

# Evaluation of Beer Fermentation with a Novel Yeast *Williopsis saturnus*

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## Summary

The aim of this study is to evaluate the potential of a novel yeast *Williopsis saturnus* var. *mrakii* NCYC 500 to produce fruity beer. Fermentation performance of *W. mrakii* and beer volatile composition were compared against that fermented with *Saccharomyces cerevisiae* Safale US-05. °Brix, sugar and pH differed significantly between the two types of beer. A total of 8 alcohols, 11 acids, 41 esters, 9 aldehydes, 8 ketones, 21 terpenes and terpenoids, 5 Maillard reaction products and 2 volatile phenolic compounds were detected. Yeast strain Safale US-05 was more capable of producing a wider range of ethyl and other esters, while yeast strain NCYC 500 produced significantly higher amounts of acetate esters. Strain NCYC 500 retained more terpenes and terpenoids, suggesting that the resultant beer could possess more of the aromatic hint of hops. This study showed that *W. saturnus* var. *mrakii* NCYC 500 could ferment wort to produce low-alcohol beer with higher levels of acetate esters, terpenes and terpenoids than yeast *S. cerevisiae* Safale US-05.

*Key words:* fermentation, beer, volatiles, esters, *Williopsis* sp., yeasts

## Introduction

Beer is traditionally brewed from malted barley, water and hops using *Saccharomyces cerevisiae*, which produces flavour-active substances during wort fermentation, giving beer its flavour profile. These substances include esters, alcohols, aldehydes and ketones, volatile sulphur compounds and fatty acids, etc. Among the flavour volatiles, esters are one of the most important aroma compounds found in beer, which provide fruity aroma (1,2).

Esters may be broadly categorised into two main groups, namely acetate esters and ethyl esters. Acetate esters are produced *via* alcoholysis from alcohols and acetyl coenzyme A (CoA) catalysed by alcohol acetyl transferases. Important acetate esters in beer include ethyl acetate, isoamyl acetate and 2-phenylethyl acetate (3). On the other hand, ethyl esters are secondary metabolites produced

by both *S. cerevisiae* and non-*Saccharomyces* yeasts. These ethyl esters can be formed by esterification or alcoholysis. Esterification forms esters *via* condensation reactions between ethanol and carboxylic acids catalysed by fatty acid ethyl ester (FAEE) synthases. In contrast, alcoholysis is a transesterification reaction between fatty acyl-CoAs and ethanol catalysed by fatty acyl transferases (4). Important ethyl esters in beer include ethyl octanoate, ethyl hexanoate and ethyl decanoate (5).

At an industrial level, *S. cerevisiae* is the preferred yeast to perform beer fermentation because of its efficient conversion of sugars to ethanol, high ethanol tolerance and ability to carry out fermentation under acidic conditions as in wort (6). However, consistence in fermentation practices results in little flavour differentiation. To drive product differentiation, various microbreweries are dedi-

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cated to the production of specialty beer. Examples include fruit, honey and herb or spiced beer. To boost the fruity aroma in fruit beer, brewers have come up with different methods and styles of brewing by adding fruit (7). Fruits can also provide flavours, aromas and colours that cannot be obtained from grains.

The resultant fruit beer often lacks traditional beer flavours (7) so research has been directed at adding fruitiness to beer by enhancing the formation of flavour-active esters with the use of *S. cerevisiae* (1) or genetically modified yeasts (4,5). However, given the controversial nature of genetic modification in food application, it would be of interest to impart fruitiness to beer using natural yeasts including non-*Saccharomyces* yeasts.

Non-*Saccharomyces* yeasts such as *Brettanomyces* sp. produce volatile phenols and play important positive roles in the spicy and smoky flavour profiles of specialty beer such as Lambic and Gueuze (8). Other non-*Saccharomyces* yeasts such as *Candida* and *Pichia* are well-known in sherry production for their role in oxidising ethanol to acetaldehyde (giving oxidised off-flavour) and have also been reported in mixed culture fermentation of acidic ales (8). Nevertheless, non-*Saccharomyces* yeasts can cause damage to beer and wine flavour, depending on the yeast and style of an alcoholic beverage.

One of the non-*Saccharomyces* yeasts that have received much research attention is from the genus *Williopsis*, which includes the saturn-shaped *Williopsis saturnus* (9). *W. saturnus* is known to possess high activities for synthesising fruity acetate esters, besides antifungal properties (10). Yilmaztekin *et al.* (11) studied the growth of *W. saturnus* var. *saturnus* on beet molasses and found that the yeast produced significant amounts of isoamyl acetate. Fermentation studies in sake using *W. saturnus* var. *mrakii* showed that it was a potent producer of acetate esters (12,13).

*Williopsis* yeasts are not usually associated with beer fermentation, but have found potential applications in wine and sake fermentations (12–14). Furthermore, the ability of *Williopsis* yeasts to produce esters, especially acetate esters, while producing small amounts of ethanol in wine suggests a feasible way for production of fruity, low-alcohol beer (14). Therefore, the aim of this study is to evaluate the potential of *W. saturnus* var. *mrakii* NCYC 500 to produce fruity beer. Fermentation performance of *W. mrakii* NCYC 500 was compared against that of *S. cerevisiae* Safale US-05. Findings of this study could offer a novel biotechnological direction in brewing extra fruity, low-alcohol beer.

## Materials and Methods

### Yeast strains and culture media

*Saccharomyces cerevisiae* Safale US-05 (Fermentis Division of S. I. Lesaffre, Marcq-en-Baroeul, France) and *Williopsis saturnus* var. *mrakii* NCYC 500 (National Collection of Yeast Cultures, Norwich, UK) were used in this study. *S. cerevisiae* Safale US-05 was stored in its unopened, new package at 4 °C. *W. saturnus* var. *mrakii* NCYC 500 was grown in malt-peptone-yeast extract-glucose broth (pH=5)

consisting of (in %, by mass per volume): glucose 2, bacteriological peptone 0.25, malt extract 0.25 and yeast extract 0.25. Precultures were prepared by incubating the yeast in the same broth at 25 °C for 48 h. Strain NCYC 500 with 20 % glycerol was maintained at –80 °C prior to use. Potato dextrose agar (PDA, 39 g/L; Oxoid, Basingstoke, Hampshire, UK) was used for enumeration.

### Wort preparation

Wort was prepared by dissolving 1.1 L of Light Malt Extract (Coopers Brewery, Regency Park, Australia) in 6 L of water. A mass of 450 g of milled specialty malt (150 g of Carapils, 150 g of Crystal 10L and 150 g of Aromatic; Briess Malt & Ingredients Co., Chilton, WI, USA) was steeped in 1.5 L of water at 70 °C for 30 min for extraction of flavour compounds from the grains. The extract was then added to the dissolved malt extract to make up 8 L of solution, which was boiled for 60 min. Once hot break had occurred, 113 g of glucose (brewing sugar) were mixed into the wort. Then, kettle hopping was carried out. At 60 min before the end of boiling, 14 g of Cascade hop pellets (iBrew, Schaumburg, IL, USA) were added to the wort. At 5 min before the end of boiling, 7 g of Perle hop pellets (iBrew) were added. After boiling, cold distilled water (Ice Mountain, Singapore) was added to the remaining wort to make up to 5 L. The wort was then rapidly cooled down. Cooled wort was then bottled into 240-mL bottles and stored at 4 °C. Before inoculation, the wort was centrifuged (29 703×g, 3-18K centrifuge; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) for 15 min and the supernatant was collected. Sterile filtration was carried out *via* peristaltic pumping of wort through a sterile 0.65-µm filter and then a sterile 0.45-µm filter.

### Fermentation conditions

Triplicate fermentations were carried out in 500-mL sterile conical flasks (plugged with cotton wool, then wrapped in aluminium foil and parafilm) containing 400 mL of sterile wort at 21 °C. Inoculation conditions were as follows. For Safale US-05 strain, yeast cream was prepared following manufacturer's instructions: 0.92 g of dry yeast was reconstituted in 10 times its mass in wort (8 mL). Yeast cream was then inoculated at 0.5 %, by volume (equivalent to approx. 10<sup>7</sup> CFU/mL). A preculture of NCYC 500 strain grown for 48 h in nutrient broth was inoculated at 1 %, by volume (equivalent to approx. 10<sup>5</sup> CFU/mL). Fermentation was carried out statically for 14 days.

### Measurement of pH and °Brix

The total soluble solids (°Brix) and pH were measured using a refractometer (ATAGO Co., Ltd, Tokyo, Japan) and a pH meter (Metrohm, Zofingen, Switzerland), respectively.

### Analysis of sugars and organic acids

Sugars were separated and quantified using a Shimadzu UFLC (Shimadzu, Kyoto, Japan) equipped with a Pinnacle II amino column (150 mm×4.6 mm; Restek, Bellefonte, PA, USA). The mobile phase consisted of acetoni-

trile and water (80:20) that was sonicated for 30 min before use. Elution was carried out at 40 °C with flow rate of 1 mL/min (isocratic mode). An evaporative light-scattering detector (ELSD; Shimadzu, Kyoto, Japan) was used as the detector.

Organic acids were determined by a Shimadzu UFLC (same as above), using a Supelcogel C-610H column (Supelco, Bellefonte, PA, USA). A photodiode array detector at UV spectrum (210 nm) was used. The mobile phase contained 0.1 % (by volume) sulphuric acid that was sonicated for 30 min before use. Elution was carried out at 40 °C with flow rate of 0.4 mL/min (isocratic mode).

### Volatile analysis by HS-SPME-GC-MS/FID

Volatiles were analysed using headspace (HS) solid-phase microextraction (SPME) and a Carboxen/PDMS fibre (85 µm; Supelco, Sigma-Aldrich, Barcelona, Spain). This was coupled with gas chromatograph-mass spectrometer (GC-MS) and flame ionisation detector (FID) (Agilent Technologies, Santa Clara, CA, USA). Sample size was 5 mL (with pH adjusted to 2.5 using 1 M HCl). Each sample was extracted by HS-SPME at 60 °C for 50 min using 250-rpm agitation. Thermal desorption of the fibre was carried out at the injection port of Agilent 7890A gas chromatograph (Agilent Technologies) coupled to Agilent 5975C triple-axis MS at 250 °C for 3 min. Elution and separation of compounds were carried out with helium as the carrier gas in a capillary column (Agilent DB-FFAP) with dimensions of 60 m×0.25 mm i.d. The column had a 0.25-µm coating of polyethylene glycol modified with nitroterephthalic acid. Oven temperature was held at 50 °C for 5 min before a gradual increase of 5 °C per min until 230 °C, which was held for 30 min. Volatiles were identified by comparing their mass spectra with NIST08 Mass Spectral Library (15) and Wiley 275 Library (16). Identification of volatiles was verified using standard compound and/or linear retention indices (LRI).

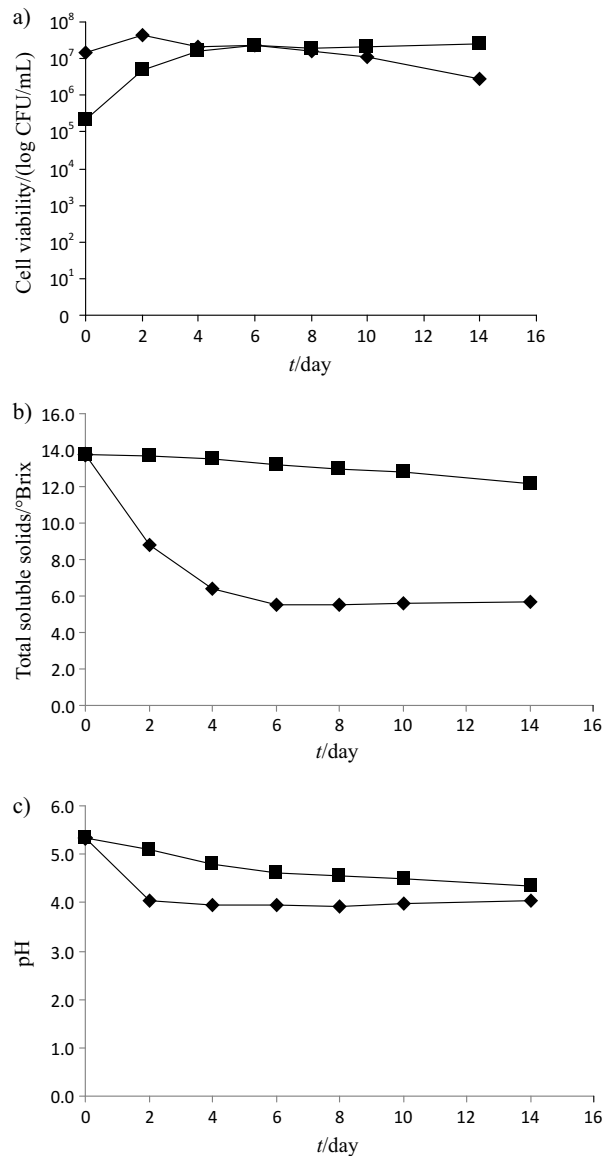
### Statistical analysis

Data collected from triplicate fermentations were subjected to statistical analysis. To compare values, analysis of variance (ANOVA) using Microsoft Office Excel (Microsoft, Redmond, WA, USA) was used. If the p-value was below 0.05, differences were deemed statistically significant.

## Results and Discussion

### Yeast growth, total soluble solids and pH changes

The two yeasts showed significant differences in terms of growth, total soluble solid content (°Brix) and pH changes (Fig. 1). *S. cerevisiae* Safale US-05 reached the maximum cell count on day 2 at  $4.44 \cdot 10^7$  CFU/mL. The maximum *W. saturnus* var. *saturnus* NCYC 500 cell count of  $2.26 \cdot 10^7$  CFU/mL was reached on day 6 (Fig. 1a). The total soluble solid content reduced only slightly in the beer fermented with NCYC 500 strain relative to that of the beer fermented with Safale US-05 strain; the latter total soluble solid content decreased from 13.8 to 5.9 % by day 6 (Fig. 1b). The pH decrease in the beer fermented



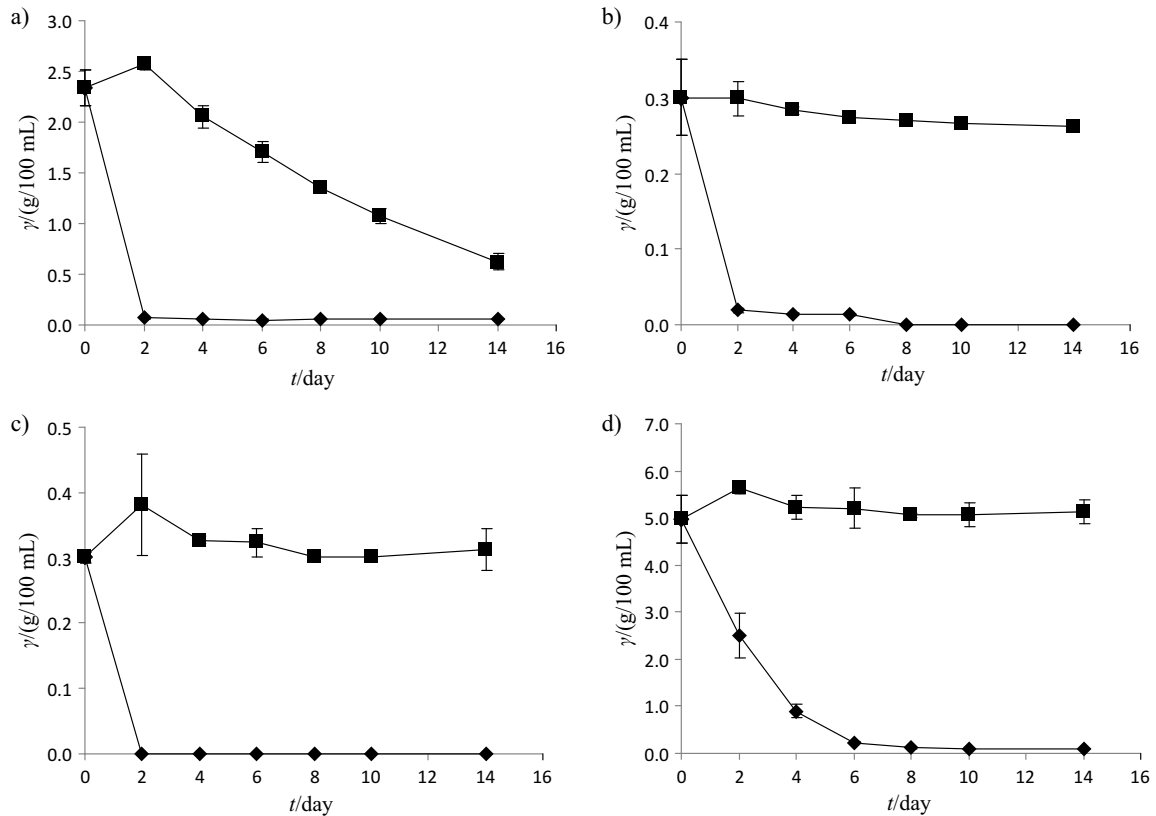
**Fig. 1.** Changes in: a) growth of yeasts, b) total soluble solids, and c) pH during ale beer fermentation by yeasts *S. cerevisiae* Safale US-05 (diamond) and *W. saturnus* var. *mrakii* NCYC 500 (square)

with NCYC 500 strain was more gradual than that of the beer fermented with Safale US-05 strain; the latter pH decreased markedly from day 0 to day 2 (Fig. 1c).

Other researchers have also observed the comparatively slower growth rates of *Williopsis* yeasts (17,18). It has been shown that the intensity of fermentation (defined as the average number of days elapsing between inoculation of the glucose fermentation tube and the reduction of gas volume in the insert) with *W. saturnus* var. *mrakii* takes about 5 days (19). Thus, growth of NCYC 500 strain observed here corresponded to that reported for this yeast.

### Sugar utilisation

Safale US-05 strain utilised glucose, fructose and sucrose rapidly by day 2 (Figs. 2a–c). This corresponded to



**Fig. 2.** Changes of: a) glucose, b) fructose, c) sucrose and d) maltose concentrations during ale beer fermentation by yeasts *S. cerevisiae* Safale US-05 (diamond) and *W. saturnus* var. *mrakii* NCYC 500 (square)

the large decreases in °Brix, pH and maximal growth of Safale US-05 strain from day 0 to day 2, indicating the efficient fermentation of these sugars by the yeast. On the other hand, the utilisation of these sugars by NCYC 500 strain was much slower, with glucose being preferred but with few disaccharides being fermented. Maltose utilisation by Safale US-05 strain was the slowest amongst the four sugars (Fig. 2d), with only about half of the sugar being utilised by day 2 as opposed to the virtually complete utilisation of the other three sugars by the yeast during that time.

Utilisation of sugars by *S. cerevisiae* is well-studied with easily fermentable monosaccharides such as D-fructose and D-glucose, which are utilised first (20). *Williopsis* yeasts have also been reported to be glucophilic, consuming glucose faster than fructose (11). Sucrose was depleted by Safale US-05 strain by day 2 (Fig. 2c). It is not transported into the yeast cell but it is hydrolysed by invertase into fructose and glucose in the periplasmic space (21). Also, sucrose may be broken down *via* acid hydrolysis into the monosaccharides followed by intracellular utilisation. Unlike Safale US-05, the wort fermented by NCYC 500 strain had a high content of residual sucrose, likely due to its weak fermentative ability.

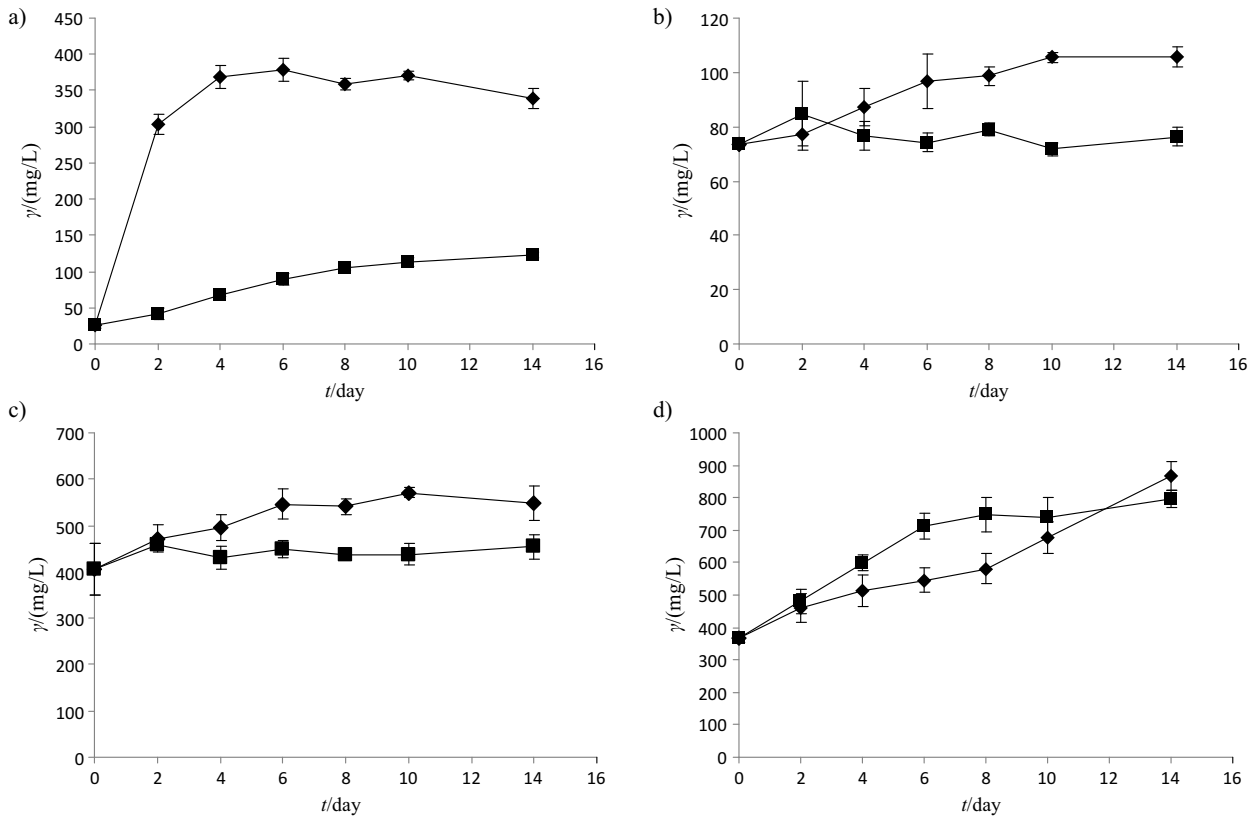
Utilisation of maltose by *S. cerevisiae* was slower than of fructose or glucose. This could be due to catabolite repression by glucose, which represses maltase gene expression (22). Fermentation of maltose with NCYC 500 strain was not significant (Fig. 2d). Non-utilisation of maltose is apparently characteristic of *Williopsis saturnus*

yeasts (23). Maltose is a quantitatively dominant sugar in wort and its utilisation (or lack of) has a direct influence on the alcohol content in the final beer, as shown in this study.

#### Organic acid metabolism

Among the organic acids in beer fermented by Safale US-05 strain, those that showed significant increases in concentration were pyruvic,  $\alpha$ -ketoglutaric, lactic and acetic acids (Fig. 3). When using NCYC 500 strain, the pyruvic and acetic acid concentrations increased significantly. The concentration of the remaining acids stayed relatively constant (Fig. 3).

Pyruvic acid concentration increased significantly in the beer fermented with Safale US-05 strain (Fig. 3a). The beer fermented with NCYC 500 strain also showed an increase in pyruvic acid concentration, but it was not as significant. In both yeasts, the increase in pyruvic acid concentration was due to the Embden-Meyerhof pathway, which converts available sugars into pyruvic acid, yielding adenosine triphosphate (ATP). Fermentation rate by Safale US-05 was much faster than by NCYC 500 strain, which might explain why pyruvic acid concentration in the Safale US-05-fermented beer increased significantly by day 2, while there was a much more gradual increase in pyruvic acid concentration in the beer fermented with NCYC 500. In addition, the higher concentration of pyruvic acid in the beer fermented with Safale US-05 could also be because *S. cerevisiae* carries out anaerobic fermentation



**Fig. 3.** Changes of major organic acid: a) pyruvic, b)  $\alpha$ -ketoglutaric, c) lactic and d) acetic acid concentrations during ale beer fermentation by *S. cerevisiae* Safale US-05 (diamond) and *W. saturnus* var. *mrakii* NCYC 500 (square)

while *W. saturnus* var. *mrakii* is more oxidative. Thus, oxidative respiration would result in a lower concentration of pyruvic acid being excreted into the medium by NCYC 500 strain.

The initial concentration of  $\alpha$ -ketoglutaric acid was low (Fig. 3b). In the beer fermented with Safale US-05 strain,  $\alpha$ -ketoglutaric acid concentration increased. In the beer fermented with NCYC 500 strain,  $\alpha$ -ketoglutaric acid concentration showed minor fluctuations.  $\alpha$ -Ketoglutaric acid is synthesised from the Krebs cycle or from amino acid catabolism and is a minor organic acid found in beer (24). Thus, it was expected that in the beer fermented with Safale US-05 the increase of the concentration of this acid was greater because amino acid catabolism was more extensive in *S. cerevisiae* than in NCYC 500 strain (data not shown).

Lactic acid concentration increased slightly throughout fermentation in the beer fermented with Safale US-05 (Fig. 3c). In the beer fermented with NCYC 500 strain, there were minor fluctuations in lactic acid concentration. Strain NCYC 500, being more oxidative, would likely carry out little, or none at all, anaerobic conversion of pyruvic to lactic acid, which might explain the insignificant concentration of lactic acid in the beer fermented with NCYC 500.

Acetic acid concentration in the beer fermented with both types of yeast increased, with the production rate being higher by NCYC-500 than by Safale US-05 (Fig. 3d). However, the final concentration of acetic acid was a little higher in the beer fermented with Safale US-05 (870 mg/L)

than in the beer fermented with NCYC 500 (800 mg/L). Acetic acid can be formed in *S. cerevisiae* by the oxidation of acetaldehyde, an intermediate in the alcoholic fermentation pathway, and co-reduction of  $\text{NAD}^+$  catalysed by aldehyde dehydrogenase. The oxidation of acetaldehyde to acetic acid acts as a redox sink for anabolic and physiological stress reactions. Pyruvic acid can also be reabsorbed by the yeast and metabolised to acetate (25). Excess acetic acid is secreted into the fermentation medium (26). Acetic acid is not desired in alcoholic beverages as it can impart a sour vinegary off-odour if its concentration is too high. Since the flavour threshold of acetic acid in beer is 130 mg/L, the detected concentrations of acetic acid in the two beers could impart a vinegary odour, although this was not discernible organoleptically in informal sensory evaluation by sniffing.

#### Changes in volatile composition

A total of 8 alcohols, 11 acids, 41 esters, 9 aldehydes, 8 ketones, 21 terpenes and terpenoids, 5 Maillard reaction products (furans, pyrans and pyrroles) and 2 volatile phenolic compounds were detected in the beer samples. Selected major volatiles in wort and beer are presented in Table 1.

The biggest group of volatiles detected in the beer samples were esters (Table 1). Fermentation altered the volatile composition of wort drastically, with a large variety of esters reduced or produced *via* the yeast metabolism. Ethyl and acetate esters were the two most significant types of esters detected.

Table 1. Selected major volatile compounds (GC-FID peak area·10<sup>6</sup>) and their relative peak areas (RPA) identified in wort and beer fermented with *S. cerevisiae* Safale US-05 or *W. saturnus* var. *mrakii* NCYC 500 after 14 days of fermentation

Compound	RI	Wort		Beer fermented with Safale US-05		Beer fermented with NCYC 500		Organoleptic properties
		Peak area	RPA/%	Peak area	RPA/%	Peak area	RPA/%	
<i>Acids</i>								
Acetic	1464	(2.2±0.3) <sup>a</sup>	1.2	(4.8±0.2) <sup>b</sup>	0.1	(7.4±0.4) <sup>c</sup>	0.7	Sour, vinegar
Isobutanoic	1576	(0.6±0.1) <sup>a</sup>	0.3	(1.2±0.3) <sup>a</sup>	0.03	(0.5±0.1) <sup>a</sup>	0.04	Acidic, unpleasant
Hexanoic	1855	(0.6±0.0) <sup>a</sup>	0.3	(4.5±0.7) <sup>b</sup>	0.1	(1.6±0.1) <sup>a</sup>	0.2	Sour, fatty, cheesy
Octanoic	2068	(1.8±0.4) <sup>a</sup>	1.0	(28.2±6.5) <sup>b</sup>	0.7	(3.6±0.1) <sup>a</sup>	0.3	Fatty, rancid, cheesy
Decanoic	2282	(0.6±0.1) <sup>a</sup>	0.3	(9.2±2.9) <sup>b</sup>	0.2	(1.2±0.1) <sup>a</sup>	0.1	Rancid, sour, fatty
Benzoic	2463	(0.2±0.1) <sup>a</sup>	0.1	(0.8±0.1) <sup>b</sup>	0.02	(0.2±0.0) <sup>a</sup>	0.02	Faint, balsam
<i>Alcohols</i>								
Ethanol	946	(8.1±0.3) <sup>a</sup>	4.3	(3052±133) <sup>b</sup>	77.4	(604±33) <sup>c</sup>	57.0	Alcoholic, ethereal
Isobutanol	1084	n.d.	0.0	(8.9±0.3) <sup>a</sup>	0.2	(5.2±0.2) <sup>b</sup>	0.5	Ethereal, winey
Active amyl alcohol	1211	(0.8±0.2) <sup>a</sup>	0.4	n.d.	0.0	(7.1±0.5) <sup>b</sup>	0.7	Pungent, fermented
Isoamyl alcohol	1214	(4.3±0.2) <sup>a</sup>	2.3	(25.4±5.4) <sup>b</sup>	0.6	(44.7±4.1) <sup>c</sup>	4.2	Fusel, alcoholic, fruity
Hexanol	1359	(0.67±0.01) <sup>a</sup>	0.4	(0.21±0.02) <sup>b</sup>	0.01	(0.8±0.1) <sup>a</sup>	0.07	Pungent, fusel oil, fruity
2-Phenethyl alcohol	2027	n.d.	0.0	(56.3±3.8) <sup>a</sup>	1.4	(1.1±0.0) <sup>b</sup>	0.1	Floral, rose, honey
<i>Aldehydes</i>								
Acetaldehyde	761	n.d.	0.0	9.7±0.4	0.3	n.d.	0.0	Pungent, ethereal, fruity
3-Methylbutanal	929	9.0±0.6	4.8	n.d.	0.0	n.d.	0.0	Ethereal, chocolate, peach
Hexanal	1067	0.91±0.07	0.5	n.d.	0.0	n.d.	0.0	Fresh, green, fatty
Nonanal	1390	n.d.	0.0	(1.7±0.2) <sup>a</sup>	0.04	(0.81±0.04) <sup>b</sup>	0.1	Fatty, orange peel
Benzaldehyde	1543	(2.7±0.2) <sup>a</sup>	1.5	(4.3±0.2) <sup>b</sup>	0.1	(4.0±0.2) <sup>b</sup>	0.4	Almond, cherry
(E)-2-Decenal	1655	n.d.	0.0	(0.36±0.02) <sup>a</sup>	0.01	(0.29±0.03) <sup>b</sup>	0.03	Waxy, coriander
2-Phenylacetaldehyde	1665	1.8±0.0	1.0	n.d.	0.0	n.d.	0.0	Green, floral, honey
<i>o</i> -Tolualdehyde	1673	n.d.	0.0	(1.7±0.2) <sup>a</sup>	0.04	(4.9±0.1) <sup>b</sup>	0.5	Cherry
<i>m</i> -Tolualdehyde	1673	6.0±0.2	3.2	n.d.	0.0	n.d.	0.0	Sweet, cherry
<i>Esters</i>								
Methyl heptanoate	1277	0.24±0.02	0.1	n.d.	0.0	n.d.	0.0	Sweet, fruity
Methyl 4-methyl-2-hexenoate	1329	n.d.	0.0	(0.55±0.06) <sup>a</sup>	0.01	(2.7±0.1) <sup>b</sup>	0.3	Sweet, fruity
Ethyl propionate	958	3.7±0.2	2.0	n.d.	0.0	n.d.	0.0	Sweet, grape, winey
Ethyl butanoate	1034	n.d.	0.0	(5.6±0.4) <sup>a</sup>	0.14	(3.4±0.1) <sup>b</sup>	0.3	Fruity, pineapple
Ethyl hexanoate	1216	n.d.	0.0	42.4±7.5	1.1	n.d.	0.0	Sweet, fruity, pineapple
Ethyl octanoate	1435	(1.6±0.3) <sup>a</sup>	0.9	(383±6.0) <sup>b</sup>	9.7	(3.05±0.02) <sup>a</sup>	0.3	Fruity, wine, sweet
Ethyl decanoate	1643	(2.2±0.8) <sup>a</sup>	1.2	(182±41.5) <sup>b</sup>	4.6	(5.46±0.05) <sup>a</sup>	0.5	Sweet, fruity, apple, grape
Ethyl 9-decenoate	1694	(0.5±0.1) <sup>a</sup>	0.2	(45.1±8.9) <sup>b</sup>	1.1	(0.28±0.03) <sup>a</sup>	0.03	Fruity, fatty
Ethyl laurate	1850	(0.4±0.2) <sup>a</sup>	0.2	(16.7±5.6) <sup>b</sup>	0.4	(3.0±0.2) <sup>a</sup>	0.3	Sweet, waxy, soapy
Isobutyl isobutanoate	1070	(7.3±0.1) <sup>a</sup>	3.9	n.d.	0.0	(0.44±0.03) <sup>b</sup>	0.04	Pineapple, tropical
Isobutyl 2-methylbutanoate	1148	0.29±0.04	0.2	n.d.	0.0	n.d.	0.0	Sweet, fruity
Isoamyl propionate	1165	(4.6±0.1) <sup>a</sup>	2.4	n.d.	0.0	(3.3±0.2) <sup>b</sup>	0.3	Sweet, fruity, pineapple
Amyl isobutanoate	1170	1.5±0.1	0.8	n.d.	0.0	n.d.	0.0	Fruity, apricot, pineapple
2-Methylbutyl isobutanoate	1172	8.0±0.1	4.3	n.d.	0.0	n.d.	0.0	Fruity, ethereal
Isoamyl octanoate	1455	n.d.	0.0	0.49±0.02	0.01	n.d.	0.0	Sweet, pineapple, coconut
Propyl octanoate	1516	n.d.	0.0	0.66±0.06	0.02	n.d.	0.0	Coconut, cacao, gin
2-Methylbutyl octanoate	1660	n.d.	0.0	2.6±0.5	0.07	n.d.	0.0	Apple, fruity
Propyl decanoate	1725	n.d.	0.0	0.22±0.05	0.01	n.d.	0.0	Fruity, fatty, green
Isobutyl decanoate	1760	n.d.	0.0	0.30±0.07	0.01	n.d.	0.0	Brandy, apricot, cognac
Isoamyl decanoate	1867	n.d.	0.0	1.3±0.2	0.03	n.d.	0.0	Fruity, sweet

Table 1. – continued

Compound	RI	Wort		Beer fermented with Safale US-05		Beer fermented with NCYC 500		Organoleptic properties
		Peak area	RPA/%	Peak area	RPA/%	Peak area	RPA/%	
Ethyl acetate	905	(19.7±0.7) <sup>a</sup>	10.42	(14.7±0.9) <sup>a</sup>	0.4	(155±9) <sup>b</sup>	14.6	Ethereal, fruity, sweet, weedy, green
Isobutyl acetate	1015	n.d.	0.0	n.d.	0.0	3.8±0.3	0.4	Sweet, fruity, ethereal
Isoamyl acetate	1095	(45.8±1.0) <sup>a</sup>	24.3	(7.8±0.6) <sup>b</sup>	0.2	(148±14.5) <sup>c</sup>	14.0	Sweet, fruity, banana
2-Phenylethyl acetate	1832	(0.8±0.0) <sup>a</sup>	0.4	(2.9±0.5) <sup>a</sup>	0.07	(12.9±1.8) <sup>b</sup>	1.2	Floral, rose, sweet
<i>Ketones</i>								
4-Methyl-2-pentanone	1012	3.2±0.1	1.7	n.d.	0.00	n.d.	0.00	Sharp, green, fruity
2-Octanone	1280	(0.37±0.03) <sup>a</sup>	0.2	n.d.	0.00	(0.16±0.01) <sup>b</sup>	0.01	Earthy, woody
6-Methyl-5-hepten-2-one	1338	(1.05±0.04) <sup>a</sup>	0.6	(0.34±0.05) <sup>b</sup>	0.01	(0.220±0.004) <sup>b</sup>	0.02	Mushroom, blackcurrant
2-Nonanone	1389	(2.1±0.3) <sup>a</sup>	1.1	(0.41±0.08) <sup>b</sup>	0.01	n.d.	0.00	Fruity, sweet, waxy
<i>Maillard products</i>								
2-Acetylpyrrole	1996	(0.11±0.01) <sup>a</sup>	0.06	(0.17±0.02) <sup>b</sup>	0.00	(0.150±0.004) <sup>b</sup>	0.01	Musty, nutty
Furfural	1481	(9.6±0.3) <sup>a</sup>	5.1	(0.53±0.05) <sup>b</sup>	0.01	(1.69±0.04) <sup>c</sup>	0.2	Sweet, caramellic
2-Acetylfuran	1523	(0.50±0.07) <sup>a</sup>	0.3	(0.23±0.02) <sup>b</sup>	0.01	(0.67±0.04) <sup>c</sup>	0.06	Sweet, caramel, coffee
<i>Phenolic compounds</i>								
4-Vinyl-2-methoxy-phenol	2225	(0.080±0.004) <sup>a</sup>	0.04	(0.15±0.02) <sup>b</sup>	0.00	(0.100±0.002) <sup>a</sup>	0.01	Spicy, clove, smoky
2,4-di-tert-Butylphenol	2322	(0.90±0.02) <sup>a</sup>	0.48	(0.22±0.02) <sup>b</sup>	0.01	(0.49±0.01) <sup>c</sup>	0.05	Phenolic
Dimethyl disulfide	1061	1.17±0.04	0.62	n.d.	0.00	n.d.	0.00	Sulfurous, cabbage
<i>Terpenes and terpenoids</i>								
α-Phellandrene	1126	1.6±0.2	0.8	n.d.	0.00	n.d.	0.00	Citrus, green
Sabinene	1128	(0.6±0.1) <sup>a</sup>	0.3	(0.26±0.05) <sup>b</sup>	0.01	(1.79±0.01) <sup>c</sup>	0.17	Woody, citrus
α-Terpinene	1142	(0.40±0.05) <sup>a</sup>	0.2	(0.15±0.02) <sup>b</sup>	0.00	(0.38±0.01) <sup>a</sup>	0.04	Citrusy, woody
Limonene	1161	n.d.	0.0	(0.39±0.07) <sup>a</sup>	0.01	(0.45±0.15) <sup>a</sup>	0.04	Citrus, fresh, sweet
cis-Ocimene	1208	1.2±0.4	0.6	n.d.	0.00	n.d.	0.00	Citric, floral, sweet
trans-β-Ocimene	1229	(0.93±0.05) <sup>a</sup>	0.5	(0.33±0.11) <sup>b</sup>	0.01	(0.66±0.05) <sup>c</sup>	0.06	Sweet, herbal
p-Cymene	1253	n.d.	0.0	(0.20±0.07) <sup>a</sup>	0.00	(0.68±0.06) <sup>b</sup>	0.06	Fresh, citrus, woody
α-Terpinolene	1262	(0.37±0.04) <sup>a</sup>	0.2	n.d.	0.00	(0.24±0.02) <sup>b</sup>	0.02	Fresh, woody, citrus
Linalool oxide	1448	(0.27±0.05) <sup>a</sup>	0.14	n.d.	0.00	(0.130±0.003) <sup>b</sup>	0.01	Floral, herbal, green
Linalool	1550	(14.8±0.3) <sup>a</sup>	7.9	(0.99±0.11) <sup>b</sup>	0.03	(10.9±0.2) <sup>c</sup>	1.03	Citrus, floral, waxy
β-Caryophyllene	1597	0.33±0.05	0.2	n.d.	0.00	n.d.	0.00	Sweet, woody, clove
4-Terpineol	1611	(0.65±0.03) <sup>a</sup>	0.4	n.d.	0.00	(0.17±0.01) <sup>b</sup>	0.02	Woody, mentholic, citrus
Citronellyl acetate	1664	n.d.	0.0	(0.65±0.01) <sup>a</sup>	0.02	(0.43±0.02) <sup>b</sup>	0.04	Floral, green, rose, citrus
α-Terpineol	1710	(1.46±0.08) <sup>a</sup>	0.8	(0.44±0.05) <sup>b</sup>	0.01	(0.96±0.05) <sup>c</sup>	0.09	Pine, citrus, floral
α-Selinene	1730	0.12±0.04	0.07	n.d.	0.00	n.d.	0.00	Amber
Geranyl acetate	1762	n.d.	0.0	n.d.	0.00	0.44±0.05	0.04	Floral, rose, green
trans-β-Damascenone	1835	(1.83±0.09) <sup>a</sup>	1.0	(0.56±0.03) <sup>b</sup>	0.01	(1.38±0.08) <sup>c</sup>	0.13	Apple, rose, honey

Statistical analysis at 95 % confidence level with same letters in superscript indicates no significant difference; RI=experimentally determined retention index on the DB-FFAP column, relative to C5–C40 hydrocarbons; n.d.=not detected

In the wort, there were only a few ethyl esters detected. However, Safale US-05 strain produced a wider variety of ethyl esters in higher concentrations relative to NCYC 500 strain after fermentation. Major ethyl esters were ethyl octanoate, decanoate, 9-decenoate and laurate. Fatty acid ethyl esters are secondary metabolites produced by *S. cerevisiae* (4). Esterification of the medium-chain fatty acids that are thought to be toxic to yeast can be considered as a

means to allow the diffusion of these toxic compounds out of the cell (4). In terms of flavour, ethyl octanoate with a very low detection threshold is expected to contribute significantly to the desired fruity aspect of beer (3).

Acetate esters were the next major type of esters formed by strains Safale US-05 and NCYC 500. The major acetate esters were ethyl, isoamyl and 2-phenylethyl acetate. The concentrations of ethyl and isoamyl acetate de-

tected in the beer fermented with NCYC 500 strain were significantly higher than those detected in the beer fermented with Safale US-05. *Williopsis* yeasts are able to produce high concentrations of acetate esters, in particular isoamyl, ethyl and 2-phenylethyl acetate (11,12). The concentration of isoamyl acetate in the beer fermented with Safale US-05 was approx. 20 times lower than in that fermented with NCYC 500. 2-Phenylethyl acetate production by Safale US-05 was 5 times lower than that by NCYC 500 strain. Thus, usage of *Williopsis* to ferment wort would result in a beer with a more distinct fruity, banana-like and floral aroma imparted by higher concentrations of acetate esters (11). However, the higher production of ethyl acetate by NCYC 500 strain could lead to a glue-like off-flavour in beer (27).

Methyl esters were produced at low concentrations in both beer samples fermented with Safale US-05 and NCYC 500. These methyl esters were likely products of the esterification of methanol and organic acids. The presence of methanol in fruit and grain-based beverages has been attributed to the demethylation of methylated pectins, possibly due to acid hydrolysis (28) or enzyme action.

The major alcohol produced by both yeasts was ethanol, with Safale US-05 strain producing about 5 times more ethanol than NCYC 500 strain (Table 1). Based on °Brix approximation, the ethanol volume fraction in the beer fermented with Safale US-05 was 6.9 %, while that of the beer fermented with NCYC 500 strain was 1.7 %. This result was expected, as NCYC 500 strain has been reported to carry out fermentation with low ethanol production (11,29). *Williopsis* yeasts seem to predominantly utilize sugars *via* oxidative respiration, producing carbon dioxide and water, but only little ethanol (29). Other major alcohols detected in both beer samples included isobutanol, active amyl, isoamyl and 2-phenethyl alcohol, which are mainly produced *via* branched-chain and aromatic amino acid metabolism (30).

Short- to medium-chain fatty acids such as hexanoic, octanoic and decanoic acids were detected in the highest concentrations in both beer samples (Table 1). The fatty acids may be products of yeast metabolism such as  $\beta$ -oxidation of higher fatty acids (31). These fatty acids are important for beer flavour because they themselves are odour-active and can lead to rancid off-flavour at high concentrations. Moreover, they are also one of the starting materials for the synthesis of volatile esters (32). In general, fatty acid production by Safale US-05 was higher than by NCYC 500 strain.

Terpenes and terpenoids in wort are mainly derived from added hops. Terpene and terpenoid concentrations generally decreased from day 0 to day 14 in the beer fermented with either Safale US-05 or NCYC 500, with some terpenes becoming undetectable (Table 1). The exception was *p*-cymene, which was not detected in the wort, but was detected in both beer samples fermented with US-05 or NCYC 500 strain.

The most significant terpenoid detected in the wort and the fermented beer samples was linalool (Table 1). Linalool is one of the key odourants that impart a floral, fruity and citrus note to the Cascade and Perle hops

(33,34) used in this study. More importantly, linalool is also one of the hop-derived odourants in hopped beer, indicating that the presence of this terpenoid in beer comes at least partly from wort (35,36). However, the concentration of linalool found in the beer can be affected by yeast metabolism (37). *S. cerevisiae* is able to convert the odourless linalyl glycoside to linalool *via* the action of  $\beta$ -glucosidases (38). Yeast has been shown to be able to convert geraniol to linalool (37,39), although geraniol was not detected in the wort.

Linalool content could also decrease due to yeast metabolism, as indicated by our results (Table 1). It has been shown that linalool can be converted into other terpenoids such as  $\alpha$ -terpineol (37,39), which was detected in the final beer samples albeit at levels lower than those in the wort. Linalool could also have been transformed into linalool oxide detected in the beer fermented with NCYC 500 strain. Hence, it must be pointed out that the final concentration of linalool is a net balance of production and utilisation.

Two terpenoid esters were detected in the fermented beer, but not in the wort. Specifically, citronellyl acetate was detected in both beer samples fermented with Safale US-05 and NCYC 500, but geranyl acetate was detected only in the beer fermented with NCYC 500 strain (Table 1). Citronellol was not detected in the wort, but it is possible that citronellyl acetate could have been formed *via* reduction of geranyl acetate (37). In addition, although geraniol was also not detected in all the samples, geranyl acetate was still formed. It has been shown that the geraniol to linalool biotransformation reaction is reversible; thus, some linalool present in the wort could have been converted into geraniol *via* yeast metabolism (37). Strain NCYC 500, with its higher acetate ester-synthesising activity than that of Safale US-05, could then form geranyl acetate, whereas the latter could not.

Acetaldehyde was the major aldehyde detected in the beer fermented with Safale US-05 strain, but it was not detected in the beer fermented with NCYC 500 strain (Table 1). In *S. cerevisiae*, anaerobic catabolism of sugars to produce ethanol involves production of acetaldehyde as an intermediate. Although most of the acetaldehyde is metabolised, some molecules would still diffuse out of the cell (40). Strain NCYC 500 has a low anaerobic fermentative ability; hence, the alcoholic fermentative pathway would not be carried out as extensively as by Safale US-05, resulting in only trace amounts of acetaldehyde formed.

Benzaldehyde was also another major aldehyde detected in the beer fermented by either Safale US-05 or NCYC 500 (Table 1). Benzaldehyde may originate from the oxidative degradation of phenylpyruvic acid during phenylalanine metabolism of yeast (41). 3-Methylbutanal, hexanal, 2-phenylacetaldehyde and *m*-tolualdehyde (3-methylbenzaldehyde) were detected in the wort but not in the beer, probably due to yeast-mediated reduction of aldehydes to alcohols. Nonanal, (E)-2-decenal and *o*-tolualdehyde (2-methylbenzaldehyde) identified in both beer samples could be the result of autoxidation of unsaturated fatty acids.



## Conclusions

The fermentation of sweet wort by a brewer's yeast and a *Williopsis saturnus* yeast was compared. *W. saturnus* var. *mrakii* NCYC 500 was able to produce higher concentrations of acetate esters and retain better the original terpenoid content. Fermentation with this yeast could be a novel biotechnological way of increasing the desirable fruity and floral flavour of beer while retaining the characteristic hop aroma of the wort. Given that its fermentative ability of sugars is low, this could be a way of brewing extra-fruity low-alcohol beer.

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