is desirable since it influences positively the sensory quality of alcoholic beverages, while not detrimental for alcohol production. Glycerol imparts a slightly sweet taste and contributes to the mouth-feel and complexity of wine flavour at low levels [35, 36].

Production of higher alcohols

Higher alcohols need to be kept low since they are mostly detrimental, with few exceptions, to the sensory quality of fermented beverages. In our experiments, a higher production of total higher alcohols in agitated culture (620.9 mg/L) was observed, in comparison with the static culture (486.0 mg/L). This behavior has been also described for non-Saccharomyces yeasts (K. apiculata, H. uvarum and S. ludwigii) under similar culture conditions [1, 2, 4]. Nevertheless, an opposite effect was evidenced with S. cerevisiae [24]. Hence, considering the present results together with previous ones from our group [1-4, 24], it is possible to suggest that the production of higher alcohols depends on yeast type and culture conditions. From the sensory point of view, higher alcohols are identified by a strong, pungent smell and taste [37, 38]. For instance, total concentrations higher than 400 mg/L negatively contribute to the organoleptic quality of wines [39]. Except for 2-phenylethanol, which imparts a floral aroma [40], the rest of higher alcohols provide unpleasant sensory characteristics [39, 40].

Production of esters

Esters are the most important aroma compounds in alcoholic beverages. They confer pleasant sensory characteristics under certain concentrations. Among them, ethyl acetate is present in most fermented beverages in higher amounts as compared to the rest of ester compounds. It imparts a light-fruity or solvent-like aroma depending on its concentration. Mean values of experiments carried out in triplicate showed that agitation enhanced the production of ethyl acetate (176.0 ± 5.0 mg/L), ten times higher than in static culture (17.0 ± 1.5 mg/L, p < 0.05). These results are opposite to those by Yoshioka and Hashimoto [19],
Table 1. Compounds of sensory value produced by *Candida stellata* RIVE 3-16-1 during fermentation of apple juice at 28 ºC under static and agitated cultivation, as compared to typical compounds found in ciders fermented with *Saccharomycyes cerevisiae*.

<table>
<thead>
<tr>
<th>Compounds (mg/L)</th>
<th>Reference values in apple juice (Mean ± SD)</th>
<th><em>Candida stellata</em> culture</th>
<th>Compounds analyzed in ciders fermented with <em>Saccharomyces cerevisiae</em> (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol†</td>
<td>0</td>
<td>52.48 ± 1.9</td>
<td>51.07 ± 0.4 [31]</td>
</tr>
<tr>
<td>Glycerol†</td>
<td>0.75 ± 0.04 [30]</td>
<td>131.0 ± 4.0</td>
<td>22.17 ± 0.08 [32]; 34.8 ± 8.9 [31]</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>6.99 ± 0.04 [32]; 6.1 ± 0.7 [31]</td>
<td>16.66 ± 1.0</td>
<td>32.6 ± 1.7 [31]</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>0.01 ± 0.00 [32]; 0.0 [28]</td>
<td>90.0 ± 3.5</td>
<td>282.93 ± 16.9 [30]</td>
</tr>
<tr>
<td>2-Butanol</td>
<td>2.4 ± 0.5 [30]</td>
<td>154.0 ± 6.5</td>
<td>137.0 ± 3.5</td>
</tr>
<tr>
<td>2-Methyl-propanol</td>
<td>0.46 ± 0.02 [30]</td>
<td>154.0 ± 4.5</td>
<td>98 ± 0.3 [32]; 131.5 ± 55.3 [31]</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.57 ± 0.03 [30]</td>
<td>176.0 ± 5.0</td>
<td>231.06 ± 33.09 [32]; 114.6 ± 35.5 [31]</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.01 ± 0.00 [30]</td>
<td>90.0 ± 3.5</td>
<td>282.93 ± 16.9 [30]</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>0.17 ± 0.15 [28]</td>
<td>150.0 ± 6.5</td>
<td>200.0 ± 30.0 [32]; 0.5 ± 0.06 [29]</td>
</tr>
</tbody>
</table>

* Values reported in references [28], [29] and [30] are average values. Values from reference [31] were found in ciders prepared from a mixture of cider apples. Values from references [32] and [33] are direct values. Reference values for 2-Methyl-butanol in references [31] and [43] correspond to a mix of amyllic alcohols 3-Methyl-butanol + 2-Methyl-butanol [31, 32].

Sensory evaluation of fermentation product

Significant differences (p < 0.05) were found for the two fermentation beverages (static vs agitated) when comparing the mean values of the score of 10 judges who evaluated them attending to flavour, taste and odour. Panelists described the beverage fermented statically with a slightly taste of solvent-like, little acidic, with an acceptable balanced sensory profile. On the other hand, they referred to the beverage fermented under agitation as of strong taste to solvent-like and with unbalanced sensory profile. The beverage fermented statically had an overall acceptability, based on the attributes evaluated, which is consistent with the traditional fermentation process.

Batch cultivation in bioreactor under constant air flow

Batch cultivation with constant air flow regime allows constant supply of oxygen to the medium. Oxygen is the most important parameter determining the balance between fermentative and respiratory activity in most yeasts. Respiratory rate becomes dependent on oxygen availability in the medium at concentrations lower than the critical value, this parameter very low for yeasts at about 0.12 mg/L at 20 ºC [45]. Oxygen transfer into the liquid phase depends on several parameters, such as: the bioreactor geometry, type of impeller, culture medium viscosity, agitation speed, number of baffles, temperature, among others. Only the oxygen dissolved in the liquid phase is available for yeast cells.

Therefore, the aim was to evaluate the impact of oxygenation on the fermentative behavior of *C. stellata* RIVE 3-16-1 yeast. For that purpose, apple juice from the single Rubin variety was used and following the routine procedure of juice extraction. Oxygen consumption, cell growth, pH shift and the synthesis and utilization of metabolism byproducts were monitored at a constant air flow rate of 25.0 L/h (0.2 mol O₂/h) during the whole fermentation time (232 h). As shown...

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in figure 1, after 10 h of culture, the concentration of dissolved oxygen dropped to zero and remained at this value the whole cultivation period. During this time the oxygen transferred to the liquid phase was totally consumed. Since this value does not give information about the rate of oxygen consumption, it was required to measure this parameter in the outlet gas of the bioreactor. This was also observed in previous reports of our group, for H. anomala, S. ludwigii and Brettanomyces intermedius yeasts when cultured under conditions similar to the ones tested [2, 3, 46]. Nevertheless, oxygen consumption by S. cerevisiae was reported to follow a different behavior after 50 h in culture. Almost the same pattern of oxygen consumption was followed by K. apiculata and H. uvarum [1, 4, 24]. The oxygen consumed by these yeasts is used in the respiration process for glucose oxidation, and also in non-respiratory pathways such as synthesis of sterols and unsaturated fatty acids, which are essential components of the cellular membrane [47].

The course of the whole fermentation process in terms of sugar consumption, ethanol, glycerol and acetic acid production is shown in figure 2. Fermentation can be divided in two stages: one characterized by sugar consumption (glucose and fructose) and production of ethanol and glycerol until 70 h, followed by a second one in which fermentation byproducts (ethanol and glycerol) are consumed. Regarding yeast growth kinetics, it reached 19.6 g/L, with a specific growth rate of 0.13 h⁻¹ at the end of culture. In this regard, Ciani et al. [12] reported that the presence of oxygen increased growth rate in approximately three-fold (from 0.05 to 0.16 h⁻¹) when C. stellata was cultured in shake flasks at 150 rpm in synthetic medium and synthetic grape juice, as compared to anaerobic cultivation experiments. The low biomass production seen in our experiment may be due to the use of fermentable sugar by C. stellata RIVE 3-16-1 in cell maintenance reactions, as cultures were run at a low pH value (3.2-3.8). It was also observed that once sugars were consumed, ethanol and glycerol were simultaneously used as carbon source, followed by acetic acid.

As shown for sugar consumption (Figure 2), glucose was the first substrate to be depleted (up to 70 h) and then fructose (up to 90 h), this last with a higher consumption rate. This can be explained due to the fact that C. stellata is a fructophilic yeast using fructose as a preferential substrate. This sugar depletion up to 90 h was also observed previously in experiments with S. cerevisiae under similar culture conditions [24].

Ethanol and glycerol production were mainly produced in the first stage (Figure 2), the former at lower levels than in previous studies with S. cerevisiae under similar culture conditions [24]. On the other hand, acetic acid production was observed at the end of culture when ethanol was the main carbon source. In fact, it was generated by C. stellata RIVE 3-16-1 at levels three-fold lower than those attained with S. cerevisiae [24].

Simultaneously, sugar consumption resulted in maximal production of ethanol (27.0 g/L) and glycerol (1.2 g/L), these compounds serving as carbon source to maintain cell growth once sugars were depleted (Figure 2). From this stage on, the dissolved oxygen concentration dropped to zero and it remained constant toward the end of the cultivation period. Finally, acetic acid was produced toward the end of cultivation during ethanol assimilation.

In the case of glycerol, its production by yeast may constitute a response against the high osmolarity of the environment (osmoregulation) [48]. Apple juice normally contains high sugar concentrations and it can increase the osmolarity in C. stellata RIVE 3-16-1 cells, which could synthesize glycerol as compatible intracellular solute to compensate this parameter. In fermentation studies with Candida magnolia, it was shown that cell growth and glycerol production were strongly affected by oxygen supply [49]. Apart from the effect on glycerol synthesis, the 0.13 h⁻¹ growth rate seen (Table 2) was similar to that reported by us for other yeasts cultured under similar conditions [3, 24]. It is common knowledge that the energy...
generated from glucose metabolism may be mostly used in cell growth during glucose-limited and fully aerobic fermentations.

Additionally, in in-batch cultivation under constant air flow, *C. stellata* RIVE 3-16-1 was able to utilize 48.2% of the total malic acid present in apple juice. This finding is interesting since yeasts with high capability to degrade malic acid are desired. The presence of oxygen in the medium would enhance its utilization. It was also observed that degradation of malic acid started after sugar depletion and at the same time with ethanol assimilation (Figure 2 and 3). This behaviour was also observed in previous studies with *S. cerevisiae* cultured under similar conditions as used in this study [24] and, it may indicate that malic acid serves as carbon source to support the cell growth in both yeasts. Malic acid is the most important organic acid present in apple juice. It contributes to the total acidity in ciders. The decrease of its content to values lower than 5 g/L gives ciders with better sensory quality. Wine yeast in general cannot effectively degrade malic acid during alcoholic fermentation. Commercial wine yeasts (*S. cerevisiae*) are able to degrade only 18% of the total malic acid [50], we previously reported degradation levels of up to 36% of the total malic acid present in apple juice [24].

The other major metabolic compound, succinic acid, is the main organic acid generated by yeasts during metabolism of the carbon source, contributing to the taste of many alcoholic beverages [38, 44]. Here we found that *C. stellata* RIVE 3-16-1 produced it at 1.4 g/L. Moreover, the variability found for succinic acid synthesis throughout the entire fermentation process could be explained by its continuous excretion and reimport into yeast cells [38, 48].

Finally, other byproducts such as higher alcohols and acetate esters were also identified. They were confirmed among trace compounds remaining at the end of culture (Table 2). Probably, at the second fermentation stage (aerobic assimilation of ethanol), these higher alcohols could have served as carbon source for cell biomass increase, their low concentrations at the end of cultivation supporting this hypothesis (Table 2). From the technological point of view, fermentations should be stopped once sugars get depleted or when ethanol concentrations reach the intended value. Overall, our results are in line with common knowledge on alcoholic fermentation, regarding that excessive aeration during fermentation leads to increased cell biomass and consequently decreased ethanol production.

Conclusions

The concentration of oxygen and sugars are the most important factors that affect the metabolism of *C. stellata* RIVE 3-16-1. At high sugar concentration, alcoholic fermentation takes place even with insufficient oxygen amounts. In culture under agitation in Erlenmeyer flasks it was found oxygen transfer is a limiting factor, probably leading to very low oxygen concentration in the medium. These oxygen-limited conditions influences the synthesis of fermentation byproducts by *C. stellata* RIVE 3-16-1, particularly glycerol and increases the overall production of higher alcohols (especially 3-methyl-butanol and 2-phenylethanol) and acetate esters such as ethyl acetate. Simultaneously, acetic acid diminishes under agitation, something desirable for alcoholic beverages, due to low volatile acidity. From the sensory point of view, the static culture conditions provide the best acceptability criteria, providing additional evidence for static culture as optimal for apple juice fermentation.

Conversely, constant air flow conditions supporting oxygen consumption for three days led to sugar depletion and enhanced yeast growth and fermentation rate, but affecting ethanol yields. Hence, excessive aeration should be avoided to improve ethanol yields, since *C. stellata* RIVE 3-16-1 normally produces ethanol, glycerol and acetic acid during aerobic fermentation, but these products are metabolized with continuous oxygen supply once the sugars are aerobically depleted. Therefore, care must be taken when main carbon sources (sugars) are exhausted, to avoid the consumption of glycerol and acetic acid as energy sources, as well as malic acid, affecting the sensory quality of the final beverage. In this case, the fermentation process should be stopped once the maximum ethanol concentration has been attained.

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### Table 2. Compounds utilized and produced by Candida stellata RIVE 3-16-1 during batch cultivation under constant air flow regime at 18 °C

<table>
<thead>
<tr>
<th>Initial compounds</th>
<th>Concentration (g/L)</th>
<th>Higher alcohols and ethyl acetate produced</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugars (fructose + glucose + sucrose)</td>
<td>129.05</td>
<td>1-propanol</td>
<td>Traces</td>
</tr>
<tr>
<td>Malic acid</td>
<td>5.02</td>
<td>Propyl acetate</td>
<td>0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>4.3</td>
<td>2-methyl propanol</td>
<td>1.7 ± 0.04</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0</td>
<td>3-methyl butanol</td>
<td>5.6 ± 0.05</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0</td>
<td>2-methyl butanol</td>
<td>3.0 ± 0.05</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>1.0 ± 0.3</td>
<td>Ethyl acetate</td>
<td>13.1 ± 0.06</td>
</tr>
<tr>
<td>Malic acid</td>
<td>2.6 ± 0.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. Course of malic acid degradation and succinic acid synthesis in batch cultivation of Candida stellata RIVE 3-16-1 under constant air flow regime (25 L/h) at 18 °C.
From the technological point of view, small amounts of oxygen would be suitable in order to control the production of ethanol and the synthesis of fermentation byproducts.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.