

Article

# Screening and Application of *Cyberlindnera* Yeasts to Produce a Fruity, Non-Alcoholic Beer

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**Abstract:** Non-alcoholic beer (NAB) is enjoying growing demand and popularity due to consumer lifestyle trends and improved production methods. In recent years in particular, research into the application of non-*Saccharomyces* yeasts to produce NAB via limited fermentation has gained momentum. Non-*Saccharomyces* yeasts are known to produce fruity aromas, owing to a high ester production. This trait could be harnessed to mask the often-criticized wort-like off-flavor of NAB produced via limited fermentation. Six *Cyberlindnera* strains were characterized and screened in wort extract. Four of the six strains produced a pleasant, fruity aroma while exhibiting low ethanol production. The strain *Cyberlindnera subsufficiens* C6.1 was chosen for fermentation optimization via response surface methodology (RSM) and a pilot-scale (60 L) brewing trial with subsequent sensory evaluation. A low fermentation temperature and low pitching rate enhanced the fruitiness and overall acceptance of the NAB. The NAB (0.36% ABV) produced on pilot-scale was significantly more fruity and exhibited a significantly reduced wort-like off-flavor compared to two commercial NABs. This study demonstrated the suitability of *Cyberlindnera subsufficiens* to produce a fruity NAB, which can compete with commercial NABs. The outcome strengthens the position of non-*Saccharomyces* yeasts as a serious and applicable alternative to established methods in NAB brewing.

**Keywords:** brewing; *Cyberlindnera*; NABLAB; non-alcoholic beer; non-conventional yeast; non-*Saccharomyces* yeast; response surface methodology

## 1. Introduction

While the overall market growth of beer is slowing down, non-alcoholic and low alcohol beer (NABLAB) is growing in volume and popularity, owed to stricter legislation, lifestyle trends and improved production methods [1]. The increasing interest has fueled research in NABLAB production methods, especially in recent years, aimed at overcoming taste deficits compared to regular beer and consequently improving consumer acceptance. The two major production methods, physical dealcoholization and limited fermentation, both compromise the taste of the beer. Dealcoholized beer is often criticized for its lack of body and aromatic profile, a consequence of the removal of volatile esters and higher alcohols in conjunction with ethanol. Apart from a sweet taste due to residual sugars, one of the main points of criticism of NAB produced by limited fermentation is its wort-like off-flavor caused by aldehydes present in the wort [2]. In regular beer, ethanol significantly increases aldehyde retention, reducing the perceptibility of the wort-like flavor. However, in NAB produced by limited fermentation, the low ethanol content and higher levels of mono- and disaccharides intensify this undesired off-flavor [3].

It is known that esters, which yeast produce as a by-product of alcoholic fermentation, are extremely important for the flavor profile of beer [4,5]. The lack thereof, as well as their overproduction, can significantly compromise the flavor. Aside from strain-specific differences, the process parameters such as the fermentation temperature, pitching rate and wort gravity have been shown to have a significant influence on ester formation [4,6]. In non-alcoholic beers, ester concentrations are lower compared to regular beer, independent of the production method [7,8]. While physical dealcoholization removes esters that were previously produced, limited fermentation adversely affects the production of substantial amounts in the first place.

Non-*Saccharomyces* yeasts are known for their important contribution to the flavor profile of fermented foods and beverages and have therefore been investigated for their targeted application in bioflavoring and, not least, NABLAB brewing [1,9,10]. Species that have been mentioned in the context of NABLAB production belong to the genera *Cyberlindnera*, *Hanseniasspora*, *Lachancea*, *Mrakia*, *Pichia*, *Torulasporea*, *Saccharomyces*, *Scheffersomyces* and *Zygosaccharomyces* [1,11–16]. In particular, the *Cyberlindnera* species are known for their high ester production, which was shown in studies with *Cyberlindnera saturnus* (formerly *Williopsis saturnus*), *C. mrakii* (formerly *Williopsis saturnus* var. *mrakii*) and *C. subsufficiens* (formerly *Williopsis saturnus* var. *subsufficiens*) [17–20]. Furthermore, it has been proposed to use yeasts with high production of flavor compounds (i.e., esters, higher alcohols) to mask the wort-like flavor of NAB produced by limited fermentation. However, research in that direction is sparse [21,22]. In addition, such yeasts are capable of reducing aldehydes to their correspondent alcohol, which can also enhance the reduction of the often-criticized wort-like off-flavor [23,24].

In this study, six strains of the genus *Cyberlindnera* were investigated to create a fruity NAB. After identification, the strains were characterized for their substrate utilization, flocculation behavior and stress responses. A screening in diluted wort extract was performed to investigate the strains' potential to produce a pronounced fruity flavor without the production of high concentrations of ethanol. Interspecific differences in sugar consumption and the production of volatile fermentation by-products was investigated by means of high-performance liquid chromatography (HPLC) and gas chromatography (GC). The most promising strain was studied further to determine the optimal fermentation conditions to enhance the fruity flavor, which was performed by means of response surface methodology (RSM). Finally, a non-alcoholic beer was produced on pilot-scale (60 L), and its analytical attributes, aroma, and taste compared to two commercial NABs were examined.

## 2. Materials and Methods

### 2.1. Materials

All reagents used in this study were at least analytical grade from Sigma-Aldrich (St Louis MO, USA) unless stated otherwise. The wort extract applied in this study was spray-dried wort from 100% barley malt (Spraymalt Light, Muntons plc, Suffolk, UK). For the pilot-scale brewing, pilsner malt and acidulated malt were sourced from Weyermann (Malzfabrik Weyermann, Bamberg, Germany).

### 2.2. Yeast Strains

#### Strain Origin and Identification

Strain 837A was isolated from a brewery cellar, NT Cyb originates from a dried fermentation starter for rice wine, strain C6.1 originates from a coconut, and L1 from "Lulo", the fruit of *Solanum quitoense*. The type strains CBS 1707 and CBS 5763 originate from soil samples. For identification, the D1/D2 domain of the 26S rRNA gene was amplified, sequenced and compared to publicly available sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The DNA of the yeast isolates was extracted using an extraction kit (Yeast DNA Extraction Kit, Thermo Fisher Scientific, Waltham MA, USA). To amplify the D1/D2 domain

of the 26S rRNA gene, the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') were used. Polymerase chain reaction (PCR) was performed using the temperature protocol: 95 °C/2 min; 30 cycles of 95 °C/30 s, 56 °C/15 s; 72 °C/60 s; 72 °C/5 min. Stock cultures were kept in 50% (v/v) glycerol at −80 °C.

### 2.3. Yeast Characterization

#### 2.3.1. Flocculation Assay and Phenolic Off-Flavor (POF) Test

The flocculation test was performed using a slightly modified Helm's assay [25,26]. Essentially, all cells were washed in ethylenediaminetetraacetic acid (EDTA) and the sedimentation period was extended to 10 min. Wort was composed of 75 g/L spray-dried malt extract (Spraymalt Light, Muntons plc, Suffolk, UK) adjusted to 15 International Bitterness Units (IBU) (15 mg/mL iso- $\alpha$ -acids; from 30% stock solution; Barth-Haas Group, Nürnberg, Germany).

The phenolic off-flavor test was performed according to Meier-Dörnberg et al. [27]. In short, yeast strains were spread on yeast and mold agar plates (YM-agar) containing only one of the following precursors: either ferulic acid, cinnamic acid or coumaric acid. After three days of incubation at 25 °C, plates were evaluated by a trained panel by sniffing to detect any of the following aromas: clove-like (4-vinylguajacol), Styrofoam-like (4-vinylstyrene) and medicinal-like (4-vinylphenol). *Saccharomyces cerevisiae* LeoBavaricus—TUM 68® (Research Center Weihenstephan for Brewing and Food Quality, Freising-Weihenstephan, Germany) was used as a positive control.

#### 2.3.2. Substrate Utilization

To analyze substrate utilization by the *Cyberlindnera* strains, the test kit API ID 32C (BioMérieux, Marcy-l'Étoile, France) was used. Preparation of the inoculum and inoculation of the strips were performed according to the manufacturers' instructions. Colonies for the inoculum were grown on yeast extract peptone dextrose (YPD) agar plates for 48 h at 27 °C. After inoculation, API ID 32C strips were incubated for 2 days at 28 °C. The samples were evaluated visually for turbidity in the wells, differentiating positive (+), negative (−), and weak (w) growth.

#### 2.3.3. Stress Tests

Stress tests were performed via the measurement of yeast growth in a microplate, through the repeated measurement of absorbance over a time period of 96 h (Multiskan FC, Thermo Scientific, Waltham, MA, USA). The substrate for the hop sensitivity test was sterile-filtered wort extract (75 g/L Muntons Spraymalt Light) adjusted to 0, 50 and 100 mg/L iso- $\alpha$ -acids (1 mg/L = 1 International Bitterness Unit, IBU), respectively, by using an aliquot of a stock solution of 3% iso- $\alpha$ -acids in 96% (v/v) ethanol (Barth-Haas Group, Nürnberg, Germany). For testing ethanol sensitivity, the sterile-filtered wort extract was adjusted to 0%, 2.5%, 5% and 7.5% ABV with an aliquot of 100% (v/v) ethanol. For testing pH sensitivity, the sterile-filtered wort extract was adjusted to the following pHs with 2 M HCl: 5.5 (control without addition of HCl): 5.0, 4.0 and 3.0. For inoculation, strains were grown in sterilized wort extract for 24 h at 25 °C under aerobic conditions. The microtiter plate wells were inoculated with a concentration of 10<sup>5</sup> cells/mL. The wells contained 200  $\mu$ L of the respective wort substrates. Plates were incubated at 25 °C, and absorbance was measured every 30 min at 600 nm without shaking over a time period of 96 h (Multiskan FC, Thermo Scientific, Waltham, MA, USA). Stress tests were performed in triplicate.

### 2.4. Yeast Screening

#### 2.4.1. Propagation

Single colonies of the respective strains were taken from yeast extract peptone dextrose (YPD) agar plates after 72 h growth at 25 °C and transferred into a 250 mL sterile Duran glass bottle (Lennox

Laboratory Supplies Ltd, Dublin, Ireland) containing 150 mL propagation wort consisting of 75 g/L spray-dried malt (Spraymalt light, Muntons plc, Suffolk, UK) and 30 g/L glucose (Gem Pack Foods Ltd., Dublin, Ireland), sterilized at 121 °C for 15 min. The bottles were covered with sterile cotton and placed in an incubator with orbital shaker (ES-80 shaker-incubator, Grant Instruments (Cambridge) Ltd, Shepreth, UK) and incubated for 24 h at an orbital agitation of 170 rpm at 25 °C (Strain 837A was incubated for 48 h). Cell count was performed using a Thoma Hemocytometer with a depth of 0.1 mm (Blaubrand, Sigma-Aldrich, St. Louis, MO, USA).

#### 2.4.2. Fermentation

Fermentation wort was prepared by dissolving 75 g/L spray-dried malt extract (Munton Spraymalt light) in 1 L of brewing water and sterilizing at 121 °C for 15 min, followed by filtration through a sterile grade 1V Whatman filter (Whatman plc, Maidstone, UK) to remove hot trub formed during sterilization. The analytical attributes of the fermentation wort for the yeast screening trial and RSM trial is shown in Table 1.

**Table 1.** Attributes of screening wort from wort extract.

Attribute	Unit	Value
Real Extract	°P	6.97 ± 0.00
pH	–	5.20 ± 0.01
Free amino nitrogen (FAN)	mg/L	115 ± 1
Maltotriose	g/L	8.12 ± 0.15
Maltose	g/L	32.37 ± 0.57
Sucrose	g/L	0.83 ± 0.04
Glucose	g/L	5.68 ± 0.91
Fructose	g/L	1.45 ± 0.10

Fermentation trials were carried out in 1 L sterile Duran glass bottles, equipped with an air lock. Per yeast strain, triplicate bottles were filled with 400 mL of wort and left untouched throughout the fermentation. Yeast cells for pitching were washed by centrifugation at 900 g for 5 min and resuspended in sterile water to ensure no carryover of sugars from the propagation wort into the fermentation wort. Pitching rate was  $3 \times 10^7$  cells/mL. Fermentation temperature was 25 °C. Fermentation was performed until no change in extract could be measured for two consecutive days.

#### 2.5. Scanning Electron Microscopy (SEM)

Yeast cultures for scanning electron microscopy (SEM) were prepared following the protocol for cultured microorganisms by Das Murtey and Ramasamy [28]. Single colonies were taken from a YPD agar plate and grown in YPD broth for 24 h at 25 °C. One milliliter of sample was centrifuged at 900 g for 2 min for pellet formation and resuspended in 5% glutaraldehyde solution prepared in 0.1 M phosphate buffer (pH 7.2) for fixation. After 30 min, the sample was centrifuged, the supernatant was discarded, and the pellet was washed twice in 0.1 M phosphate buffer. Consequently, the pellet was resuspended in 1% osmium tetroxide prepared in 0.1 M phosphate buffer. After 1 h, cells were again washed twice in 0.1 M phosphate buffer. The sample was then dehydrated through an ethanol series of 35%, 50%, 75%, 95%, absolute ethanol, and hexamethyldisilazane (HDMS), with 30 min per step (last two ethanol steps twice), centrifuging and discarding the supernatant at each change. Lastly, the second HDMS was discarded and the sample left drying overnight in a desiccator.

The dehydrated yeast sample was mounted onto plain aluminum stubs using carbon double surface adhesive and coated with a 5 nm gold-palladium (80:20) layer using a Gold Sputter Coater (BIO-RAD Polaron Division, SEM coating system, England), then observed under a constant accelerating voltage of 5 kV under a JEOL scanning electron microscope type 5510 (JEOL, Tokyo, Japan).

## 2.6. Response Surface Modeling (RSM)

To investigate optimal fermentation conditions for C6.1 to produce a fruity, non-alcoholic beer, response surface methodology (RSM) was performed using DesignExpert 9 software (StatEase, Minneapolis, MN, USA). A two-factorial, face-centered, central composite design with single factorial points and 5 replications of the center point was chosen. The predictor factors were temperature (17, 22, 27 °C), and pitching rate (10, 35, 60 × 10<sup>6</sup> cells/mL).

Spray-dried malt extract (Spraymalt light, Muntons plc, Suffolk, UK) served as the substrate. Wort preparation, propagation and inoculation were carried out as outlined in 2.4.1. The wort used was the same as in the screening (Table 1). Fermentation volume was 150 mL in 250 mL Duran glass bottles equipped with an air lock. Fermentation was performed until no change in extract could be measured for two consecutive days. Table 2 shows the experimental design.

**Table 2.** Response surface methodology (RSM) experimental design: Two-factorial, face-centered, central composite design with five repetitions of the center point. Factor 1, A: temperature, range 17, 22, 27 °C. Factor 2, B: pitching rate, range 10, 35, 60 × 10<sup>6</sup> cells/mL.

Run	Factor 1	Factor 2
	A: Temperature (°C)	B: Pitching Rate (×10 <sup>6</sup> cells/mL)
1	22	60
2	22	10
3	17	35
4	27	35
5 *	22	35
6 *	22	35
7	17	60
8 *	22	35
9 *	22	35
10 *	22	35
11	17	10
12	27	10
13	27	60

\* Center point.

Models were produced applying backward elimination regression of insignificant model terms with  $\alpha$  to exit of 0.1 (detailed report in supplementary Data Sheet S1). For significant models with insignificant lack of fit (LOF), 3D response surface plots were produced. Fermentations for model validation were performed in the same wort with propagation as outlined in 2.4.1 and fermentation as outlined above.

## 2.7. Pilot-Scale Brewing

### 2.7.1. Wort Production

Wort for the pilot brew was produced in a 60 L pilot-scale brewing plant consisting of a combined mash-boiling vessel, a lauter tun and whirlpool (FOODING Nahrungsmitteltechnik GmbH, Stuttgart, Germany). The grain bill comprised 6.65 kg Weyermann Pilsner Malt and 0.35 kg Weyermann Acidulated Malt (Malzfabrik Weyermann, Bamberg, Germany). Grains were milled with a two-roller mill ("Derby", Engl Maschinen, Schwebheim, Germany) at a 0.8 mm gap size. The crushed malt was mashed-in with 30 L of brewing water at 50 °C. The following mashing regime was employed: 20 min at 50 °C, 20 min at 62 °C, 10 min at 72 °C and mashing out at 78 °C. The mash was pumped into the lauter tun, and lautering was performed after a 15 min lauter rest, employing four sparging steps of 5 L hot brewing water each. Boil volume was 50 L at a gravity of 1.030 (7.0 °P), and total boiling time was 60 min. Thirty minutes into the boil, 15 g of Magnum hop pellets (14% iso- $\alpha$ -acids) were added for

a calculated IBU content of 9. After boiling, gravity was readjusted to 1.030 (7.0 °P) with hot brewing water, and hot trub precipitates and hop residue were removed in the whirlpool with a rest of 20 min. Clear wort was pumped through a heat exchanger and filled into 60 L cylindroconical fermentation vessels at a temperature of 17 °C.

#### 2.7.2. Propagation, Fermentation and Aftercare

A first propagation step was employed as described in 2.4.1. A second propagation step was performed by transferring the small-scale propagated wort into a 5 L carboy filled with 2 L of sterile wort extract at 7 °P and closed with sterile cotton. The second propagation step was conducted for 24 h under constant agitation at ambient temperature ( $20 \pm 2$  °C).

Yeast was pitched into the fermenter at a pitching rate of  $10^7$  cells/mL. Fermentation was carried out in cylindroconical fermentation vessels with a capacity of 60 L, at ambient pressure and at a glycol-controlled fermentation temperature of 17 °C. Samples were withdrawn every day. Fermentation was carried out until no change in extract could be measured for two consecutive days. The beer was then filled into a 50 L keg and carbonated by repeated pressurization with CO<sub>2</sub> to 1 bar at 2 °C. After 5 days, the carbonated beer was filled into 330 mL brown glass bottles with a counter-pressure hand-filler (TOPINCN, Shenzhen, China) and capped. Bottles were pasteurized in a pilot retort (APR-95; Sundry, Abadiano, Vizcaya, Spain) with spray water at 65 °C for 10 min resulting in approximately 23 pasteurization units (PU). The successful pasteurization was confirmed by plating the pasteurized NAB on agar plates. Beer bottles were stored at 2 °C in a dark place for further analysis and sensory evaluation.

#### 2.8. Sensory Evaluation

The sensory evaluation of the samples produced during yeast screening and RSM trial were judged by a panel of 12–15 experienced tasters. Samples were given at ambient temperature (20 °C) with a three-digit code. Each panelist evaluated the samples in an individual booth at ambient temperature (20 °C). The tasters were asked to describe the sample in their own words, followed by evaluation of the intensity of a fruity smell and the overall acceptance of the smell of the sample on a hedonic scale from 0 (“not fruity”/“dislike extremely”) to 5 (“extremely fruity”/“like extremely”) according to MEBAK Sensory Analysis 3.2.1 “Simple Descriptive Test” and 3.2.2 “Profile Test”, respectively.

The non-alcoholic beer samples (C6.1 pilot scale and commercial samples) were tasted and judged by a sensory panel of ten experienced and certified (DLG International Certificate for Sensory Analysis—beer and beer-based mixed drinks; Deutsche Landwirtschafts-Gesellschaft e.V.) panelists. A “Simple Descriptive Test” and “Profile Test” were performed according to MEBAK Sensory Analysis 3.2.1 and 3.2.2, respectively. Attributes for the aroma were “wort-like”, “floral”, “fruity”, “citrus-like” and “tropical”. A taste attribute “sweet taste” was also included. Panelists were asked to evaluate the attributes in their intensity on a line-marking scale from 0, “not perceptible”, to 5, “strongly perceptible”. Before the evaluation of the intensity, a descriptive sensory was performed, where the panelists were asked to describe the aroma of the samples in their own words. Samples were provided in dark glasses with a three-digit code and evaluated at a temperature of 20 °C in order to evaluate the full flavor profile (following DLG guidelines). The commercial samples NAB A and NAB B were non-alcoholic beers produced by limited fermentation [29] and “dialysis technology” [30], respectively. Each panelist tasted the samples in an individual booth at ambient temperature (20 °C). The amount of sample tasted was 50 mL per sample.

#### 2.9. Wort and Beer Analyses

##### 2.9.1. HPLC Analyses

Sugars and ethanol were determined by HPLC Agilent 1260 Infinity (Agilent Technologies, Santa Clara CA, USA) equipped with a refractive index detector (RID) and a Sugar-Pak I 10 µm,

6.5 mm × 300 mm column (Waters, Milford MA, USA), with 50 mg/L Ca-EDTA as mobile phase and a flow rate of 0.5 mL/min at 80 °C. Differentiation of maltose and sucrose was achieved with a Nova-Pak 4 µm, 4.6 mm × 250 mm column (Waters, Milford MA, USA), with acetonitrile/water 78:22 (v/v) as mobile phase and a flow rate of 1.0 mL/min. Quantification was achieved by external standards in a calibration range of 0.5 to 30 mM.

### 2.9.2. GC Analyses

Free vicinal diketones were quantified by a Clarus 500 gas chromatograph (Perkin-Elmer, Waltham MA, USA) with a headspace unit and Elite-5 60 m × 0.25 mm, 0.5 µm column using a 2,3-hexandione internal standard. Fermentation by products (esters, higher alcohols) was quantified using a Clarus 580 (Perkin-Elmer, Waltham MA, USA) gas chromatograph with a headspace unit and INNOWAX cross-linked polyethylene-glycol 60 m × 0.32 mm, 0.5 µm column (Perkin-Elmer, Waltham MA, USA). Vials containing beer samples were equilibrated for 25 min at 60 °C. The samples were injected at 50 °C, rising to 85 °C after one minute by heating at 7 °C/min. A temperature of 85 °C was maintained for one minute and then elevated to 190 °C at a heating rate of 25 °C/min.

### 2.9.3. Other

Glycerol was determined via enzymatic assay kit (glucokinase method), following the recommended procedure (K-GCROLGK, Megazyme, Bray Co. Wicklow, Ireland). The method is based on the use of ADP-glucokinase and an increase in absorbance on conversion of NAD<sup>+</sup> to NADH, and is performed at ambient temperature at a sample volume of 2 mL.

Free amino nitrogen (FAN) was measured using a ninhydrin-based dyeing method, where absorbance is measured at 570 nm against a glycine standard (ASBC Method Wort-12 A). The method is performed at a total volume of 10 mL. Following the color reaction at 95 °C, the samples are measured at ambient temperature.

Extract (apparent and real) and ethanol (for fermentation monitoring) were analyzed via density meter DMA 4500M with AlcoLyzer Beer ME (Anton-Paar GmbH, Graz, Austria) at 20 °C and a sample volume of 30 mL.

The pH was determined using a digital pH meter (Mettler Toledo LLC, Columbus, OH, USA).

### 2.10. Statistical Analyses

Screening fermentations and analyses were carried out in triplicate. Statistical analysis was performed using RStudio, Version 1.1.463 with R version 3.5.2 (RStudio Inc, Boston, MA, USA; R Core Team, r-project). One-way analysis of variance (ANOVA) was used to compare means, and Tukey's post hoc test with 95% confidence intervals was applied for the pairwise comparison of means. When available, values are given as the mean ± standard deviation. Statistical analyses during the RSM trials were performed using the DesignExpert 9 software (StatEase, Minneapolis, MN, USA).

## 3. Results and Discussion

### 3.1. Yeast Strain Characterization

To identify the species of the yeast strains, amplification of the D1/D2 domain via PCR was performed and sequenced. The obtained sequences were compared to publicly available sequences in the NCBI nucleotide database via BLAST. The results of the strain identification are shown in Table 3.

**Table 3.** Yeast strain designation, species and origin of yeast strains used in this study.

Strain Designation	Species	Origin	Yeast Bank
837A	<i>Cyberlindnera misumaiensis</i>	Brewery cellar	FZW BLQ <sup>1</sup> , Weihenstephan, Germany
NT Cyb	<i>Cyberlindnera fabianii</i>	Dried yeast starter for rice wine	FZW BLQ <sup>1</sup> , Weihenstephan, Germany
L1	<i>Cyberlindnera jadinii</i>	Fruit of <i>Solanum quitoense</i> , “Lulo”	UCC Culture Collection, Cork, Ireland
C6.1	<i>Cyberlindnera subsufficiens</i>	Coconut	UCC Culture Collection, Cork, Ireland
CBS 1707 <sup>T</sup>	<i>Cyberlindnera mrakii</i>	Soil	Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands
CBS 5763 <sup>T</sup>	<i>Cyberlindnera subsufficiens</i>	Soil	Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands

<sup>1</sup> Research Centre Weihenstephan for Brewing and Food Quality, Technische Universität München; <sup>T</sup> Type strain.

The yeast strains were found to belong to the species *Cyberlindnera misumaiensis* (837A), *C. fabianii* (NT Cyb), *C. jadinii* (L1), and *C. subsufficiens* (C6.1). The *Cyberlindnera mrakii* type strain CBS 1707 (former *Williopsis saturnus* var. *mrakii*; synonym NCYC 500) was included in this study as a strain that has previously been investigated for the production of a low alcohol beer with high levels of esters [20]. The *Cyberlindnera subsufficiens* type strain CBS 5763 was included as an example to investigate potential intraspecific differences from C6.1.

### 3.2. API Substrate Utilization

Before considering non-conventional yeasts for NABLAB brewing, their behavior regarding utilization of important wort sugars like maltose and sucrose should be investigated. An API ID 32C test was performed to investigate the utilization of those sugars and to show general, interspecific differences between the strains. The results of the API test are shown in Table 4.

**Table 4.** Results of the API ID 32C substrate utilization test of the individual strains. Substrates without brewing relevance, which were negative for all strains, are not shown. “+” positive, “-” negative, “w” weak.

Substrate	837A	NT Cyb	L1	C6.1	CBS 1707	CBS 5763
Cycloheximide (Actidione)	+	-	-	-	-	-
D-Cellobiose	+	+	+	+	+	+
D-Galactose	-	-	w	-	-	-
D-Glucose	+	+	+	+	+	+
D-Maltose	-	+	+	-	+ <sup>1</sup>	- <sup>1</sup>
D-Mannitol	+	+	w	w	w	w
D-Melibiose	-	-	-	-	-	-
D-Melezitose	-	+	+	-	+	-
D-Raffinose	-	+	+	+	+	+
D-Sorbitol	+	+	w	+	-	-
D-Sucrose	-	+	+	+	-	+
D-Trehalose	-	+	-	-	+	-
D-Xylose	-	+	+	+	+	+
Esculin Ferric Acid	+	+	+	+	+	+
Glucosamine	-	-	-	w	-	-
Glycerol	+	+	+	+	+	+
Lactic Acid	-	+	+	+	+	+
Levulinic Acid	-	w	w	w	w	+
L-Sorbose	-	-	-	-	-	+
Methyl-αD-Glucopyranoside	-	+	-	-	-	-
N-Acetyl-Glucosamine	-	-	w	-	w	-
Palatinose	-	+	+	-	+	-
Potassium Gluconate	w	w	-	+	w	+

<sup>1</sup> Growth “variable” according to Kurtzman et al. [31].

Maltose utilization was positive for NT Cyb, L1 and CBS 1707, in accordance with the reported literature, although assimilation of maltose by CBS 1707 is classified as “variable” [31]. Sucrose utilization was positive for four of the six strains and negative for 837A and CBS1707. The results suggest that in brewers’ wort, where maltose is the most abundant fermentable sugar, only NT Cyb, L1 and CBS 1707 have the capability to achieve high attenuations. However, the API test investigates substrate utilization under aerobic conditions. Sugar consumption during fermentation, under anaerobic conditions, can differ significantly [31], which is also known as the Kluyver effect [32]. Due to the inability of 837A and CBS 1707 to utilize sucrose, lower attenuations in fermentations in wort could be expected.

### 3.3. Stress Tests

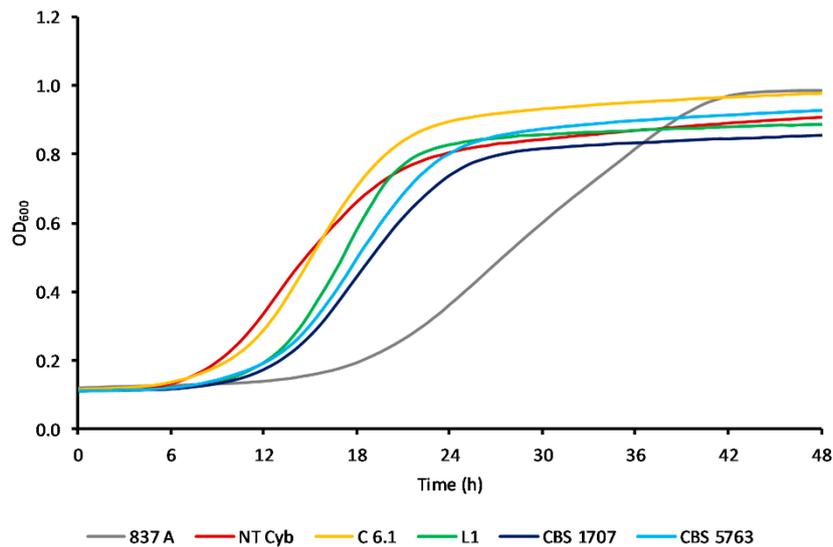
When considering non-*Saccharomyces* yeast strains for brewing purposes, several brewing-relevant parameters such as flocculation behavior, POF production and stress responses should be investigated [33]. The flocculation behavior can give initial indications regarding yeast handling in terms of potential bottom cropping. POF behavior is important because in most beer styles, POF is not desired. Substances like hop-derived iso- $\alpha$ -acids, ethanol content, or the pH value of the wort can have significant influences on yeast activity, manifesting mainly in a prolonged lag time, and even complete growth inhibition [33–35]. With the investigated yeast strains, iso- $\alpha$ -acid concentrations of up to 100 IBU had no significant effect on the yeast growth (data not shown), which is in accordance with previous reports on seven different non-*Saccharomyces* species [34,35]. However, Michel et al. [33] reported a minor prolongation in the lag time of *Torulasporea delbrueckii* strains in concentrations of up to 90 IBU. The results of the investigated characterization attributes are shown in Table 5.

**Table 5.** Characterization of yeast strains for flocculation behavior, phenolic off-flavor (POF) production and lag time in wort with and without a stressor at different concentrations. “—” no growth.

Characterization Attributes	Unit	837A	NT Cyb	L1	C6.1	CBS 1707	CBS 5763
Flocculation	%	78 ± 3	22 ± 2	35 ± 4	32 ± 1	85 ± 2	51 ± 4
POF	-	negative	negative	negative	negative	negative	negative
Ethanol	0% ABV	18	6	9	6	9	9
	2.5% ABV	120	12	18	18	12	18
	5% ABV	—	24	36	24	48	—
	7.5% ABV	—	42	—	—	126	—
pH	5.5	18	6	9	6	9	9
	5	18	6	9	6	9	9
	4	66	6	9	6	9	9
	3	—	12	24	18	78	42

CBS 1707 exhibited the strongest flocculation behavior, at 85%, followed by 837A and CBS 5763, at 78% and 51%, respectively. NT Cyb, L1 and C6.1 exhibited very low flocculation of below 35%. All strains were negative for POF behavior. NT Cyb and C6.1 exhibited the fastest growth in wort (without a stress factor), overcoming the lag time after only 6 hours, followed by L1 and the CBS strains after 9 hours. Strain 837A exhibited a long lag phase of 18 hours (Figure 1). Concentrations of 2.5% ABV ethanol in the wort affected the lag time of all investigated strains. 837A was especially susceptible, with a prolonged lag phase of 120 hours. The remainder of the strains showed an extension of the lag phase of 3 to 12 hours. At 5% ABV, growth was fully inhibited for 837A and CBS 5763, while the other strains again exhibited an extension of the lag phase, of up to a maximum of 48 hours in CBS 1707. Complete growth inhibition was observed for L1 and C6.1 at 7.5% ABV, while the lag phase of NT Cyb and CBS 1707 was prolonged to 42 and 126 hours, respectively. All strains except 837A, which showed a significant extension of the lag phase to 66 hours, remained unaffected by a lower pH of 4. Only at pH 3 were lag times affected, while 837A was fully inhibited. Growth at low pH is important when

considering the yeast for sour beer production, where the yeast must withstand pH values of below 4 [36]. However, it has been shown that organic acids like lactic acid can have a stronger inhibitory effect on yeasts and other microorganisms than HCl, which is caused by its chemical properties as a weak acid [35,37]. Inhibition by lactic acid could therefore be more pronounced than the HCl inhibition observed in this study. Figure 1 shows the growth of the investigated yeast strains in wort without the addition of a stressor.



**Figure 1.** Growth of yeast strains in 7 °P wort extract at 25 °C without a stressor. Growth curves shown are the mean of a triplicate.

### 3.4. Screening

To investigate interspecific differences in the fermentation of wort, fermentation trials were performed in a diluted wort extract of 7 °P. Previous studies have shown that extract contents of around 7 °P will yield ethanol concentrations of around 0.5% ABV, a popular legal limit for NAB [7], in fermentations with maltose-negative yeast strains [1,14,34,38]. After aerobic propagation for 24 hours, NT Cyb exhibited the highest number of cells, at  $2 \times 10^9$  cells/mL, more than four-fold the amount of cells compared to L1, C6.1, and the CBS strains with counts between  $3.4$  and  $4.9 \times 10^8$  cells/mL (Table 6). Due to a delayed growth (compare Figure 1), 837A had to be propagated for 48 hours, reaching a cell count of  $6.1 \times 10^8$  cells/mL. For the screening in wort, yeast cells were added at a concentration of  $3 \times 10^7$  cells/mL, after a gentle washing step in water to prevent carry-over of propagation wort sugars. The results from the yeast screening are shown in Table 6. The fermentations were carried out until no change in extract could be measured for two consecutive days.

**Table 6.** Results of the screening of the investigated *Cyberlindnera* strains in wort extract.

Non-Alcoholic Beer (NAB) Attributes		Unit	837A	NT Cyb	L1	C6.1	CBS 1707	CBS 5763
Propagation	Cell count (24 h) <sup>1</sup>	× 10 <sup>6</sup> cells/mL	611 ± 34 <sup>1</sup>	2055 ± 21	486 ± 27	445 ± 4	338 ± 25	386 ± 48
Fermented wort	Real Extract	°P	6.53 ± 0.03 <sup>b</sup>	6.40 ± 0.04 <sup>ab</sup>	6.45 ± 0.05 <sup>ab</sup>	6.36 ± 0.03 <sup>a</sup>	6.57 ± 0.10 <sup>b</sup>	6.35 ± 0.10 <sup>a</sup>
	Attenuation	%	18 ± 1 <sup>ab</sup>	23 ± 1 <sup>bc</sup>	21 ± 2 <sup>abc</sup>	24 ± 1 <sup>c</sup>	17 ± 3 <sup>a</sup>	24 ± 3 <sup>c</sup>
	Ethanol	% ABV	0.55 ± 0.01 <sup>a</sup>	0.63 ± 0.01 <sup>b</sup>	0.66 ± 0.00 <sup>cd</sup>	0.63 ± 0.00 <sup>bc</sup>	0.54 ± 0.01 <sup>a</sup>	0.67 ± 0.02 <sup>d</sup>
	pH	-	4.41 ± 0.02 <sup>ab</sup>	4.51 ± 0.02 <sup>c</sup>	4.44 ± 0.01 <sup>bc</sup>	4.38 ± 0.03 <sup>b</sup>	4.37 ± 0.06 <sup>ab</sup>	4.33 ± 0.01 <sup>a</sup>
	FAN	mg/L	88 ± 1 <sup>b</sup>	83 ± 2 <sup>ab</sup>	80 ± 1 <sup>a</sup>	81 ± 4 <sup>a</sup>	78 ± 1 <sup>a</sup>	84 ± 5 <sup>ab</sup>
Sugar consumption	Maltotriose	%	3 ± 2	6 ± 1	4 ± 1	5 ± 1	3 ± 1	5 ± 3
	Maltose	%	4 ± 2	4 ± 0	3 ± 1	4 ± 0	4 ± 1	4 ± 4
	Sucrose	%	2 ± 10	100	100	100	2 ± 2	100
	Glucose	%	100	100	100	100	100	100
	Fructose	%	81 ± 1	75 ± 1	100	80 ± 1	73 ± 8	83 ± 2
Fermentation by-products	Glycerol	g/L	0.25 ± 0.05 <sup>ab</sup>	0.23 ± 0.04 <sup>a</sup>	0.36 ± 0.03 <sup>b</sup>	0.30 ± 0.04 <sup>ab</sup>	0.18 ± 0.05 <sup>a</sup>	0.21 ± 0.05 <sup>a</sup>
	Acetaldehyde	mg/L	9.70 ± 2.83 <sup>b</sup>	8.05 ± 1.48 <sup>b</sup>	2.60 ± 0.14 <sup>a</sup>	3.37 ± 0.71 <sup>a</sup>	3.83 ± 0.45 <sup>a</sup>	2.57 ± 0.21 <sup>a</sup>
	Ethyl acetate	mg/L	65.70 ± 14.57 <sup>b</sup>	22.55 ± 2.90 <sup>a</sup>	9.27 ± 3.23 <sup>a</sup>	4.90 ± 0.85 <sup>a</sup>	8.10 ± 0.28 <sup>a</sup>	5.17 ± 0.29 <sup>a</sup>
	Isoamyl acetate	mg/L	0.90 ± 0.14 <sup>ab</sup>	<LOD	0.15 ± 0.07 <sup>a</sup>	1.60 ± 0.62 <sup>b</sup>	1.67 ± 0.12 <sup>b</sup>	1.03 ± 0.23 <sup>ab</sup>
	Ethyl formate	mg/L	0.53 ± 0.04 <sup>a</sup>	0.31 ± 0.06 <sup>a</sup>	0.57 ± 0.09 <sup>a</sup>	0.25 ± 0.03 <sup>a</sup>	2.70 ± 0.57 <sup>c</sup>	1.45 ± 0.07 <sup>b</sup>
	Ethyl propionate	mg/L	0.13 ± 0.04 <sup>a</sup>	0.13 ± 0.01 <sup>a</sup>	<LOD	<LOD	0.16 ± 0.01 <sup>a</sup>	0.17 ± 0.03 <sup>a</sup>
	Isoamyl alcohols	mg/L	11.20 ± 0.14 <sup>a</sup>	16.40 ± 0.57 <sup>b</sup>	23.15 ± 0.92 <sup>c</sup>	11.67 ± 1.74 <sup>a</sup>	11.93 ± 0.93 <sup>a</sup>	10.50 ± 0.14 <sup>a</sup>
	n-Propanol	mg/L	4.03 ± 0.84 <sup>ab</sup>	3.73 ± 0.21 <sup>ab</sup>	4.40 ± 0.62 <sup>b</sup>	3.27 ± 0.15 <sup>ab</sup>	2.93 ± 0.29 <sup>a</sup>	3.33 ± 0.15 <sup>ab</sup>
	Isobutanol	mg/L	7.57 ± 1.24 <sup>ab</sup>	7.70 ± 0.36 <sup>b</sup>	8.27 ± 1.38 <sup>b</sup>	8.03 ± 0.40 <sup>b</sup>	5.33 ± 0.55 <sup>a</sup>	7.20 ± 0.20 <sup>ab</sup>
	Σ Esters	mg/L	67.26 ± 14.79 <sup>b</sup>	22.99 ± 2.87 <sup>a</sup>	9.99 ± 3.31 <sup>a</sup>	6.75 ± 0.61 <sup>a</sup>	12.62 ± 1.48 <sup>a</sup>	7.82 ± 0.30 <sup>a</sup>
Σ Alcohols	mg/L	22.80 ± 0.14 <sup>a</sup>	27.83 ± 0.64 <sup>b</sup>	35.82 ± 1.48 <sup>c</sup>	22.97 ± 1.97 <sup>a</sup>	20.20 ± 0.17 <sup>a</sup>	21.03 ± 0.21 <sup>a</sup>	
Sensory	Aroma	-	Solvent-like, unpleasant	Cabbage-like, unpleasant	Fruity, pleasant	Fruity, pleasant	Fruity, pleasant	Fruity, pleasant

<sup>1</sup> Cell count after 48 h due to delayed growth compared to other strains (compare Figure 1). LOD 'limit of detection'. Different superscripts of values within a row indicate a significant difference ( $p \leq 0.05$ ).

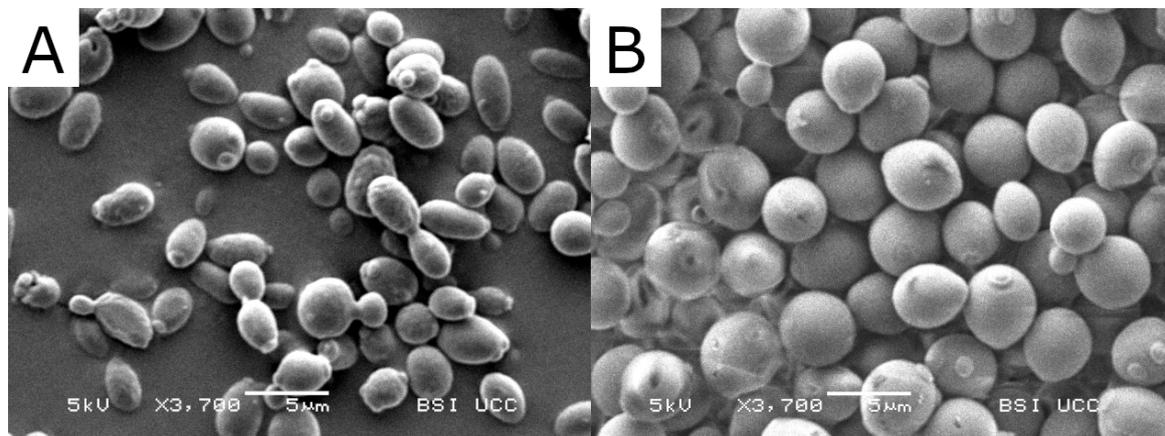
Strains 837A and CBS 1707 exhibited the lowest attenuation of only 18% and 17%, respectively, owing to their inability to utilize sucrose (Table 4), which was confirmed by the lack of sucrose consumption. Liu and Quek [20] also reported the absence of sucrose utilization by CBS 1707. The other strains, which depleted sucrose completely, reached attenuations of 21% to 24%. Consequently, 837A and CBS 1707 also produced, at 0.55% and 0.56% ABV, the lowest amounts of ethanol ( $p \leq 0.05$ ) compared to the remaining strains, where ethanol concentrations ranged from 0.63% to 0.67% ABV. The final pH of the fermented samples ranged from 4.33 (CBS 5763) to 4.51 (NT Cyb). Residual FAN ranged from 78 (CBS 1707) to 88 mg/L (837A). As expected, none of the strains consumed maltotriose. Maltose consumption was also neglectable in all strains, although the species *Cyberlindnera fabianii* (like NT Cyb) has been reported to be able to ferment maltose [31,39]. The observations also underlined that results from the API substrate utilization test (where NT Cyb, L1 and CBS 1707 were positive for maltose) are not necessarily reflected in practice, especially since sugar utilization during respiration and fermentation can differ [31,32,40]. While glucose was depleted by all strains, fructose was only fully depleted by L1. The remaining strains exhibited glucophilic behavior and consumed only 73% to 83% of fructose during fermentation. Regarding fermentation by-products, glycerol concentrations were low, ranging from 0.18 to 0.36 g/L. The strains 837A and NT Cyb accumulated significantly higher amounts of acetaldehyde, at 9.7 and 8.1 mg/L, respectively, compared to 2.6 to 3.8 mg/L in the remaining samples. The sample fermented with *Cyberlindnera misumaiensis* 837A exhibited extremely high values of ethyl acetate, at 65.7 mg/L, twice the flavor threshold concentration in beer [2,41]. Ethyl acetate is described to have a fruity, estery character but also solvent-like, especially in high concentrations. The remaining strains exhibited ethyl acetate production between 4.9 (C6.1) and 22.6 mg/L (NT Cyb). Isoamyl acetate, which is predominantly described as having a fruity, banana-like aroma, has a much lower flavor threshold of only 1.4–1.6 mg/L [2,41]. The strains C6.1 and CBS 1707 produced the highest amounts of isoamyl acetate, at 1.67 and 1.60 mg/L, followed by CBS 5763, 837A and L1, at 1.03, 0.90 and 0.15 mg/L, respectively. NT Cyb did not produce detectable amounts of isoamyl acetate. Concentrations of ethyl formate and ethyl propionate in the fermented samples were low, ranging from undetectable to 2.7 mg/L. Ethyl butyrate and ethyl caproate were not detected in either of the samples (data not shown). The strain L1 produced a significantly higher amount of higher alcohols, at 35.8 mg/L, followed by NT Cyb, at 27.8 mg/L, and the remaining strains at 20–23 mg/L. During sensory evaluation, the high ethyl acetate concentration in the sample fermented with 837A was indeed perceptible and described as an unpleasant, solvent-like aroma. The sample fermented with NT Cyb was described as having an unpleasant, cabbage-like aroma. The remaining samples were characterized by a pleasant, fruity aroma.

The unpleasant, solvent-like aroma in the sample fermented with 837A was attributed to the very high ethyl acetate concentration, well above the flavor threshold. However, the cabbage-like aroma, which is generally associated with sulfides or thiol compounds [41], that was detected in the sample fermented with NT Cyb could not be linked to the volatile by-products that were measured. Interestingly, ethyl acetate concentrations in the remaining samples, characterized by a pleasant, fruity aroma, were low, at only 2.6–3.8 mg/L. However, C6.1, CBS 1707 and CBS 5763 exhibited higher amounts of isoamyl acetate, a desired ester in beer (particularly ales) [42], when compared to the samples with unpleasant aroma. The concentrations of 1.0–1.6 mg/L are within the reported flavor threshold in beer of 0.5–2.0 mg/L [43]. Additionally, it is well known that synergistic effects between esters occur that can push the concentration of perception below their individual flavor thresholds [42,44,45]. Isoamyl acetate could therefore have been a cause of the fruity aroma in the samples fermented with C6.1, CBS 1707 and CBS 5763. However, the sample fermented with L1, which was also characterized by a fruity aroma, only contained a very low isoamyl acetate concentration of 0.15 mg/L. It is noteworthy, however, that the L1 sample contained a significantly higher amount of isoamyl alcohol, at 23.2 mg/L, which is described as having an alcoholic, fruity and banana-like flavor [2]. The results have confirmed that not a high amount of esters, but rather a balanced profile will lead to a pleasant, fruity aroma [5].

Based on the results from the screening, *Cyberlindnera subsufficiens* C6.1 was chosen for optimization of fermentation conditions by means of response surface methodology, followed by an up-scaled brewing trial at 60 L to create a fruity, non-alcoholic beer ( $\leq 0.5\%$  ABV). Strains 837A and NT Cyb were eliminated because of their poor flavor characteristics. CBS 1707 was eliminated due to its inability to ferment sucrose, which apart from the lower attenuation, would remain in the wort after fermentation, acting as an additional sweetening agent and potential contamination risk. *Cyberlindnera jadinii* strain L1 was eliminated due to its very low isoamyl acetate production (Table 6) and due to its maltose utilization when oxygen was present (Table 4). The decision between the two similarly performing *Cyberlindnera subsufficiens* strains C6.1 and CBS 5763 was made in favor of C6.1 due to a more pleasant fruitiness. In addition, C6.1 showed increased tolerance towards stress caused by ethanol or low pH (Table 5).

### 3.5. Response Surface Methodology (RSM)

To find the optimal fermentation conditions for C6.1 for an up-scaled application to produce a fruity, non-alcoholic beer, RSM was performed. Michel et al. [46] applied RSM to optimize the fermentation conditions of a *Torulasporea delbrueckii* strain for brewing purposes. They found that the pitching rate and fermentation temperature were crucial parameters, which influenced the flavor character of the final beer. The optimal fermentation conditions were shown to be at 21 °C with a high pitching rate of  $60 \times 10^6$  cells/mL. Especially for non-*Saccharomyces* yeasts, the pitching rate can be crucial since most non-*Saccharomyces* species have comparably smaller cell sizes [46]. Figure 2 shows an example of the differing cell size between *Cyberlindnera subsufficiens* strain C6.1 (A) and the brewers' yeast strain *Saccharomyces cerevisiae* WLP001 (B) at identical magnification.



**Figure 2.** Scanning electron microscopy (SEM) picture of *Cyberlindnera subsufficiens* strain C6.1 (A) and the brewers' yeast strain *Saccharomyces cerevisiae* WLP001 (B) at a magnification of  $\times 3700$ . Size of bar: 5  $\mu\text{m}$ .

It is also known that temperature and pitching rate have an influence on ester production, though strain-specific differences also play a role [4,6]. Previously reported fermentation temperatures of *Cyberlindnera subsufficiens* and other *Cyberlindnera* spp. range from 20 to 25 °C [12,17,19,20,47]. Consequently, a two-factorial, face-centered central composite design was chosen with Factor A: fermentation temperature (17, 22, 27 °C), and Factor B: pitching rate (10, 35,  $60 \times 10^6$  cells/mL). The individual experiment runs are listed in Table 2. The wort extract applied in the RSM trial was the same as that used for the screening, at an extract content of 7 °P (Table 1). Fermentation was conducted until no change in extract could be measured for two consecutive days. With the measured response values, significant models could be produced. The significant response models, with their respective minima and maxima and a summary of the model statistics, are shown in Table 7. Insignificant response models are not shown, and response models with a significant lack of fit will not be discussed in this

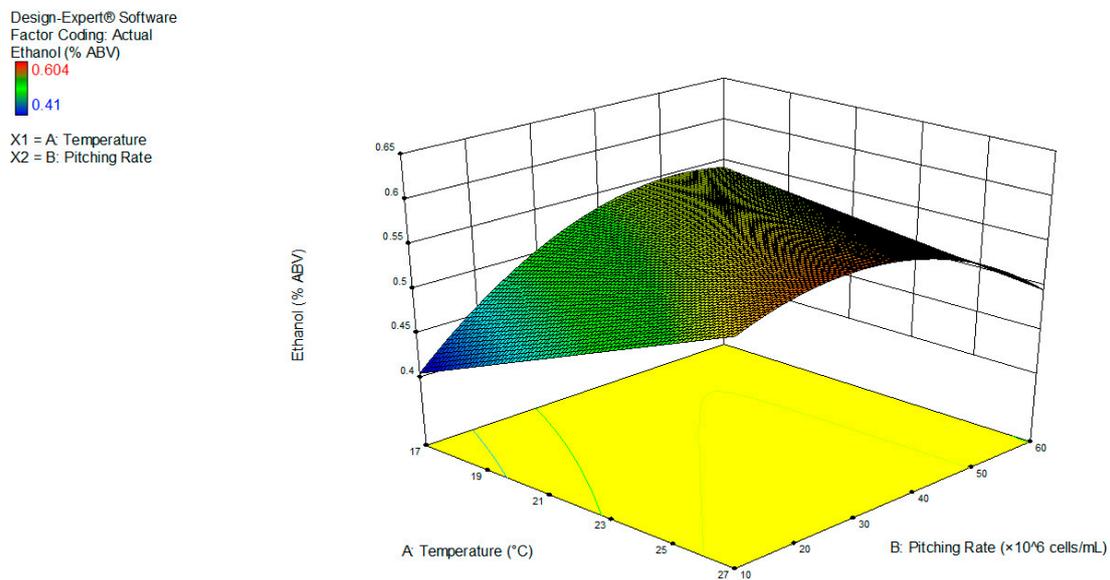
study but are included in the visualized data for the sake of a complete picture. For a full report on model statistics and response values, refer to the supplementary Data Sheet S1.

**Table 7.** Analysis of variance (ANOVA) results for response models of the response surface methodology (RSM) trial.

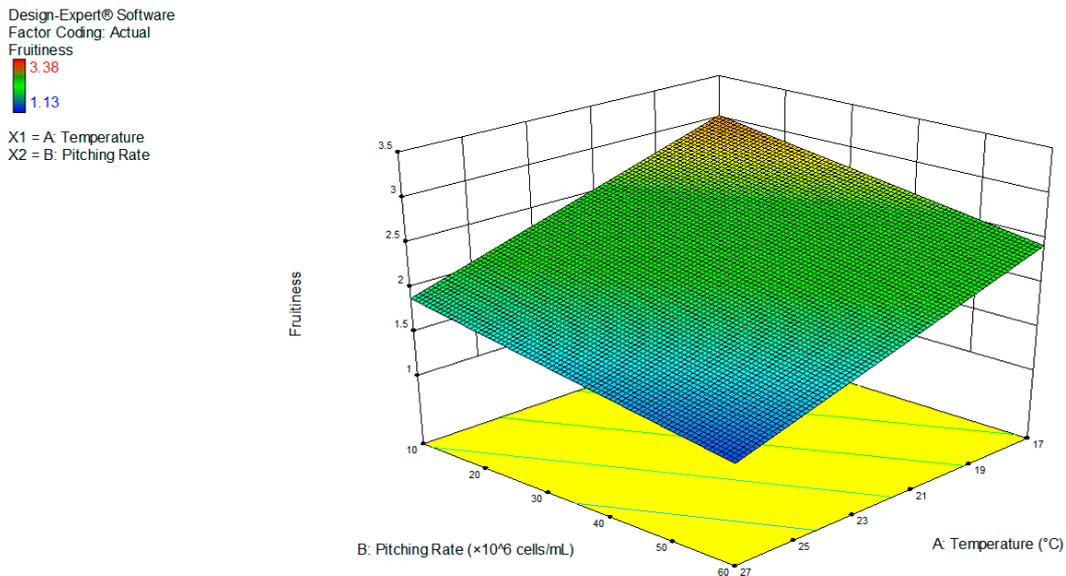
Response	Unit	Minimum	Maximum	Model	P-Value	LOF P-Value
Ethanol	% ABV	0.41	0.60	RQuadratic	$2.80 \times 10^{-3}$ **	0.648
Ethyl acetate	mg/L	3.4	9.3	2FI	$3.12 \times 10^{-2}$ *	0.007 **
Isoamyl acetate	mg/L	0.8	2.2	RQuadratic	$1.42 \times 10^{-2}$ *	0.046 *
Acetaldehyde	mg/L	1.9	3.4	RLinear	$1.35 \times 10^{-3}$ **	0.337
n-Propanol	mg/L	3.2	4.5	2FI	$9.03 \times 10^{-3}$ **	0.029 *
Isobutanol	mg/L	3.2	6.7	RQuadratic	$4.30 \times 10^{-9}$ ***	0.145
Isoamyl alcohols	mg/L	7.3	13.3	Quadratic	$2.67 \times 10^{-5}$ ***	0.270
Σ Esters	mg/L	4.2	11.1	RQuadratic	$1.48 \times 10^{-2}$ *	0.018 *
Σ Alcohols	mg/L	13.7	22.9	RQuadratic	$3.28 \times 10^{-8}$ ***	0.339
Glycerol	g/L	0.17	0.37	RQuadratic	$4.85 \times 10^{-5}$ ***	0.034 *
Acceptance	-	1.08	3.38	Linear	$1.31 \times 10^{-2}$ *	0.377
Fruitiness	-	1.13	3.38	Linear	$7.31 \times 10^{-3}$ **	0.484

Model terminology: “RQuadratic” Reduced Quadratic; “2FI” Two-Factor Interaction; “RLinear” Reduced Linear. “LOF” Lack of Fit. ANOVA significance codes: \*\*\*  $p \leq 0.001$ , \*\*  $p \leq 0.01$ , \*  $p \leq 0.05$ .

It was possible to create significant models for 12 responses (Table 7). However, five also exhibited significant lack of fit (LOF), rendering them unusable for predictions. The aim of the RSM was to investigate the optimal fermentation conditions to create a fruity, non-alcoholic beer. The three-dimensional response surface plots of the interactive effects of temperature and pitching rate on the final ethanol content and the fruitiness of the produced NAB are shown in Figures 3 and 4.



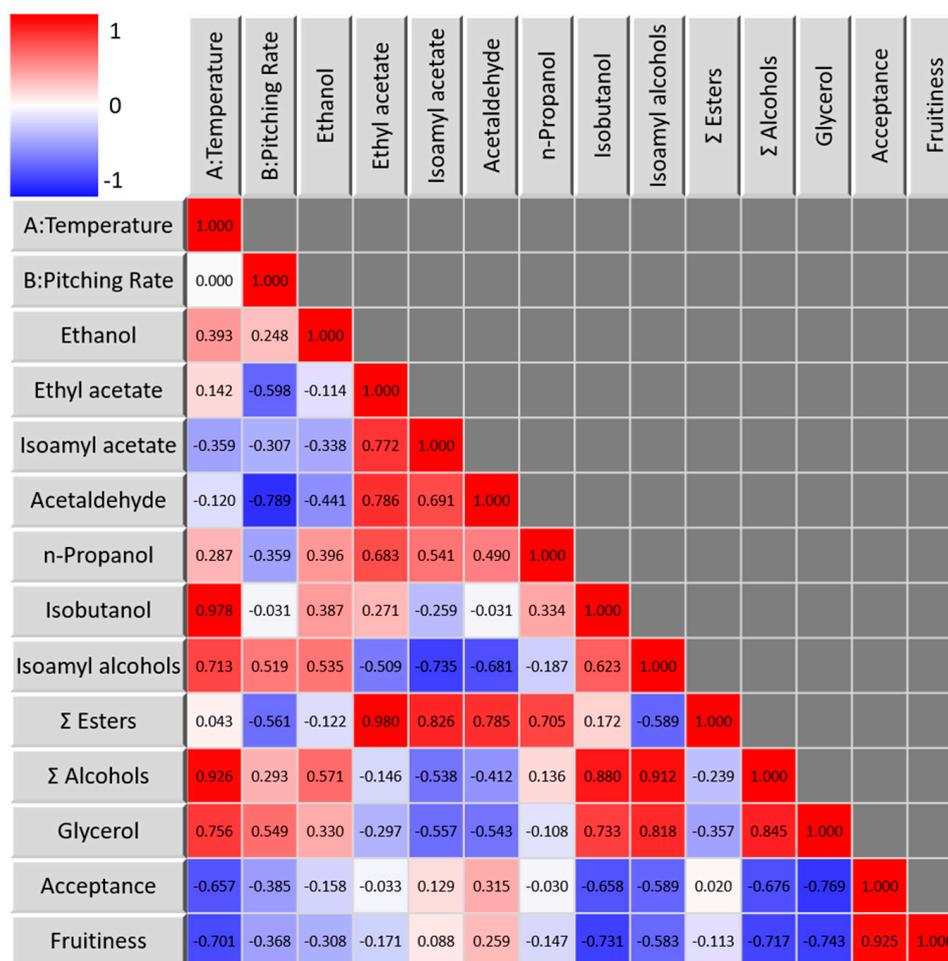
**Figure 3.** Three-dimensional response surface plot of the interactive effects of temperature and pitching rate on the ethanol content of the produced non-alcoholic beer ( $p < 0.01$ ).



**Figure 4.** Three-dimensional response surface plot of the effects of temperature and pitching rate on the fruitiness of the produced non-alcoholic beer ( $p < 0.01$ ).

Ethanol content was lowest at a low temperature of 17 °C and low pitching rate ( $10^7$  cells/mL), and it went up with increasing temperature and pitching rate, but lowered again at a high pitching rate combined with a high fermentation temperature (Figure 3). The minimum and maximum values were 0.41% and 0.60% ABV. Sugar analysis revealed that at 17 °C and  $10^7$  cells/mL, about 0.5 g/L of glucose was remaining after fermentation, while it was fully depleted in worts fermented at higher pitching rates and higher temperatures (data not shown). The residual sugar explained the lower final ethanol concentration. Fructose was only fully depleted in the samples that were fermented at 27 °C. At 22 °C, fermented samples exhibited residual fructose concentrations between 0.2 and 0.5 g/L, and at 17 °C, fermented samples showed remaining fructose concentrations between 0.2 and 0.7 g/L. Acetaldehyde concentrations were only dependent on the pitching rate, with increasing amounts of acetaldehyde found at lower pitching rates (Figure A1). This result correlates with other studies that found a decrease in acetaldehyde with increasing pitching rate in wort fermentations with brewers' yeasts [48,49]. However, overdosing yeast ( $>5 \times 10^7$  cells/mL) can lead to an increase in acetaldehyde again, as observed by Erten et al. [50]. The temperature did not have a significant effect on the acetaldehyde concentration and was therefore excluded from the model ( $p = 0.39$ ; supplementary Data Sheet S1). However, regarding higher alcohols, the fermentation temperature had a stronger effect, with increasing amounts of higher alcohols found at higher temperatures (Figures 5 and A2), which is consistent with the literature [4,5]. Isoamyl acetate concentrations were generally high and ranged from 0.8 to 2.2 mg/L. Although the model was significant ( $p < 0.05$ ), it was unsuitable for value prediction due to a significant lack of fit ( $p = 0.046$ ).

Interestingly, the production of the esters ethyl acetate and isoamyl acetate did not show a clear correlation to temperature, which underlines that the general rule of thumb, that higher fermentation temperatures lead to increased ester production, is not valid for all yeast strains (Figure 5) [4]. Furthermore, the amount of esters that were quantified in this study did not correlate with the perceived fruitiness of the NAB, which tentatively suggests that the fruity flavor profile was caused by yet unidentified compounds (Figure 5).



**Figure 5.** Map visualizing correlations of response surface methodology (RSM) factors and responses based on the Pearson Correlation Coefficient. 1 signifies strong positive correlation, 0 signifies no correlation, and -1 signifies a strong negative correlation.

In terms of fruitiness, a low fermentation temperature paired with a low pitching rate led to the highest perceived fruitiness. Indeed, the highest fruitiness was recorded at 17 °C and  $1 \times 10^7$  cells/mL and the lowest at 27 °C and  $6 \times 10^7$  cells/mL, following a linear model. General acceptance showed a strong positive correlation with the fruitiness, indicating that the panel preferred fruity samples (Figures 5 and A3).

Due to the ideal combination of lowest ethanol content and highest fruitiness and acceptance, the fermentation temperature of 17 °C and pitching rate of  $1 \times 10^7$  cells/mL were chosen as the optimal fermentation conditions for application to produce a fruity, non-alcoholic beer.

A small-scale fermentation at the optimal conditions (17 °C,  $10^7$  cells/mL) was conducted to validate the RSM model. Table 8 shows the predicted mean including 95% prediction intervals (PI) and the measured (“observed”) mean with standard deviation.

Although predicted by a significant model, the observed means for ethanol, acetaldehyde and isobutanol values were not within the 95% prediction interval. Sugar analysis revealed the complete depletion of glucose in the experimental fermentation trial at optimal conditions compared to the RSM model prediction, which explained the increased ethanol production (data not shown). The moderate success in model validation demonstrates the limitations in the application of RSM to optimize fermentations, where small differences in substrate and process conditions can have significant influences on the outcome. Because wort is a very complex substrate, comprising a complex mixture of different sugars, nitrogen sources, minerals and vitamins, among others, any interpretation or the

transfer of the RSM results to other substrates (even different wort substrates) should be made with caution. In particular, a different sugar composition will have a significant effect on the responses when applying maltose-negative yeasts. However, the improved fruitiness and therefore higher acceptance of the NAB produced at low temperature and low pitching rate, the main goal from the optimization, was significant and reproducible (Table 8).

**Table 8.** Response surface methodology (RSM) model validation via predicted value vs. observed value.

Response	95% PI Low	Predicted Mean	95% PI High	Observed Mean	Std. Dev.
Ethanol *	0.33	0.40	0.48	0.53	0.01
Ethyl acetate	0.89	4.74	8.60	6.83	0.59
Isoamyl acetate	0.78	1.63	2.47	2.50	0.10
Acetaldehyde *	2.19	2.97	3.74	1.27	0.29
n-Propanol	2.68	3.28	3.88	3.57	0.06
Isobutanol *	2.91	3.23	3.54	2.80	0.10
Isoamyl alcohols	5.78	7.03	8.29	4.10	0.10
SUM Esters	3.01	7.10	11.19	9.33	0.68
SUM Alcohols *	12.84	13.74	14.64	10.47	0.31
Glycerol	0.13	0.18	0.22	0.27	0.01
Acceptance *	2.12	3.23	4.34	3.75	0.62
Fruitiness *	2.02	3.03	4.05	3.58	0.87

\* Significant model with insignificant lack of fit. 'PI' Prediction interval.

### 3.6. Pilot-Scale Brewing

Despite the limited model validation, the fermentation parameters were successfully optimized to enhance the fruity character of the NAB. Therefore, the pilot-scale brewing trial was conducted with the optimized conditions of 17 °C fermentation temperature and a pitching rate of  $10^7$  cells/mL.

The grain bill of the wort for the pilot-scale brewing trial consisted of 95% pilsner malt and 5% acidulated malt to lower the starting pH of the wort, to account for the reduced pH drop during fermentations with non-*Saccharomyces* yeasts compared to brewers' yeast. A low beer pH is desired to prevent microbial spoilage and to ensure good liveness of the beer [51,52]. The analytical attributes of the wort produced at pilot-scale are shown in Table 9.

**Table 9.** Attributes of the wort produced on pilot-scale.

Wort attributes	Unit	Value
Extract	°P	7.00 ± 0.01
pH		4.86 ± 0.01
FAN	mg/L	107 ± 3
Glucose	g/L	6.01 ± 0.08
Fructose	g/L	0.80 ± 0.01
Sucrose	g/L	2.13 ± 0.03
Maltose	g/L	31.59 ± 0.44
Maltotriose	g/L	9.32 ± 0.13

To assess the suitability of *Cyberlindnera subsufficiens* C6.1 to produce a fruity NAB, it was compared to two commercial NABs. NAB A was a commercial non-alcoholic beer produced by limited fermentation [29], and NAB B was a non-alcoholic beer produced by "dialysis technology" [30]. The NABs were analyzed for their extract, ethanol, FAN and glycerol content as well as their sugar composition and concentration of volatile fermentation by-products. The results are shown in Table 10.

**Table 10.** Attributes of the non-alcoholic beer (NAB) produced with C6.1 compared to two commercial NABs, NAB A and NAB B.

NAB Attributes	Unit	C6.1 NAB	NAB A	NAB B
Extract (real)	°P	6.60 ± 0.01	6.76 ± 0.07	7.05 ± 0.03
Extract (apparent)	°P	6.46 ± 0.02	6.57 ± 0.06	6.86 ± 0.01
Ethanol	% ABV	0.36 ± 0.00	0.50 ± 0.03	0.49 ± 0.04
pH		4.45 ± 0.01	4.29 ± 0.02	4.29 ± 0.04
FAN	mg/L	96 ± 2	86 ± 6	24 ± 0
Glycerol	g/L	0.30 ± 0.02	0.33 ± 0.01	1.40 ± 0.03
Glucose	g/L	2.77 ± 0.05	2.74 ± 0.04	5.61 ± 0.04
Fructose	g/L	1.65 ± 0.03	1.96 ± 0.03	0.19 ± 0.00
Sucrose	g/L	<LOD	<LOD	<LOD
Maltose	g/L	30.27 ± 0.62	30.11 ± 0.50	17.69 ± 0.24
Maltotriose	g/L	8.67 ± 0.24	8.31 ± 0.21	1.84 ± 0.03
Acetaldehyde	mg/L	10.55	2.40	0.70
Ethyl acetate	mg/L	12.00	<0.10	2.70
Isoamyl acetate	mg/L	0.80	<0.1	0.70
Isoamyl alcohols	mg/L	4.00	4.80	17.40
n-Propanol	mg/L	2.20	<0.5	2.50
Isobutanol	mg/L	3.60	1.00	4.90
Diacetyl	mg/L	<0.01	0.02	0.04
2,3-Pentandione	mg/L	<0.01	<0.01	<0.01
Σ Esters	mg/L	12.8	<0.1	3.4
Σ Alcohols	mg/L	9.8	5.8	24.8

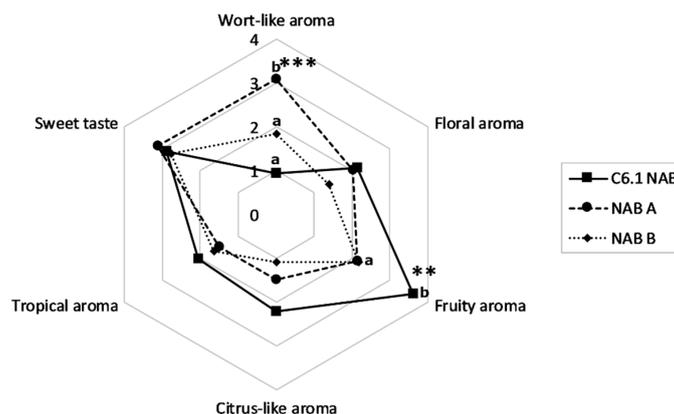
The C6.1 NAB reached final attenuation after 13 days of fermentation at 17 °C, at an ethanol content of 0.36% ABV. At the end of fermentation, 2.77 g/L glucose was remaining in the wort and sucrose was fully depleted. Compared to the initial sugar concentration of the wort (Table 9), fructose concentrations in the final beer were significantly higher, at 1.65 g/L, twice as high as the starting concentration in the wort. Since sucrose was fully depleted, it can be assumed that it was converted to glucose and fructose by the yeast's invertase. The high residual fructose could therefore be attributed to the previously observed glucophilic character of the C6.1 strain in the screening and RSM trial. As a result, fructose was not consumed by the yeast due to the permanent presence of glucose until fermentation came to a halt. As expected, maltose and maltotriose consumption was negligible. Despite the limited fermentation, C6.1 produced a relatively high amount of esters, at 12.8 mg/L, the majority of which was ethyl acetate (12 mg/L). NAB A had an ethanol content of 0.50% ABV. Interestingly, the sugar composition was very similar to that of the C6.1 NAB. Regarding fermentation by-products, however, NAB A exhibited very low concentrations, at about half the amount of higher alcohols and a total lack of the esters ethyl acetate and isoamyl acetate. NAB B had an ethanol content of 0.49% ABV. Owing to its fundamentally different production method, the analyzed attributes were very different from those of the two NABs produced solely by limited fermentation. The low FAN content together with a high glycerol content compared to the other NABs were indicators of a more extensive fermentation, with subsequent removal of ethanol. However, NAB B still exhibited high amounts of monosaccharides, which suggested that the production of the NAB either also entailed a limited fermentation, or the dealcoholized beer was blended with wort (or other means of sugar addition). The increased amounts of higher alcohols in NAB B, at 24.8 mg/L, are uncommon for beers dealcoholized via dialysis, since the process commonly reduces their content in the final NAB by 90%–95% [7]. Despite the addition of acid malt during the wort production for the C6.1 NAB, the final pH after fermentation was, at 4.45, higher compared to 4.29 in the commercial NABs.

Due to the high amounts of residual sugars, proper pasteurization is essential for non-alcoholic beers produced by limited fermentation to avoid microbial spoilage [1,38,53]. After bottling, C6.1 NAB was therefore pasteurized with approximately 23 PU, and the successful pasteurization was confirmed

by plating the pasteurized NAB on agar to check for microorganism growth, which was found to be negative.

### 3.7. Sensory Evaluation

For a holistic evaluation of the C6.1 NAB compared to the two commercial NABs, a sensory trial was conducted with 10 trained and experienced panelists. The panel was asked to describe the flavor of the beer in their own words, followed by an assessment of several intensity attributes. The mean score values of the parameters wort-like, floral, fruity, citrus-like and tropical aroma, as well as sweet taste, of the NABs are shown in Figure 6.



**Figure 6.** Spider web with the means of the descriptors from the sensory trial of the NAB produced with *Cyberlindnera subsufficiens* C6.1 and the two commercial NABs. Different letters next to data points indicate a significant difference as per Tukey's *post hoc* test. Significance codes: \*\*\*  $p \leq 0.001$ , \*\*  $p \leq 0.01$ .

The NAB produced with C6.1 was described as very fruity with aromas of pear, banana, mango and maracuja together with a slightly wort-like character. NAB A was described as malty, wort-like and hoppy, while NAB B was described as wort-like and caramel-like. The C6.1 NAB was indeed evaluated as being significantly more fruity than the commercial NABs ( $p \leq 0.01$ ), at an average of 3.6 out of 5 compared to 2.1 and 2.2 out of 5, scoring also higher in citrus-like and tropical aromas. Consequently, the wort-like aroma, one of the most criticized flaws of NABs produced by limited fermentation [1,2,52], was least pronounced in the NAB produced with C6.1 with an average of 1 out of 5, followed by NAB B with 1.8 out of 5. NAB A exhibited, at an average of 3.2, a significantly more pronounced wort-like aroma ( $p \leq 0.001$ ). A sweet taste, caused by a high amount of residual sugars, is another major point of criticism for NABs produced by limited fermentation [1,2,52]. All NABs scored similarly in sweet taste without significant differences. NAB B scored lower for "floral" compared to the other NABs. However, the difference was not statistically significant. When the panelists were asked for their favorite sample, 40% chose C6.1 NAB, 40% chose NAB A, and 20% chose NAB B. Similarly, Strejc et al. [3] investigated the production of a non-alcoholic beer (0.5% ABV) by a cold contact process (characterized by a low temperature and high pitching rate) with a mutated lager yeast strain (*Saccharomyces pastorianus*). The strain's targeted mutation resulted in an overproduction of isoamyl acetate and isoamyl alcohols. The authors reported that the fruity flavour of the NAB produced with the mutated strain was "partially able to disguise" the typical wort-like off-flavor [21]. However, the isoamyl acetate concentration of the resulting NAB was, at 0.5 mg/L, lower than the concentration in the C6.1 NAB in this study (Table 10). Furthermore, the complex mutation and isolation procedure paired with a potentially limited stability of the mutation limits its applicability in practice. Saerens and Swiegers [22] reported the successful production of a NAB at 1000 L scale with a *Pichia kluyveri* strain, owing to its high production of isoamyl acetate (2–5 mg/L), which reportedly gave the NAB a fruity flavor that was more like that of a regular beer than commercial NABs. In accordance, the results of the sensory indicated that a strong fruity aroma can mask the wort-like off flavor, and that

the non-*Saccharomyces* yeasts, which produce a pronounced fruity character, can therefore be a means to produce NAB with improved flavor characteristics.

#### 4. Conclusions

The *Cyberlindnera* genus was found to be a promising non-*Saccharomyces* genus for application in the production of a fruity, non-alcoholic beer. Four of the six investigated species produced a fruity character, despite the limited fermentative capacity, which resulted in a low ethanol concentration. It was shown that through optimization of the fermentation parameters of temperature and pitching rate, the fruity character could be enhanced. Process up-scaling with *Cyberlindnera subsufficiens* strain C6.1 produced a NAB that was significantly more fruity compared to two commercial NABs. Owing to the strong fruity aroma, the often-criticized wort-like aroma could successfully be masked. Yeast handling throughout the process (i.e., propagation, yeast pitching, fermentation) proved to be suitable for pilot-scale brewing, with potential for application at industrial scale. Further studies should investigate if the masking effect was enhanced by a reduction of wort aldehydes via yeast metabolism.

This study demonstrated the suitability of the non-*Saccharomyces* species *Cyberlindnera subsufficiens* for the production of non-alcoholic beer (<0.5% ABV) with novel flavor characteristics that can compete with commercial NABs. The successful pilot-scale (60 L) brewing trial gives prospect to future studies with diverse non-*Saccharomyces* yeasts and strengthens their position as a serious and applicable alternative to established methods in non-alcoholic and low alcohol beer brewing.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2311-5637/5/4/103/s1>, Data Sheet S1: RSM response values and model statistics.

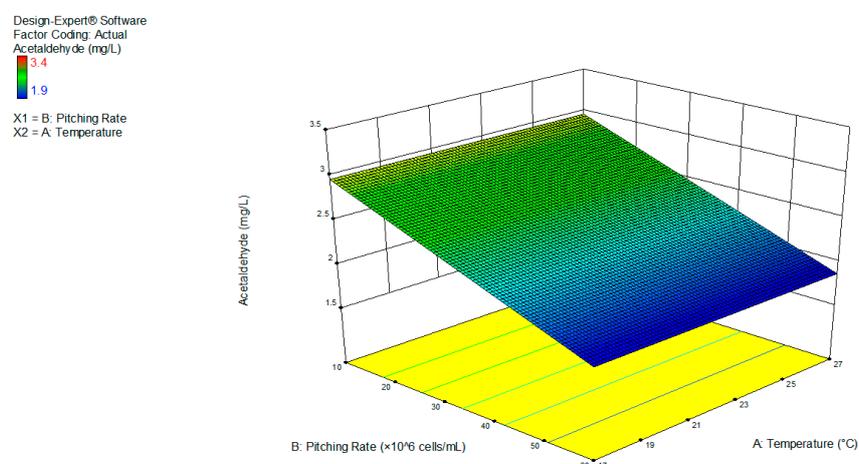
**Author Contributions:** Conceptualization, K.B., M.M., M.H., M.Z. and E.K.A.; methodology, K.B. and M.M.; investigation, K.B., J.J.A., and A.H.; resources, M.H., F.J. and E.K.A.; formal analysis, K.B.; writing—original draft preparation, K.B.; writing—review and editing, K.B., M.M., M.Z., M.H., K.M.L. and E.K.A.; visualization, K.B.; supervision, E.K.A.; project administration, E.K.A.; funding acquisition, E.K.A.

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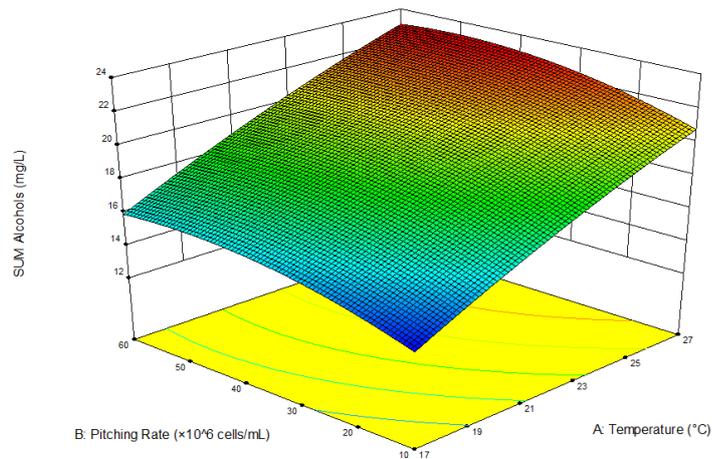
**Conflicts of Interest:** The authors declare no conflict of interest.

#### Appendix A



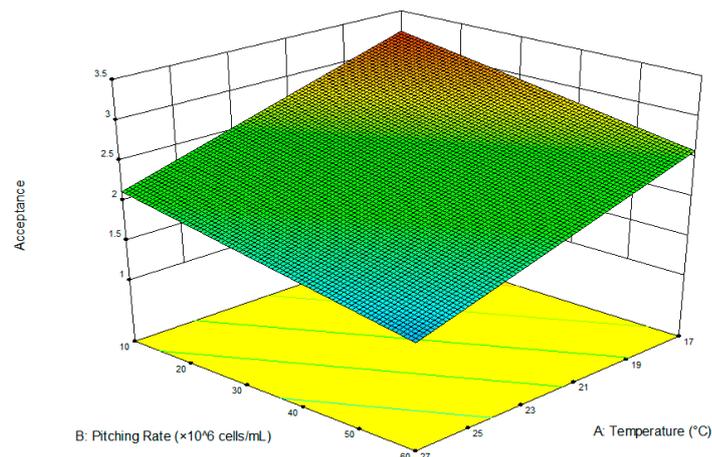
**Figure A1.** Three-dimensional response surface plot of the effect of pitching rate on the acetaldehyde content of the produced NAB ( $p < 0.01$ ). The factor temperature was excluded from the model due to insignificance ( $p = 0.39$ ; supplementary Data Sheet 1).

Design-Expert® Software  
 Factor Coding: Actual  
 SUM Alcohols (mg/L)  
 22.9  
 13.7  
 X1 = A: Temperature  
 X2 = B: Pitching Rate



**Figure A2.** Three-dimensional response surface plot of the interactive effects of temperature and pitching rate on the sum of higher alcohols of the produced NAB ( $p < 0.001$ ).

Design-Expert® Software  
 Factor Coding: Actual  
 Acceptance  
 3.38  
 1.08  
 X1 = A: Temperature  
 X2 = B: Pitching Rate



**Figure A3.** Three-dimensional response surface plot of the effects of temperature and pitching rate on the overall acceptance of the produced NAB ( $p < 0.05$ ).

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