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Screening and characterisation of β -glucosidase production strains from *Rosa roxburghii* Tratt

<https://doi.org/10.1515/ijfe-2020-0152>

Received June 24, 2020; accepted September 17, 2020;

published online November 2, 2020

Abstract: The β -glucosidase properties from one yeast isolate identified as *Wickerhamomyces anomalus* C4 were characterised. The β -glucosidase activity of *W. anomalus* C4 was 41.83 ± 0.25 mU/mL, and the optimum temperature and pH were 40 °C and 5.0, respectively. The glucose, 10% v/v of ethanol and 10 mmol/L of Cu^{2+} inhibited the β -glucosidase activities. The isolate *W. anomalus* C4 had a stronger alcohol metabolism capacity than commercial *Saccharomyces cerevisiae* X16. Besides, fermentation with *W. anomalus* C4 alone and co-fermentations with *S. cerevisiae* X16 and *W. anomalus* C4 reduced the volatile acids content and the sourness value compared to *S. cerevisiae* X16 control. Moreover, inoculation with *W. anomalus* C4 could enhance volatile aroma richness and complexity of *Rosa roxburghii* wines, regardless of type or amount thereof. Therefore, the *R. roxburghii* native yeast isolate *W. anomalus* C4 may have some application potentials for *R. roxburghii* wine-making.

Keywords: β -glucosidase; *Rosa roxburghii* Tratt; *Wickerhamomyces anomalus*; wine aroma.

1 Introduction

Aroma profile is one of the important quality characteristics of a wine [1]. The volatile compounds derived therefrom are major contributors to the typical flavour of wine; however, in many cases some bound form of non-volatile compounds (usually present as flavourless glycoconjugates) accumulated in higher amounts in fruit. These

odourless glycosidic precursors could be hydrolysed by β -glucosidase during the winemaking processes, and released the aromatic components, contributing to the varied flavour complexity of wine [2].

The β -D-glucosidases (β G; EC 3.2.1.21) are ubiquitous in oenological yeasts including *Saccharomyces* and non-*Saccharomyces* yeasts [3]. It is believed that the aroma of native fermentations could be enhanced by higher hydrolytic enzyme producing yeasts [4], therefore, the use of indigenous β -D-glucosidase from native yeasts for winemaking is a better choice when trying to improve wine aroma [5]. However, β -D-glucosidase activities from different yeast species exhibit significant variability [6]. Therefore, it is necessary to screen and characterise special glycosidases from indigenous yeast for winemaking in the initiation of fermentation.

Rosa roxburghii Tratt (*R. roxburghii*) is a plant that is widely distributed in south-western areas of China, such as Guizhou, Chongqing, Sichuan, and Guangxi [7]. The fruit of *R. roxburghii* is nutritional and contains medicinal ingredients [8]. For example, the vitamin C content is 1700–2900 mg per 100 g of edible part, which is 10 times that of kiwi fruit. The fruit of *R. roxburghii* tastes bad due to an excess of tannins and organic acids [9], making their comprehensive utilisation difficult when relying thereon for fermentation into wine.

In this study, β -D-glucosidase-producing indigenous yeasts were screened from *R. roxburghii* fruit in Longli County, Guizhou Province, China. Furthermore, β -D-glucosidase activities were also evaluated under various brewing conditions. Moreover, fermentation was performed using the *R. roxburghii* native yeast to analyse the effect of β -D-glucosidase on the aroma of *R. roxburghii* wine. To our knowledge, this is the first screening and description of β -glucosidase activity involved in *R. roxburghii* indigenous yeast and its utilisation in releasing bound-form aromas.

2 Materials and methods

2.1 Yeast strains

The commercial *Saccharomyces cerevisiae* (*S. cerevisiae*) X16 obtained from Laffort Company (France) was used as a reference strain. A total of 65 strains of yeasts isolated from *R. roxburghii* fruit in Longli County,

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Guizhou Province were analysed in this study to investigate the β -glucosidase activities. All yeasts cells were cultured on YPD solid medium (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) containing 100 mg/L of chloromycetin at 28 °C for 72 h, and then kept at 4 °C for later use.

2.2 β -glucosidase activity assay

Esculin semi-quantitative colouration analysis was applied to screen β -glucosidase production yeast strains. Yeasts were incubated in 200 μ L esculin medium with 10^5 live cells/mL in 96-well plates, and then incubated at 28 °C for 72 h. β -glucosidase activity was determined according to the colour depth.

To confirm the preliminary screening results, β -glucosidase activity was determined using *p*-nitrophenyl- β -D-glycoside (pNPG) as the substrate and the amount of *p*-nitrophenol (pNP) released from pNPG was checked. Firstly, yeasts were incubated in 50 mL YPD liquid medium with 5% (v/v) seed culture, cultured at 28 °C while being shaken at 160 rpm for 72 h, and collection via centrifugation at 3000 *g* for 10 min. The supernatant was used as an enzyme solution. Enzyme solution (0.1 mL) was mixed with pNPG solution (0.2 mL, 0.002 M) in citrate phosphate buffer (0.1 M, pH 5.0). The reaction was maintained at 40 °C for 30 min, and stopped by adding Na₂CO₃ (2.0 mL, 0.25 M). The released pNP was measured spectrophotometrically at 405 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 nmol of pNP per min under the aforementioned experimental conditions.

2.3 Identification of yeast strain

The selected isolate strain was streaked on the WL nutrient agar and incubated at 28 °C for 5 days. The micromorphology of the selected yeast was captured by using a microscope (Olympus, Tokyo, Japan) and classified according to its morphotype.

The selected isolate strain was further identified by amplifying the D1/D2 domain of 26S rDNA via polymerase chain reaction (PCR) with forward primer NL1 (5'-GCATATCAATAAGCG GAGGAAAAG-3'), and reverse primer NL4 (5'-GGTCCGTGTTCAAGACGG-3'). The sequences of the D1/D2 domain of 26S rDNA were compared by BLASTN Homology Search.

2.4 Effect of brewing conditions on β -glucosidase activity

In order to ascertain the effect of temperature or pH on β -glucosidase activity, the enzyme reactions were incubated at different temperatures over the range between 20 and 60 °C (20, 30, 40, 50 and 60 °C) at pH 5, or with citrate-phosphate buffer across the pH range from 3.0 to 7.0 (3.0, 4.0, 5.0, 6.0 and 7.0). The enzyme assay was conducted with pNPG as the substrate as previously described.

Effects of glucose or ethanol on enzyme activities were identified by using glucose concentrations over a range of 0–20% w/v or ethanol concentrations over a range of 0–20% v/v, respectively. Stock solutions of glucose or ethanol were prepared in citrate-phosphate buffer (pH 5.0) and were added to the reaction mixture resulting in final concentrations from 0 to 20% w/v or 0–20% v/v, respectively. The enzyme assay was conducted as previously described.

To verify the effect of metal ion on the β -glycosidases activity, K⁺, Na⁺, Mg²⁺, Cu²⁺ and Al³⁺ were added to citrate-phosphate buffer (pH 5.0) with the final concentration of 10 mmol/L. The reaction mixtures were incubated at 40 °C for 30 min, and then enzyme activities were measured.

2.5 Kinetic of β -glucosidase enzyme

Kinetic parameters, K_m and V_{max} , were detected from Lineweaver-Burk plots of β -glucosidase activity at pNPG concentrations ranging from 0.2 to 2.0 mM (at pH 5.0 and 40 °C). Each sample was detected in triplicate.

2.6 Laboratory-scale fermentation of *R. roxburghii* wine

The *R. roxburghii* wine fermentation was conducted as described by Huang [10] with some modifications. Fresh *R. roxburghii* was washed with sterile water and crushed to acquire its *R. roxburghii* must. The must was filtrated with microporous film, poured into a 2 L bottle and divided into three groups: an *S. cerevisiae* group, a *Wickerhamomyces anomalus* group and a mixed group. The initial *R. roxburghii* must contained 50 mg/L SO₂ and 20 mg/L pectinase at pH 3.5. Logarithmic growths of *S. cerevisiae* X16 (10^7 CFU/mL) and *W. anomalus* (10^8 CFU/mL) were inoculated into the *S. cerevisiae* and *W. anomalus* groups, respectively. The mixed group was simultaneously inoculated with 10^7 CFU/mL of *S. cerevisiae* X16 and 10^8 CFU/mL of *W. anomalus*. Fermentation was performed at 25 °C without agitation until finished. Three replicates for each group were tested.

2.7 Chemical compositions of *R. roxburghii* wine

pH measurements were conducted using a digital pH meter (Model PT-10, Sartorius AG, Germany). The concentrations of ethanol, residual sugars, total acidity and volatile acids were determined using methods recommended by the International Organization of the Vine and Wine (OIV 2005). Each sample was detected in triplicate.

2.8 Volatile compound analysis of *R. roxburghii* wine

Fermented *R. roxburghii* wine was centrifuged at 3000 *g* for 15 min and supernatant was prepared for volatile compound analysis. Cyclohexanone was added to the *R. roxburghii* wine as an internal control. A headspace solid-phase micro extraction (HS-SPME) method was performed at 40 °C for 30 min to extract volatile compounds. An Agilent TQ8040 gas chromatograph mass spectrometer was used to analyse each specimen. The flow rate of the carrier gas (helium) was maintained at 1.2 mL/min. The column temperature was programmed as follows: 40 °C for 2 min, increased to 230 °C at a rate of 5 °C/min and then held at 230 °C for 15 min. The temperatures for the injector and detector were set to 250 and 280 °C, respectively. The mass spectrometer was operated in electron-impact mode at 70 eV. Volatile compounds of orange wine were identified by comparing their linear retention indices with those of pure standards, published data or MS fragmentation patterns obtained from databases. The odour active value (OAV), as the ratio of the concentration of a flavour compound to its odour threshold, is a parameter widely used to obtain odour patterns starting from quantitative compositions. In this research,

compounds with OAV > 1 are considered to be responsible for aroma, and the higher their OAV, the more they contribute to the aroma profile.

2.9 Statistical analysis

The results were shown as mean $\pm$ SD. One-way analysis of variance, Student's *t*-test, and principal component analysis (PCA) were conducted using SPSS 25.0 statistical software (SPSS). Differences were considered significant when *P* < 0.05.

3 Results

3.1 Screening of high β -glucosidase production of yeasts

β -glucosidase production yeasts were screened from “Guinong 5” *R. roxburghii* fruit (Figure 1), obtained from Longli County, Guizhou Province, China. A total of 65 yeast isolates were chosen from YPD plates and then evaluated for β -glucosidase production using the esculin reaction method. As shown in Figure 2, eight yeast isolates (B3, B9, B11, C4, C5, C9, D8, and E9) were found to exhibit high β -glucosidase activity on account of their dark brown colour in esculin medium.

To verify the β -glucosidase activities of these eight candidate yeast isolates, quantitative analysis was undertaken using *p*-NPG colorimetry. The results demonstrated that the highest β -glucosidase activity was produced by Strain C4 (Table 1). Therefore, the C4 strain was subjected to selected for further studies.

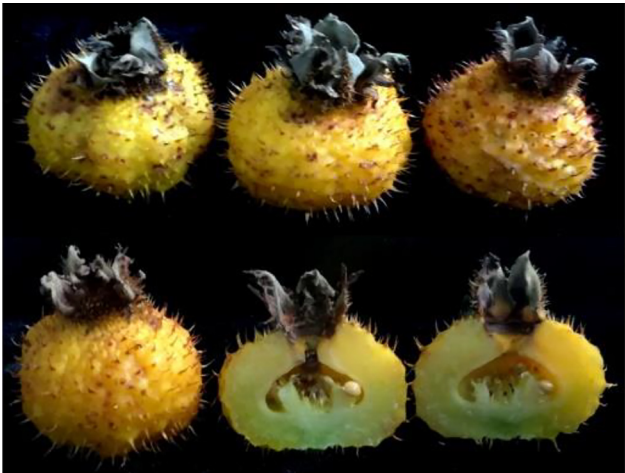


Figure 1: “Guinong 5” *R. roxburghii* fruit used in this study.

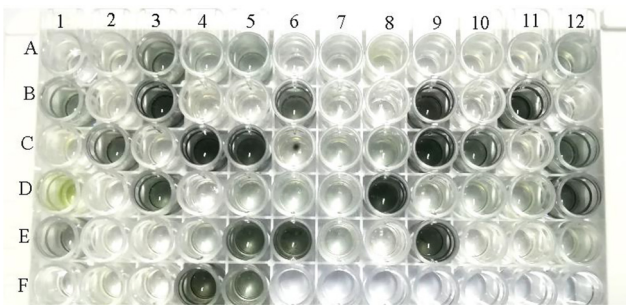


Figure 2: Detection of β -glucosidase production using the esculin reaction method.

Table 1: Quantitative analysis of β -glucosidase activities of eight strains of candidate yeast isolates.

Strain number	β -glucosidase activities (mU/mL)
B3	38.70 ± 0.40
B9	37.33 ± 0.21
B11	36.17 ± 0.25
C4	41.83 ± 0.25
C5	35.60 ± 0.20
C9	40.30 ± 0.17
D8	36.85 ± 0.23
E9	32.50 ± 0.17

3.2 Identification of selected yeast strain

Firstly, morphological characteristics were checked to identify the C4 strain. The morphology of the colony on WL (Wallerstein laboratory nutrient agar) identification medium was white on the surface and dark green within, villiform and flat. The cellular microscopic morphology was assessed after staining by crystal violet (Figure 3). These observations indicated that the yeast C4 isolate might belong to the species *W. anomalus*.

Then, a molecular biological method was applied to determine the C4 strain. The sequence of D1/D2 domain of 26S rDNA was amplified and compared by BLASTN Homology Search. The results revealed that the sequence of yeast isolate, C4, was 99.66% identical to *W. anomalus* TC2-5. Taken together, it was confirmed that the isolate C4 (GenBank accession: MT705624) was identical to *Wickerhamomyces anomalus*.

3.3 Effects of brewing conditions on β -glucosidase activity

To identify the effect of temperature on the β -glycosidases activity from the selected yeast strain C4, enzymatic

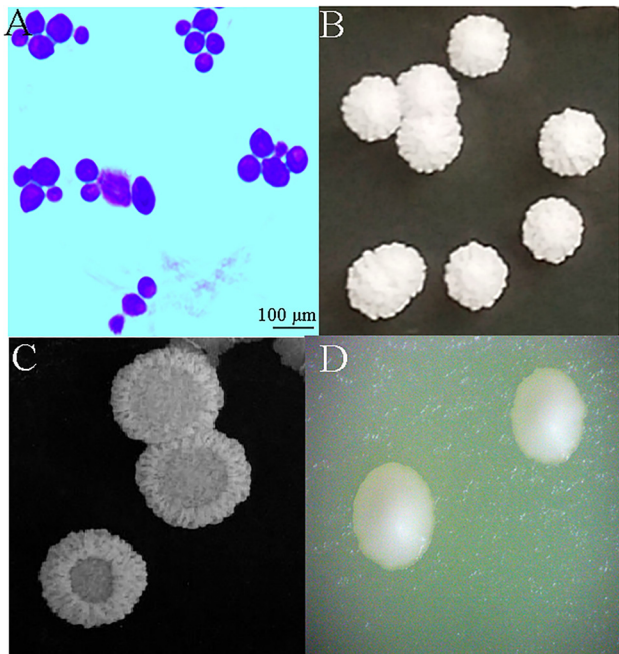


Figure 3: Morphology of cells and colonies of C4 strain. A, Microscopic morphology of C4 strain via crystal violet staining. B-D, Colony morphology of C4 strain on YPD medium (B), WL medium (C), and lysine medium (D).

activity was detected in a temperature range from 20 to 60 °C. Data indicated that the β -glycosidases of C4 strain had an optimum temperature of 40 °C (Figure 4A).

Figure 4B shows the effect of pH on enzymatic activity. The β -glucosidase extracted from *W. anomalus* C4 was strongly inhibited at pH 3.0. Maximum activity was recorded at a pH of around 5.0, therefore, the optimum pH for *W. anomalus* C4 was 5.0.

The effects of glucose concentrations in the range from 0–20% w/v on β -glucosidases were determined. The results of glucose inhibition are shown in Figure 4C. The β -glucosidase activity from *W. anomalus* C4 was strongly

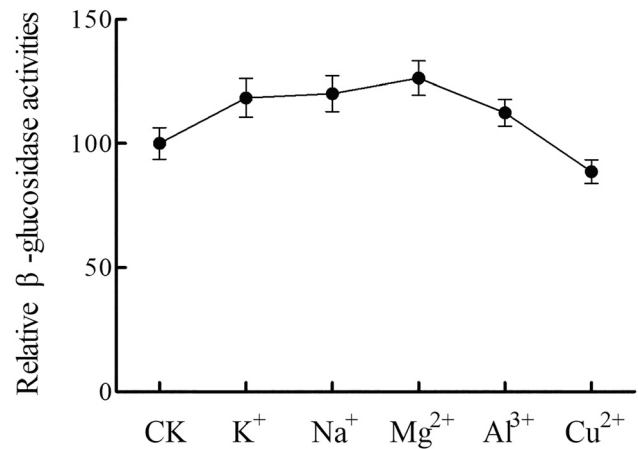


Figure 5: Effect of metal ion on β -glucosidase activity.

inhibited by 5% w/v glucose and the relative activity remained at around 50%. In the presence of 20% w/v glucose, the relative activity of this β -glucosidase was around 30%.

The effect of ethanol concentration in the range of 0–15% v/v on β -glucosidase activity was also investigated (Figure 4D). The activity of β -glucosidase from *W. anomalus* C4 was enhanced by ethanol concentrations in the range of 0–10% v/v with the maximum activity at a concentration of 10% v/v, however, when the concentration of ethanol exceeded 10% v/v, the β -glucosidase activity of *W. anomalus* C4 was inhibited.

In addition, the effect of metal ion on the β -glycosidases activity was assessed. As seen in Figure 4, K^+ , Na^+ , Mg^{2+} and Al^{3+} enhanced β -glycosidases activity, in contrast, Cu^{2+} inhibited β -glycosidases activity (Figure 5).

3.4 Kinetic parameters of β -glucosidase

The kinetic parameters, K_m and $V_{\max}$ of β -glucosidase of *W. anomalus* C4 were calculated by using *p*-nitrophenol- β -D-glucopyranoside as a substrate. The results indicated

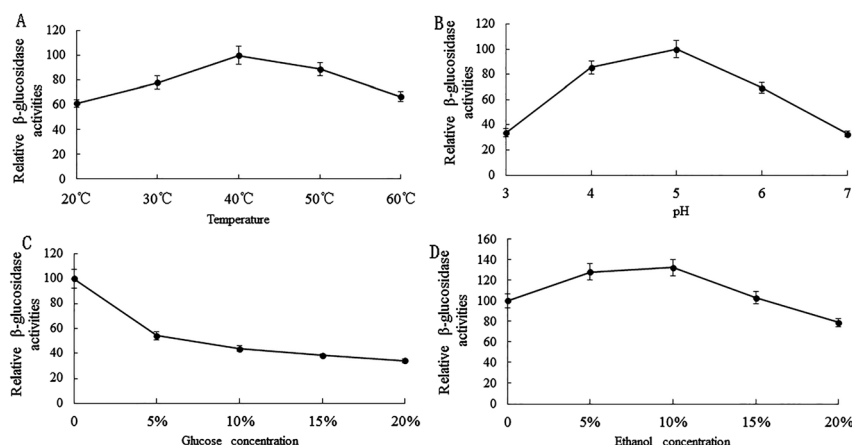


Figure 4: Effects of brewing conditions on β -glucosidase activity. A, Effect of temperature on β -glucosidase activity. B, Effect of pH on β -glucosidase activity. C, Effect of glucose on β -glucosidase activity. D, Effect of ethanol on β -glucosidase activity.

that K_m and V_{max} were 0.53 ± 0.03 mM and 1.08 U/g, respectively Table 1.

3.5 Physico-chemical characteristics of *R. roxburghii* wine fermented with different yeast strains

The physico-chemical characteristics of *R. roxburghii* wine fermented with different yeast strains, including pH, ethanol content, residual sugar total acidity, and volatile acidity were further evaluated. As shown in Table 2, a lower ethanol concentration and higher residual sugar were detected in *R. roxburghii* wines produced using *S. cerevisiae* X16 compared with pure *W. anomalous* C4, or mixed fermentation with *W. anomalous* C4 and *S. cerevisiae* X16. In addition, fermentation with *W. anomalous* C4 alone and co-fermentations with *S. cerevisiae* X16 and *W. anomalous* C4 could decrease the volatile acids content compared to *S. cerevisiae* X16 control.

An electronic tongue assay system (INSENT SS402B, Japan) was also used to compare the sensory characteristics of *R. roxburghii* wines. The results showed that there were no significant sensory differences between three *R. roxburghii* wines in bitterness, astringency, aftertaste-A, aftertaste-B, umami, richness, and saltiness except for sourness. The sourness values measured in *R. roxburghii* wines produced by *W. anomalous* C4 pure inoculation or mixed inoculation were smaller than that by *S. cerevisiae* X16 alone (Figure 6).

3.6 Volatile chemical profiles of finished *R. roxburghii* wines

A total of 64 volatile chemicals were founded in three different fermented *R. roxburghii* wines, including five volatile acids, 10 volatile alcohols, 25 volatile esters and 24 other volatile compounds (Table 3). There were 26, 45 and 58 volatile compounds detected in the three types of *R. roxburghii* wines produced by pure *S. cerevisiae* X16, *W. anomalous* C4 or co-inoculation with and *S. cerevisiae* X16 and *W. anomalous* C4, respectively. Moreover, wines

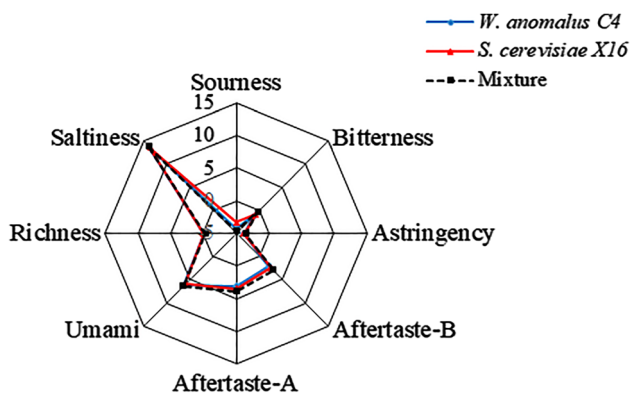


Figure 6: The *R. roxburghii* wine taste attribute radar chart.

inoculated with pure *W. anomalous* C4 or mixed with *S. cerevisiae* X16 contained lower amounts of volatile acids, increased amounts of volatile alcohols, volatile esters and other volatile compounds, compared to *S. cerevisiae* control. Taken together, inoculation with *W. anomalous* C4 could enhance the volatile aroma richness and complexity of *R. roxburghii* wines, regardless of type or amount.

The flavour active values (odour activity value, OAV) can evaluate the contribution of volatile compounds for wine aroma. When $OA V > 1$, the compound makes a significant contribution to the main aroma: $OA V < 1$ indicates that the compound does not contribute significantly thereto. The OAV values of the main volatile compounds in *R. roxburghii* wines are listed in Table 4. There were 21 and 23 compounds with $OA V > 1$ in pure *W. anomalous* C4 or in those mixed with *S. cerevisiae* X16 fermented *R. roxburghii* wine. In contrast, only 13 compounds had $OA V > 1$ in *S. cerevisiae* X16 solar-fermented *R. roxburghii* wine.

PCA was applied to analyse the chemical data pertaining to the wines obtained with each fermentation. PCA1 accounted for the 55.04% and PCA2 for an additional 44.96% of the variability (Figure 7): as seen in Figure 7A, complex odiferous compounds were clustered in the positive half-axis of PCA1, such as ethyl hexanoate, ethyl octanoate, ethyl cinnamate, isobutanol, linalool and 1-hexanol. 1-decanol and ethyl laurate, with fatty notes, were clustered in the negative half-axis of PCA1.

Table 2: Physico-chemical parameters of *R. roxburghii* wines fermentation with different yeasts.

Strains	Ethanol (% v/v)	Residual sugar (g/L)	pH	Total acidity (g/L)	Volatile acidity (g/L)
<i>Saccharomyces cerevisiae</i> X16	10.5 ± 0.14	12.58 ± 0.54	3.20 ± 0.03	4.20 ± 0.24	0.71 ± 0.01
<i>Wickerhamomyces anomalous</i> C4	$11.8 \pm 0.25^*$	$9.92 \pm 0.35^*$	3.43 ± 0.02	3.92 ± 0.00	$0.61 \pm 0.04^*$
Mixture	$11.9 \pm 0.21^{\#}$	$9.90 \pm 0.41^{\#}$	3.41 ± 0.01	4.01 ± 0.00	$0.64 \pm 0.02^{\#}$

* $P < 0.05$ versus *Saccharomyces cerevisiae* X16 group; $^{\#}P < 0.05$ versus *S. cerevisiae* X16 group.

Table 3: Volatile compounds (mg/L) in *R. roxburghii* wines fermented with different yeasts.

Number	Volatile compound	<i>Saccharomyces cerevisiae</i> X16	<i>Wickerhamomyces anomalus</i> C4	Mixture
1	Acetic acid	30.61 $\pm$ 0.51	ND	ND
2	Isobutyric acid	1.01 $\pm$ 0.19	ND	0.98 $\pm$ 0.37
3	Hexanoic acid	2.74 $\pm$ 0.14	3.55 $\pm$ 0.06	2.43 $\pm$ 0.11
4	Octanoic acid	20.11 $\pm$ 0.28	23.23 $\pm$ 2.31	19.48 $\pm$ 0.03
5	n-Decanoic acid	21.95 $\pm$ 2.53	20.69 $\pm$ 0.38	8.71 $\pm$ 0.23
	Σ Acids	76.42 $\pm$ 3.65	47.47 $\pm$ 2.75*	31.60 $\pm$ 0.74 [#]
6	Isobutanol	9.09 $\pm$ 1.07	17.59 $\pm$ 2.93	16.59 $\pm$ 0.37
7	Linalool	ND	0.83 $\pm$ 0.00	0.74 $\pm$ 0.01
8	Phenylethyl alcohol	66.11 $\pm$ 6.13	60.35 $\pm$ 1.97	81.70 $\pm$ 0.66
9	3-Hexen-1-ol, (Z)-	11.36 $\pm$ 0.48	13.74 $\pm$ 0.28	10.99 $\pm$ 0.25
10	1-Hexanol	ND	8.88 $\pm$ 0.42	7.41 $\pm$ 0.47
11	1-Decanol	3.33 $\pm$ 0.72	ND	1.72 $\pm$ 0.33
12	Isoamyl alcohol	ND	156.94 $\pm$ 17.69	188.54 $\pm$ 9.10
13	1-Propanol	ND	0.84 $\pm$ 0.09	ND
14	1-Octanol	ND	ND	1.33 $\pm$ 0.20
15	3,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-	ND	2.30 $\pm$ 0.05	1.98 $\pm$ 0.01
	Σ Alcohols	89.89 $\pm$ 8.40	261.47 $\pm$ 23.43**	311.00 $\pm$ 11.40 ^{##}
16	Hexyl acetate	ND	8.48 $\pm$ 0.19	9.69 $\pm$ 1.74
17	3-hexyl acetate	ND	8.51 $\pm$ 0.22	11.66 $\pm$ 1.81
18	Isobutyl acetate	1.57 $\pm$ 0.10	1.98 $\pm$ 0.36	2.65 $\pm$ 0.28
19	Isoamyl acetate	61.17 $\pm$ 0.01	87.01 $\pm$ 6.59	91.23 $\pm$ 9.24
20	Phenethyl acetate	13.73 $\pm$ 1.11	10.64 $\pm$ 0.20	16.30 $\pm$ 1.02
21	Ethyl acetate	ND	33.82 $\pm$ 1.10	30.52 $\pm$ 2.97
22	Ethyl butyrate	2.22 $\pm$ 0.20	2.17 $\pm$ 0.09	2.66 $\pm$ 0.15
23	Ethyl hexanoate	41.33 $\pm$ 2.43	53.48 $\pm$ 3.20	47.36 $\pm$ 1.01
24	Ethyl 2-methylpropionate	0.38 $\pm$ 0.20	0.28 $\pm$ 0.08	0.54 $\pm$ 0.10
25	Ethyl 3-hexenoate	0.91 $\pm$ 0.03	1.63 $\pm$ 0.15	1.14 $\pm$ 0.15
26	Ethyl octanoate	485.82 $\pm$ 30.11	611.70 $\pm$ 7.26	540.80 $\pm$ 45.71
27	Ethyl decanoate	477.49 $\pm$ 8.10	366.13 $\pm$ 27.95	448.68 $\pm$ 48.69
28	Ethyl laurate	29.50 $\pm$ 3.14	26.11 $\pm$ 2.58	28.52 $\pm$ 1.59
29	Ethyl cinnamate	6.12 $\pm$ 0.35	8.04 $\pm$ 0.04	7.51 $\pm$ 1.17
30	Ethenyl formate	ND	24.07 $\pm$ 2.15	1.17 $\pm$ 0.29
31	Ethyl palmitate	ND	ND	0.72 $\pm$ 0.08
32	Ethyl myristate	ND	1.87 $\pm$ 0.15	ND
33	Ethyl 9-decenoate	ND	3.45 $\pm$ 0.05	10.79 $\pm$ 0.24
34	Ethyl isobutyrate	ND	ND	2.72 $\pm$ 0.18
35	Ethyl pelargonate	ND	ND	1.03 $\pm$ 0.07
36	Isobutyl caprylate	ND	ND	1.07 $\pm$ 0.26
37	Isoamyl caprylate	ND	8.51 $\pm$ 0.23	7.19 $\pm$ 0.09
38	Isoamyl decanoate	ND	3.50 $\pm$ 0.07	2.90 $\pm$ 0.67
39	Ethyl isovalerate	ND	ND	2.76 $\pm$ 0.82
40	2-Propenoic acid, 1,7,7-trimethylbicyclo[2.2.1]hept-2-yl ester, exo-	ND	ND	0.69 $\pm$ 0.08
	Σ Esters	1120.24 $\pm$ 45.78	1261.38 $\pm$ 52.66*	1267.58 $\pm$ 118.23 [#]
41	Styrene	33.55 $\pm$ 2.96	36.54 $\pm$ 1.00	19.53 $\pm$ 1.59
42	2,4-Di-tert-butylphenol	15.96 $\pm$ 1.43	16.41 $\pm$ 0.63	12.57 $\pm$ 2.19
43	Carbamic acid, monoammonium salt	9.89 $\pm$ 1.26	27.66 $\pm$ 0.11	19.97 $\pm$ 0.33
44	Hydrazinecarboxamide	73.64 $\pm$ 1.18	89.28 $\pm$ 7.69	61.67 $\pm$ 1.53
45	Borane-methyl sulphide complex	1.63 $\pm$ 0.11	ND	ND
46	Dimethyl sulphide	1.64 $\pm$ 0.05	8.55 $\pm$ 1.52	1.24 $\pm$ 0.24
47	Ammonium acetate	ND	36.25 $\pm$ 2.59	20.00 $\pm$ 3.00
48	Acetaldehyde	ND	1.77 $\pm$ 0.00	3.93 $\pm$ 0.36
49	1-ethenyl-4-methoxybenzene	ND	1.82 $\pm$ 0.45	1.02 $\pm$ 0.02
50	2-methyl-1,5-dioxaspiro[5.5]undecane	ND	19.55 $\pm$ 1.60	19.57 $\pm$ 2.09
51	2,5-dimethyl-, 4-methoxy-, 2,3-dihydro-Furan-3-one	ND	2.39 $\pm$ 0.18	1.81 $\pm$ 0.13

Table 3: (continued)

Number	Volatile compound	<i>Saccharomyces cerevisiae</i> X16	<i>Wickerhamomyces anomalus</i> C4	Mixture
52	Nonacosane	ND	2.04 ± 0.15	ND
53	Naphthalene, 2-methyl-	ND	0.70 ± 0.02	ND
54	Benzene, 4-ethenyl-1,2-dimethoxy-	ND	2.82 ± 0.23	1.45 ± 0.16
55	4-vinyl-Guaiacol	ND	3.87 ± 0.25	1.44 ± 0.39
56	Benzofuran, 2,3-dihydro-3-Methyl	ND	8.50 ± 0.63	3.77 ± 0.15
57	3,3-dimethyl-1,5-dioxaspiro[5.5]undecane	ND	ND	0.99 ± 0.28
58	2-methyl-;2-methyl-5-dioxaspiro[5.5]undecane	ND	ND	0.63 ± 0.07
59	Butylated Hydroxytoluene	ND	ND	8.29 ± 0.75
60	2,4-Di-tert-butylphenol	ND	ND	12.57 ± 2.19
61	<i>p</i> -Mentha-1(7),8(10)-dien-9-ol	ND	ND	1.31 ± 0.30
62	Furfural	ND	ND	1.61 ± 0.15
63	5-Hydroxymethylfurfural	ND	ND	3.46 ± 0.32
64	2,3-dihydro-2,3-Benzofuran	ND	ND	3.77 ± 0.15
	Σ Other compounds	136.31 ± 6.99	$258.15 \pm 17.05^{**}$	$200.60 \pm 16.39^{##}$

* $P < 0.05$ versus *Saccharomyces cerevisiae* X16 group; ** $P < 0.01$ versus *S. cerevisiae* X16 group; # $P < 0.05$ versus *S. cerevisiae* X16 group;

$P < 0.01$ versus *S. cerevisiae* X16 group.

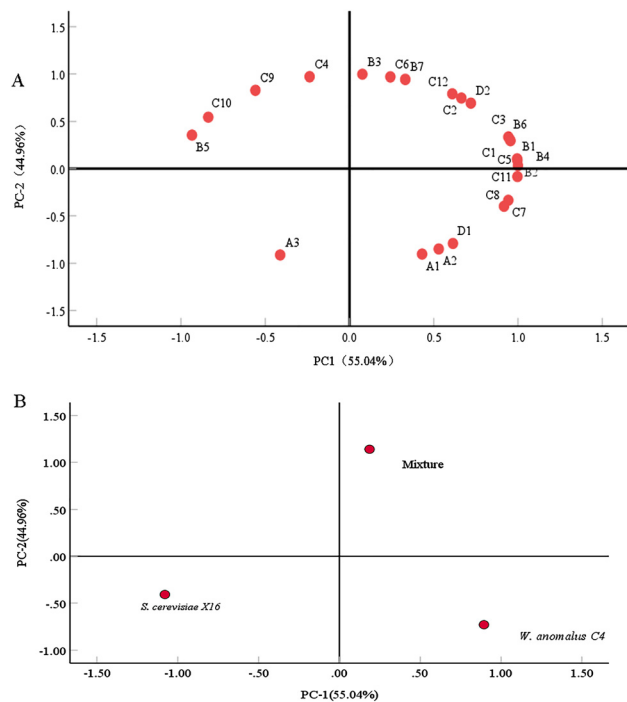


Figure 7: Principal component analysis of *R. roxburghii* wines fermentation with different yeasts. A, Principal component load plot of volatile aroma compounds. B, Principal component score of volatile aroma compounds.

The rose/floral odiferous compounds (phenylethyl alcohol, 1-octanol, and ethyl 9-decenoate) were the main components of the positive half-axis of PCA2. n-Decanoic acid and ethyl butyrate were distributed along the negative half-axis of PCA2.

This also indicated that the fruity odour (ethyl hexanoate, and ethyl octanoate) and the rose/floral odour (phenylethyl alcohol and 1-octanol) may be the main volatile characteristics of mixture fermented and pure fermented *R. roxburghii* wine made with *W. anomalus* C4, respectively (Figure 7B); however, it was difficult to identify the main volatile characteristics of *S. cerevisiae* X16 fermented *R. roxburghii* wine.

4 Discussion

The effects of β -D-glucosidases have been extensively investigated in many wine yeasts for their important role in the release of volatile aromas [11–13]. In this study, β -D-glucosidase producing indigenous yeasts was screen from *R. roxburghii* fruit, and the highest enzyme activity strain (numbered C4) was selected for further evaluation. Morphology and molecular biology data suggested that the isolate C4 was a strain of *W. anomalus*. To analyse the effect of β -D-glucosidase from *W. anomalus* C4 on the aroma of *R. roxburghii* wine, fermentation was conducted using *W. anomalus* C4 yeast alone or as a mixture. Inoculation *W. anomalus* C4 could enhance volatile aroma richness and complexity of *R. roxburghii* wines because more variety of type and higher contents of volatile chemicals in pure *W. anomalus* C4 and mixed fermented wines. It was reported that more free terpenes are released as a result of the action of β -D-glucosidase [14, 15]; however, only linalool, one type of terpene, was found in *W. anomalus*-C4-produced *R. roxburghii* wine. Three reasons might explain this phenomenon: first, the inhibition

Table 4: The odour activity values (OAVs) for main compounds of *R. roxburghii* wines fermentation with different yeasts.

Number	Volatile compounds	Odour descriptor	Odour threshold (mg/L)	OAV		
				<i>Saccharomyces cerevisiae</i> X16	<i>Wickerhamomyces anomalus</i> C4	Mixture
A1	Hexanoic acid	Cheese, fatty	3	0.91	1.18	0.81
A2	Octanoic acid	Fatty, rancid	10	2.01	2.32	1.95
A3	n-Decanoic acid	Fatty, rancid	6	3.66	3.45	1.45
B1	Isobutanol	Alcohol, solvent	75	0.12	0.23	0.22
B2	Linalool	Citrus, floral, sweet, grape-like	0.02	/	41.5	37
B3	Phenylethyl alcohol	Rose, honey	10	6.61	6.04	8.17
B4	1-Hexanol	Herbaceous, wood	1.10	/	8.07	6.74
B5	1-Decanol	Floral, fatty	0.40	8.33	/	4.35
B6	Isoamyl alcohol	Nail Polish, alcohol	60	/	2.62	3.14
B7	1-Octanol	Orange, rose, sweet herb	0.80	/	/	1.66
C1	Hexyl acetate	Fruity	0.67	/	12.66	14.46
C2	Isobutyl acetate	Sweet, fruity, apple, banana	1.60	0.98	1.24	1.66
C3	Isoamyl acetate	Banana, fruity, sweet	0.16	382.31	543.81	570.18
C4	Phenethyl acetate	Floral	1.80	7.63	5.91	9.06
C5	Ethyl acetate	Pineapple, fruity, solvent, balsamic	12	/	2.82	2.54
C6	Ethyl butyrate	Fruity	0.08	27.8	27.1	33.2
C7	Ethyl hexanoate	Fruity, green apple, banana, brandy, wine-like	0.08	516.63	668.5	592
C8	Ethyl octanoate	Sweet, floral, fruity, banana, pear, brandy	0.58	837.62	1054.66	932.41
C9	Ethyl decanoate	Brandy, fruity, grape	0.50	954.98	732.26	897.36
C10	Ethyl laurate	Fruity, fatty	3.50	8.43	7.46	8.15
C11	Ethyl cinnamate	Fruity	0.01	612	804	751
C12	Ethyl 9-decenoate	Roses	0.10	/	34.5	107.90
C13	Ethyl isobutyrate	Fruity	0.06	/	/	45.33
D1	Dimethyl sulphide	Blackcurrant, asparagus	0.03	54.67	285	41.33
D2	Acetaldehyde	Pungent	0.50	/	3.54	7.86

of β -D-glucosidase by sugars in fruit juices was common. The experimental result suggested that β -glucosidase activity from *W. anomalus* C4 was strongly inhibited by 5% w/v glucose and the relative activity remained around 50% (Figure 4C). Second, the β -glucosidase activity from *W. anomalus* C4 was only 1.65 times that of *S. cerevisiae* X16, so the ability of β -D-glucosidase of *W. anomalus* C4 to hydrolyse glucosidic bonds was not significantly enhanced. Genetic engineering or mutation breeding methods may be considered in the future to enhance the β -D-glucosidase hydrolysis ability of *W. anomalus* C4. Third, the β -D-glucosidase location of *W. anomalus* C4 remained unclear.

W. anomalus is widespread in nature and has been isolated from different environments [16]. *W. anomalus*

exhibits interesting and potentially exploitable physiological and metabolic characteristics and is of considerable importance due to its association with winemaking [17]. It was reported that *W. anomalus* is a good producer of acetate esters [18]. In this study, no ethyl acetate was detected in *S. cerevisiae* X16 fermented *R. roxburghii* wine, and 33.82 ± 1.10 mg/L and 30.52 ± 2.97 mg/L of ethyl acetate were measured in pure *W. anomalus* C4 or mixture-fermented *R. roxburghii* wine, respectively. Besides, *W. anomalus* was also a good producer of fruity acetate esters and other volatiles that exerted a positive effect on wine aroma [19, 20]. Here, the concentrations of isoamyl acetate, ethyl hexanoate, ethyl cinnamate, and ethyl octanoate founded in *W. anomalus* C4 fermented

R. roxburghii wine were higher than that of *S. cerevisiae* X16 control (Table 3).

In addition, the native yeast *W. anomalus* C4 may have a better ability to utilise sugar than commercial *S. cerevisiae* X16 originally obtained from grapes with a lower sugar content and higher ethanol content of *R. roxburghii* wine fermented with *W. anomalus* C4 (Table 2). Also, *W. anomalus* C4 could reduce *R. roxburghii* wine acidity (Table 2; Figure 6).

In conclusion, the indigenous yeasts that produced β -D-glucosidase were screened from *R. roxburghii* fruit, and the strain with the highest β -D-glucosidase activities was identified and studied. Morphology and molecular biology data suggested that the isolate C4 was a strain of *W. anomalus*. The optimum temperature and pH for β -D-glucosidase from *W. anomalus* C4 were 40 °C and 5.0 respectively. In addition, glucose, 15% v/v ethanol and Cu^{2+} inhibited β -glycosidase activity. *W. anomalus* C4 could enhance volatile aroma richness and the complexity of *R. roxburghii* wines including aroma types and content. Taken together, the *R. roxburghii* native yeast *W. anomalus* C4 may have potential for use in *R. roxburghii* wine-making.

Acknowledgments: This work were supported by the Science and Technology Program of Guizhou Province (Talents of Guizhou Science Cooperation Platform [2017]5789, [2018] 5603), Innovation Group Research Project from Guizhou Provincial Education Department (KY 2017046), and High-level Talent Research Funding Project of Guizhou Institute of Technology (XJGC20190625).

Author contribution: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: None declared.

Conflict of interest statement: None declared.

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