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Application of *Saccharomyces rouxii* for the Production of Non-alcoholic Beer

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Abstract Successive application of *Saccharomyces cerevisiae* DSM 70424 and *Saccharomyces rouxii* DSM 2535 or DSM 2531 in the production of non-alcoholic beer was investigated. The aim of the study was to consider the impact of the 2 mentioned strains of *S. rouxii* on the reduction of alcohol content in wort fermented at 12 or 24°C for 96 hr, applying periodic aeration. The 2 *S. rouxii* strains were added at the 48th hr of fermentation after thermal inactivation of *S. cerevisiae* cells. The greatest alcohol decrease rate was observed for the treatment containing *S. rouxii* DSM 2535-fermented at 24°C (from 1.56 to 0.36%). The concentration of acetaldehyde, diacetyl, and 2,3-pentandione, that have a key role in appearance of 'wort' and 'buttery' off flavors, were significantly lower in *S. rouxii*-containing treatments fermented at 24°C. *S. rouxii*-containing treatment fermented at 24°C showed slightly lower overall flavor acceptability compared to *S. cerevisiae*-containing treatment fermented at the same temperature. Such score was improved for the products obtained at 12°C.

Keywords: alcohol, brewery, non-alcoholic beer, *Saccharomyces*, yeast

Introduction

Beer is a worldwide consumed and universally popular beverage. Popularity of beer arises from its pleasant sensory attributes and favorable health characteristics as well as its lower cost compared with other types of Western and European alcoholic beverages such as wine (1). Most beers produced worldwide have alcohol content in the range of 3-6%(v/v) (2,3). Considering the alcohol content of beers, they can be found as low-alcohol/alcohol-reduced (containing about 2-3% alcohol), medium/average strength (about 5% alcohol) and high strength/strong (about 6-12% alcohol) (4). In recent years, there has been an increased market share for low-alcohol/alcohol-reduced and non-alcoholic/alcohol-free beers. The alcohol content in alcohol-reduced beers is generally recognized to be less than 2.5%(v/v). However, the legal definition of alcohol-free beer varies from country to country. For instance, this type of beer must contain maximum alcohol level of 0.1%(v/v) (in Arabic countries), 0.5% (in England, Germany, The Netherlands, and Iran), 1% (in Spain) and with no detectable amount, for example less than 0.05%(w/v) (in the US) (3-10). The alcohol concentration of 0.05% (by weight) is approximately below the usual analytical detection limits. This type of beer might be known as 'zero-alcohol' beer. The term 'nearly alcohol-free' might be used when the alcohol content is between 0.05-0.5%(v/v) (4,6). Consumers are aware of the problems that alcohol can bring about regarding civil responsibilities (1). The importance of low-alcohol or alcohol-free beers arises from several points including the safety regulations in the workplace or when driving an automobile. Also, alcohol consumption is absolutely forbidden in some countries (6,11). Alcohol-free beers are recommended for specific groups of people such as pregnant women, sporting professionals, individuals with cardiovascular and hepatic pathologies, and medicated persons (12).

Several methods have been proposed and practiced for industrial production of non-alcoholic beer. Alcohol removal/dealcoholization of the product and restricted/limited alcohol fermentation are among the most important methods. The latter can be achieved either by using yeast that can only partially ferment wort or by repressing or interrupting the fermentation through different compositional and/or process factors. By using such method, the current dealcoholization processes and their related difficulties are fully avoided (4,6,13). *Saccharomyces rouxii* spp. is considered among the weak alcohol producers in wort media (4). This is due to the fact that they can not efficiently uptake maltose (the most abundant sugar in the wort) (4). Moreover, it has been reported that *S. rouxii* spp. might be able to reduce alcohol content in media containing low sugar (7). Therefore, the present study investigated the effects of successive application of 2 brewer's yeast species, i.e., *Saccharomyces cerevisiae* 70424 and *S. rouxii* (*S. rouxii* 2535 and *S. rouxii* 2531) on the alcohol content and sensory properties of non-alcoholic beer.

Materials and Methods

Experimental design The wort with known gravity was prepared from Behnoush Brewing Co. (Tehran, Iran). The samples of wort were fermented by *Saccharomyces*

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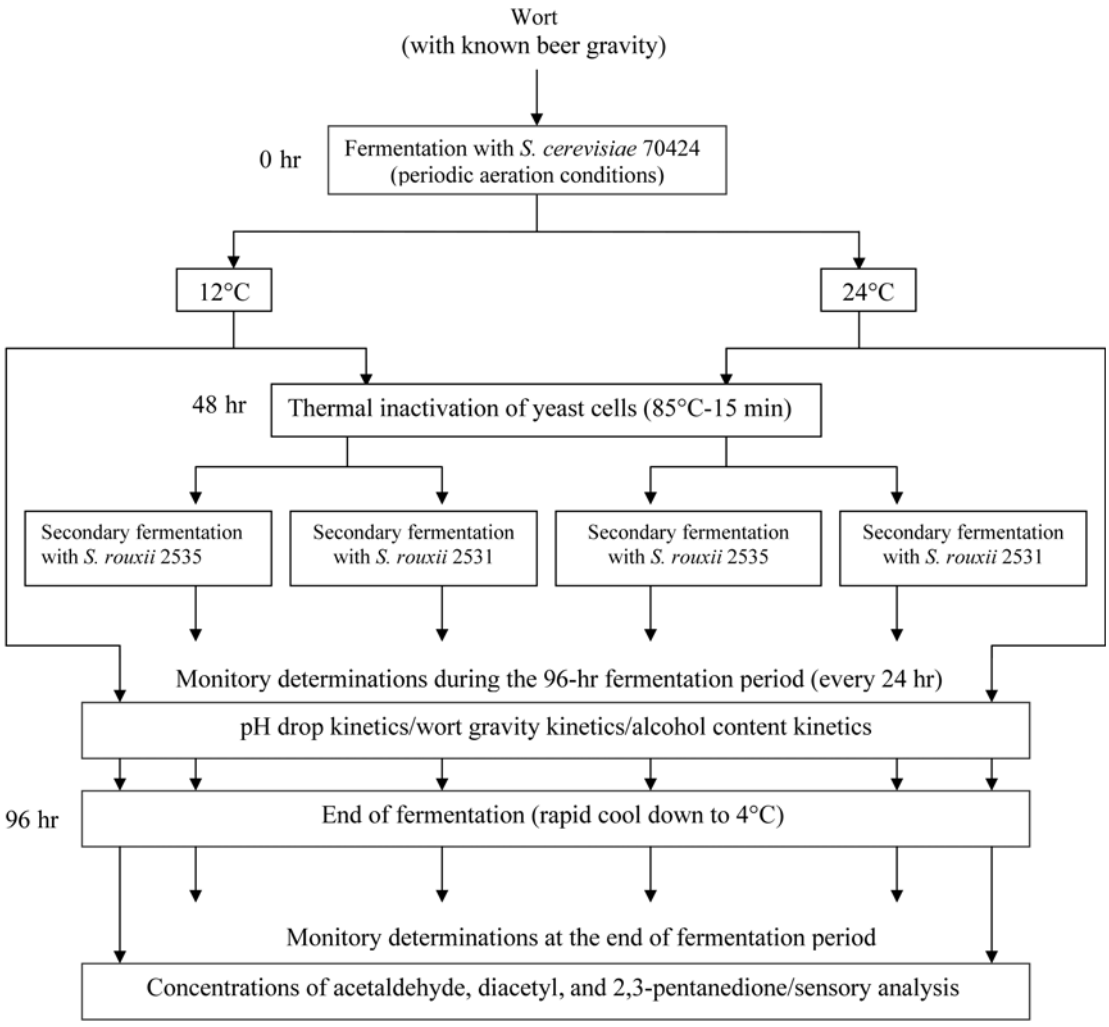

Fig. 1. Experimental design of the present study.

cerevisiae DSM 70424 (pitching rate of 10^7 CFU/mL) at 12 or 24°C for 48 hr, under periodic aeration practice (at 12 hr intervals). The periodic aeration practice was performed in order to implement restricted fermentation procedure (4). After 48 hr of fermentation, some samples were subjected to heat treatment (85°C-15 min, in a sealed container) in order to inactivate *S. cerevisiae* cells and subsequently after cooling down to 12 or 24°C, were inoculated by *Saccharomyces rouxii* DSM 2535 or DSM 2531 (pitching rate of 10^7 CFU/mL). The fermentation process continued up to 48 (at 24°C) or 96 hr (at 12°C) under periodic aeration. Six fermentation conditions as follows were applied: *S. cerevisiae* DSM 70424 at 12°C, *S. cerevisiae* DSM 70424 at 24°C, *S. cerevisiae* DSM 70424/*S. rouxii* DSM 2535 at 12°C, *S. cerevisiae* DSM 70424/*S. rouxii* DSM 2535 at 24°C, *S. cerevisiae* DSM 70424/*S. rouxii* DSM 2531 at 12°C and *S. cerevisiae* DSM 70424/*S. rouxii* DSM 2531 at 24°C. Some experimental parameters (including pH drop kinetics, wort gravity kinetics, alcohol increase, and decrease kinetics) were analyzed (at 24 hr intervals during the fermentation). Concentration of acetaldehyde, diacetyl and 2,3-pentanedione as well as sensory comparison among the treatments) were measured at the end of fermentation period. The experimental design of present study is shown in Fig. 1.

Yeast starter cultures *S. cerevisiae* DSM 70424 as well as *S. rouxii* DSM 2531 and DSM 2535 were supplied by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) in both freeze-dried and slant forms, respectively. The starters were propagated in Yeast-Mold (YM) broth (Merck, Darmstadt, Germany) followed by cultivation on YM-agar. The cultures were kept in refrigerator (5°C) until used. Culture transfers were performed after every 2 weeks using YM-agar.

Chemical determinations Alcohol concentration and the gravity (°P) of wort/beer were analyzed using a digital beer analyzer (Anton Par, Graz, Austria). pH of wort was measured using an MA235 pH meter by Mettler (Schwerzenbach, Switzerland).

Concentration of acetaldehyde was determined by direct injection into a gas chromatograph (Thermo Electron Scientific Instrument Division, Dreieich, Germany) according to Lechanmeier and Sohnius (14). Substances were separated on a CP-Wax52CB fused silica capillary column (60 m× 0.32 mm i.d., film thickness 0.5 μm, Varian Deutschland

GmbH, Darmstadt, Germany). Column temperature programming started at 40°C, where it was held for 15 min, followed by a ramp at 4°C/min up to 200°C, a hold for 10 min and another ramp at 15°C/min up to 230°C and a final hold for 10 min. The temperature for the injection port was set at 260°C. After addition of an internal standard (*n*-amyl alcohol), the samples were injected (2 μL) using split injection mode (1:5). Helium with a constant flow rate of 6.5 mL/min was used as the carrier gas.

Vicinal diketones (diacetyl and 2,3-pentanedione) were measured using a gas chromatograph (Thermo Electron Scientific Instrument Division) equipped with an electron capture detector and an automated head-space injector according to Mathis *et al.* (15). A glass column (4 m×1/4 in.) packed with Chromosorb W, 80/100 mesh (Varian Deutschland GmbH) impregnated with Carbowax 1540 (10%) was used for the separation purposes. The following temperature settings was applied: oven 80°C, injector 180°C, detector 150°C. Carriers gas in nitrogen quality U. The yeast-free sample was neutralized with NaOH and immediately analyzed. 1,3-Dichloropropane was used as internal standard.

Precursors were determined after their thermal conversion (at 60°C for 1 hr) into their corresponding diketones. Therefore, the measured diketones were composed of all free vicinal diketones originally present in the sample and those obtained by the above reaction.

Sensory evaluation Trained panelists were used for the sensory evaluation of the beer products. Several pairs of treatments were compared for minor differences using paired comparison test (duo-test) (16). *S. cerevisiae* 70424/*S. rouxii* 2535 at 12°C with *S. cerevisiae* 70424-*S. rouxii* 2531 at 12°C and *S. cerevisiae* 70424/*S. rouxii* 2535 at 24°C with *S. cerevisiae* 70424-*S. rouxii* 2531 at 24°C. The preferred treatments from the above stages were also compared against each other and the one with higher score was further compared with the corresponding treatment from *S. cerevisiae* 70424 (alone) at 12 or 24°C.

The parameters compared were flavor fullness (maturity), taint, and overall acceptability of beer. Assessors were asked to detect significant/insignificant differences between the pairs of the treatments. Analysis of the results was carried out using the relevant 'Significance Table' ($p<0.05$ or $p>0.05$) (16).

To perform statistical analysis, experiments were performed in triplicate and significant differences among the means were analyzed using the analysis of variance (ANOVA) test from Minitab software (version 13, 2002).

Results and Discussion

Alcohol content, pH drop kinetics, and wort gravity kinetics in beer treatments during fermentation period Figure 2 represents kinetics parameters (pH drop kinetics; wort gravity drop kinetics, and alcohol contents kinetics) in different treatments during 96 hr of fermentation period, at 24 hr intervals. Independent of starter composition of fermenting media, the greatest pH drop rates ($p<0.05$) during the fermentation period was related to the treatments fermented at 24°C compared to those fermented at 12°C (Fig. 2A). This can be related to the greater growth rate of starter yeasts (especially *S. cerevisiae*) at the higher temperature resulting in a higher production rate of carboxylic acids and also a higher consumption rate of nitrogen compounds (17). Among all treatments, the greatest pH drop rate was observed for *S. cerevisiae*-containing treatments fermented at 24°C. At constant fermentation temperatures (12 or 24°C), mixed starter-containing treatments (*S. cerevisiae* DSM 70424/*S. rouxii* DSM 2535 or *S. cerevisiae* DSM 70424/*S. rouxii* DSM 2531) did not show significantly different pH drop rates ($p>0.05$). Considering Fig. 2A, the greatest drop in the pH was observed within 24-48 hr of fermentation for treatments fermented at 12°C and 0-24 hr for those at 24°C. The reason for such difference is that at higher fermentation temperatures, *S. cerevisiae* cells enter a logarithmic/exponential phase of growth considerably sooner. With the combined *S. rouxii*-containing and *S. cerevisiae* treatments, after 48 hr of fermentation, the pH drop rate was milder when compared with those containing only *S. cerevisiae*. This phenomenon can be attributed to the very slower growth rate of *S. rouxii* strains compared to that of *S. cerevisiae*. The former does not display considerable growth rate in wort. Apart from its inherent slow growth characteristic, *S. rouxii* is unable to effectively uptake maltose (the most abundant fermentable sugar in wort) (4).

According to Fig 2B, towards the end of the fermentation period, the changes in the gravity of wort were milder, which was due to the decrease in the growth rate and as a result the lower rate of sugar uptake. The greatest wort gravity drop rate was observed for the fermentation with *S. cerevisiae* at 24°C.

Alcohol production rate was also influenced by the fermentation temperature. Within the 48 hr of fermentation, alcohol was produced at slightly higher rates when the fermentation occurred at 24°C compared to that at 12°C. However, after 48 hr of fermentation, alcohol contents started to decrease for treatments containing *S. rouxii* (Fig. 2C). The greatest decrease in alcohol content was observed for the treatment containing *S. rouxii* DSM 2535 fermented at 24°C (alcohol reduction from 1.56% to as low as 0.36%) and *S. rouxii* DSM 2531 fermented at the same temperature (alcohol reduction from 1.56 to 0.40%). In general, *S. rouxii* DSM 2535 compared to *S. rouxii* DSM 2531 decreased the amount of alcohol more effectively (especially between 48 and 65 hr of fermentation), at both fermentation temperatures. Under aerobic conditions, *S. rouxii* spp. is able to consume part of the alcohol produced in the previous stages especially when the level of fermentable sugars in the wort is low (7,13). During the first 48 hr of fermentation simple sugars of the wort (including sucrose, fructose, and maltose, particularly the first and second ones) were consumed by *S. cerevisiae* DSM 70424 (4) while *S. rouxii* spp. was unable to uptake maltose (the most abundant sugar in the wort). Therefore, the lack of fermentable sugars in the wort can be justified. Also, periodic aeration process (section 2.1) provided aerobic growth conditions for *S. rouxii*. Table 1 exhibits the data relevant to decrease in alcohol content due to the presence of *S. rouxii* strains in the treatments. The alcohol contents at the end of 96 hr fermentation period by *S. cerevisiae* were 2.75 at 12°C and 1.91%(v/v) at 24°C (Fig. 2C).

Table 1. Effect of *S. rouxii* strains on the decease in the alcohol content in *S. rouxii*-containing treatments

Treatment	Decrease in alcohol content (%) from 48 to 96 hr	Rate of decrease in alcohol content (alcohol%/day) from 48 (day 4) to 96 hr (day 8)
S. cer./S. rxi 2535 (12°C)[1)]	0.88[c2)]	0.22[c]
S. cer./S. rxi 2535 (24°C)	1.29[a]	0.32[a]
S. cer./S. rxi 2531 (12°C)	0.78[cd]	0.20[cd]
S. cer./S. rxi 2531 (24°C)	1.20[ab]	0.30[ab]

[1)]*S. cer.*=*Saccharomyces cerevisiae*; *S. rxi*= *Saccharomyces rouxii*.
[2)]Means shown in the same column with different letters are significantly different (p<0.05).

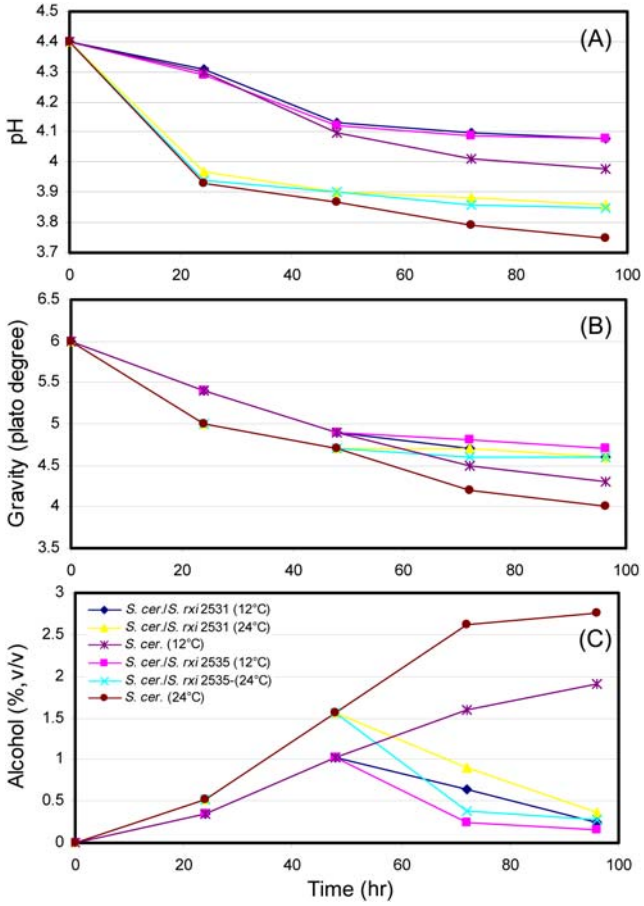

Fig. 2. Changes in the pH (A), gravity (B), and alcohol content (C) at different fermentation conditions. *S. cer.*=*Saccharomyces cerevisiae; S. rxi*=*Saccharomyces rouxii*.

Concentrations of acetaldehyde, diacetyl, and 2,3-pentanedione in treatments at the end of fermentation period The concentrations of acetaldehyde, diacetyl, and 2,3-pentanedione at the end of the fermentation period (96 hr) are shown in Fig. 3 for different treatments of this study. As shown, the concentrations of the 3 odorous compounds were significantly lower in treatments containing only *S. cerevisiae* when compared with those containing *S. rouxii*. Their concentrations were also significantly lower in treatments fermented at 24°C compared with those fermented at 12°C. Acetaldehyde, diacetyl, and 2,3-pentanedione have been known to have a key role in the typical 'wort/worty/wort-like' and 'buttery' off flavors in the beer (17,18). Wort-like off flavor is due to the presence of aldehydes produced during the wort boiling and fermentation, which were not reabsorbed by the yeast cells for alcohol production (11,19). Yeast secrete α-acetohydroxy acids e.g., α-acetolactate and α-acetohydroxybutyrate during the fermentation process (19). These compounds would then convert to vicinal diketones (diacetyl and 2,3-pentanedione, respectively) through a non-enzymatic pathway. Above certain levels, vicinal diketones (VDKs) are known as off flavor compounds in the wort more than a level to be determined (19,20).

Diacetyl at optimum (sub-threshold) concentration contributes in the flavor maturity (light flavor characteristic) along with 2,3-pentanedione of beer, while at higher amounts, it results in the off flavor (21,22). In the production of non-alcoholic beer by the restricted fermentation practice, aldehydes accumulate in the wort because they are not properly reabsorbed back into the yeast cells for further metabolism. As mentioned earlier (section 3.1), *S. rouxii* are naturally a slowly-growing species in the wort. Furthermore, depletion of fermentable carbohydrates after 48 hr of fermentation (when *S. rouxii* inoculated into wort) as well as applying periodic aeration resulted in restrictive situation for the yeast fermentation. Therefore, the accumulated volatile compounds in the wort due to their ineffective elimination after 48 hr of fermentation led to the higher concentrations of these compounds in *S. rouxii*-containing treatments compared with those containing *S. cerevisiae* only. It has been reported that using mutated strains of *S. cerevisiae* (that are unable to effectively uptake regular fermentable sugars in the wort) in the manufacturing of alcohol-free beer results in the production of relatively high amounts of acetaldehyde in the wort and its relevant 'worty' off odor (23). Regardless of its off flavor, acetaldehyde at higher levels disrupts the metabolism of yeast cells (23). Periodic aeration as practiced in the present study should have a reducing effect on the aldehydes concentration in the wort. Because, besides their re-absorption into yeast cells, aldehydes are utilized under aerobic conditions to produce unsaturated fatty acids necessary for the growth of yeast cells (24,25). In the production of alcohol-free beer by the restricted fermentation practice attempts are made to regulate the process in a way that wort aldehydes are reduced as much as possible by the activity of yeast alcohol dehydrogenase (ADHs) (18). This way, the characteristic flavor profile of beer can be achieved without the production of high amounts of alcohol and certain off flavors such as diacetyl (contributing to the buttery off flavor of beer) (18,26,27). The fact that the concentrations of the 3 odor compounds in this study (Fig. 3) were significantly lower in the treatments fermented at 24°C (compared with those fermented at 12°C) can be justified in this way that higher fermentation temperatures intensify

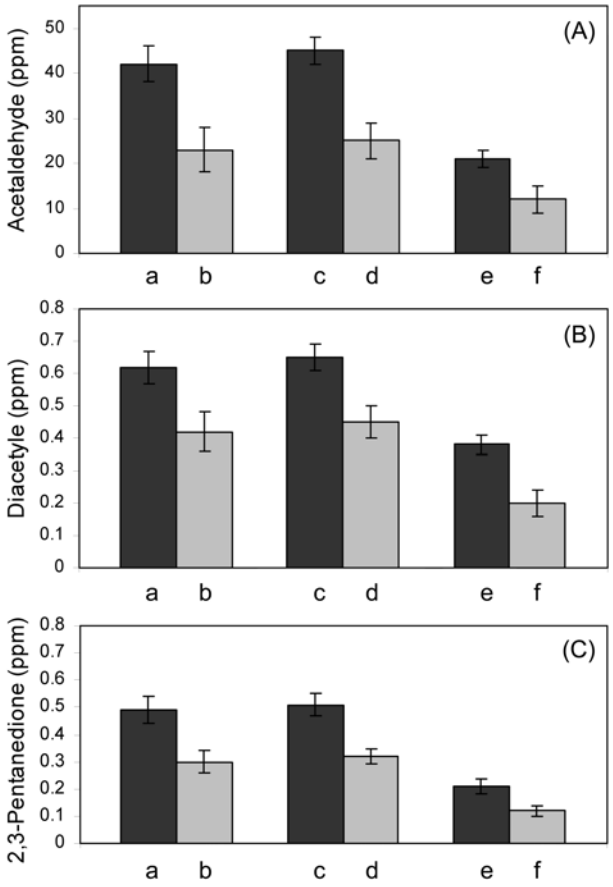

Fig. 3. Concentrations of acetaldehyde (A), diacetyl (B), and 2,3-pentanedione (C) in different treatments at the end of fermentation period. *S. cer.=Saccharomyces cerevisiae; S. rxi= Saccharomyces rouxii*. a, *S. cer./S. rxi* 2531 (12°C); b, *S. cer./S. rxi* 2531 (24°C); c, *S. cer./S. rxi* 2535 (12°C); d, *S. cer./S. rxi* 2535 (24°C); e, *S. cer.* (12°C); f, *S. cer.* (24°C).

the elimination of those compounds by vaporization during the fermentation (28). Performing fermentation at temperatures higher than the boiling point of acetaldehyde (21°C) has been reported to have a remarkable impact on the depletion of this compound. This operation decreases the concentration of acetaldehyde less than its odor threshold in beer, i.e., about 25 ppm (28). In the present study, for *S. rouxii*-containing treatments fermented at 12°C (data not shown) acetaldehyde concentrations were greater than the above limit. No significant differences were observed in the concentrations of volatile compounds between the 2 *S. rouxii*-containing treatments (*S. rouxii* DSM 2535 and DSM 2531) (Fig. 3). This indicates that the 2 strains have similar performance in the above metabolism.

Duo sensory comparison among the treatments at the end of the fermentation period Paired comparisons of flavor maturity/fullness, taint and overall acceptability of the beers at the end of fermentation period are shown in Table 2. According to the results, *S. cerevisiae* DSM 70424/*S. rouxii* DSM 2535 (12°C) and *S. cerevisiae* DSM 70424-*S. rouxii* DSM 2531 (12°C) treatments as well as those of *S. cerevisiae* DSM 70424/*S. rouxii* DSM 2535 (24°C) and *S. cerevisiae* DSM 70424-*S. rouxii* DSM 2531-(24°C) did not show significant differences ($p>0.05$). The treatment of S. *cerevisiae* DSM 70424/*S. rouxii* DSM 2535 (24°C) exhibits significantly greater overall acceptability than *S. cerevisiae* DSM 70424/*S. rouxii* DSM 2535 (12°C) ($p<0.05$). Also, the treatment of *S. cerevisiae* DSM 70424 (24°C) possesses greater overall acceptability than *S. cerevisiae* DSM 70424/*S. rouxii* DSM 2535 (24°C) ($p<0.05$).

No significant differences in the sensory attributes were found between the 2 treatments containing *S. rouxii* DSM 2535 and DSM 2531 fermented at the same temperature (12 or 24°C). In contrast, the *S. rouxii*-containing treatments fermented at 12°C served more taint and less overall acceptability than those fermented at 24°C; because as discussed previously, at higher fermentation temperatures volatile compounds contributing in the off flavor of wort can be eliminated more effectively. In *S. rouxii*-containing treatments fermented at 12°C, the concentrations of acetaldehyde were higher than its odor threshold (25 ppm) and therefore the 'worty' off flavor was evident. Beers produced by the same yeasts (*S. rouxii* DSM 2535 or DSM 2531) but fermented at 24°C (instead of 12°C), taint off flavor was not as remarkable as those obtained at 12°C. On the other hand, treatments containing only *S. cerevisiae* resulted in beers with more satisfactory flavor attributes (Table 2). This can be attributed to the difference in flavor profile of beer produced by the whole 96 hr-fermentation of *S. cerevisiae* compared with that produced by half 96 hr -fermentation of the mentioned yeast as well as the higher amount of alcohol produced in former treatments.

Generally, the nonalcoholic beer exhibits poor and immature flavor (also called artificial or dull flavor) compared with normal beer (17). Other than giving a warming sensation (from mouth to stomach) after ingestion, ethanol can also act as a flavor enhancer in some cases (10). Ethanol has also been considered as a key agent for exhibiting the characteristic background flavor of beer (10). Fusel alcohols (butyl, amyl, and iso-amyl alcohols) have low impact on the flavor profile, but take part in the producing mouth-stomach warm sensation (10,17). Therefore, alcohol-free beer does not have the mentioned characteristics.

Ethanol is also a precursor for flavor-active esters (19). Hence, in the absence of ethanol, beer serves immature flavor (19). Therefore, alcohol-free beer does not constitute the flavor components produced via the fermentation in an appropriate concentration and balance (harmony) (6-8,15). Although in nonalcoholic beers the covering effect of ethanol on the perception of worty off flavor is also missing (19), such taint is perceived at considerably lower odor threshold levels. It is noteworthy that the flavor characteristics of beers from *S. rouxii*-containing treatments and those from *S. cerevisiae* alone (fermented at 24°C) were not too much different. But, those from *S. rouxii*-containing treatments fermented at 12°C differed at a larger level.

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Table 2. Paired comparisons of flavor maturity/fullness, taint, and overall acceptability of the beer among the different treatments applied in this study

Comparing treatments	Statistical differences in parameters		
	Flavor maturity/fullness	Taint	Overall acceptability
S. cer./S. rxi 2535 (12°C) & *S. cer./S. rxi* 2531 (12°C)[1)]	(p>0.05)	(p>0.05)	(p>0.05)
S. cer./S. rxi 2535 (24°C) & *S. cer./S. rxi* 2531 (24°C)	(p>0.05)	(p>0.05)	(p>0.05)
S. cer./S. rxi 2535 (24°C) & *S. cer./S. rxi* 2535 (12°C)	(p>0.05)	*S. rxi* 2535 (12°C)> *S. rxi* 2535 (24°C) (p<0.05)	*S. rxi* 2535(24°C)> *S. rxi* 2535 (12°C) (p<0.05)
S. cer (24°C) & *S. cer./S. rxi* 2535 (24°C)	*S. cer* (24°C)> *S. cer./S. rxi* 2535 (24°C) (p<0.05)	*S. cer./S. rxi* 2535 (24°C)> *S. cer* (24°C) (p<0.05)	*S. cer* (24°)> *S. cer./S. rxi* 2535 (24°C) (p<0.05)

[1)]*S. cer.=Saccharomyces cerevisiae*; *S. rxi=Saccharomyces rouxii*

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