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Isolation of aromatic yeasts(non-*Saccharomyces cerevisiae*) from Korean traditional *nuruks* and identification of fermentation characteristics

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ABSTRACT

Ethyl caproate and isoamyl alcohol are important for the quality of *Yakju,* one of the Korean traditional alcoholic beverages. From Korean traditional fermentation agent, *Nuruk*, we have isolated 8 yeasts which produced rich aromatic compounds using YEPD agar plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) containing 50 uL cerulenin at 30℃. The isolated aromatic yeasts are identified as *Pichia anomala* (4 strains), *Pichia fabianii* (2 strains), *Pichia farinose* (1 strains), *Geotrichum candidum* (1 strains). We conducted alcohol fermentation with each of the aromatic yeasts and the compounds (ethyl caproate and isoamyl alcohol) producing range were 59.5 - 193.2 ppm and 10.8 - 91.6 ppm respectively. As a control, Fermivin®, famous aromatic wine yeast, was 89.4 ppm and 16.2 ppm respectively. We also find that the isolated *Pichia anomala* could produce higher level ethanol (4.2% - 5.0%, v/v) than other species (0.4% - 2.2%, v/v). Using the aromatic yeasts in fermentation industries, we expect to improve the quality of traditional alcoholic beverages.

Keywords: Aromatic Yeast; Pichia sp.; Yakju; Korean Traditional Alcoholic Beverage; Ethyl Caproate

1. INTRODUCTION

Many Korean alcoholic beverages are made using *nuruk*, a traditional Korean fermentation agent, which enables the hydrolysis of the raw materials and is known to produce richly flavored components in addition to alcohol, giving the final product a pleasant taste [7]. During the fermentation process, the yeast cells create a number of spectrums of volatile molecules, such as esters, organic acids, aldehydes, higher alcohols and carbonyl compounds offering sensory characteristics for the beverages [5]. The representative components of flavors in traditional Korean alcoholic beverages are ethyl caproate, producing an apple like flavor, isoamyl alcohol (i-AmOH), exhibiting a banana like flavor and 2-phenyl alcohol, which provides for a rose like essence; all compounds which normally originate from alcohol fermentation yeasts [2]. We aimed to screen for highly productive aromatic compound strains with high levels of ethyl caproate (primarily), i-AmOH and 2-phenylalcohol, amongst others. Finding out the potential of the isolated yeast for use in the brewing of *yakju*, a variety of traditional Korean alcoholic beverage, was also an important objective. Thus, we embarked on the isolation and identification of 76 wild yeast strains from traditional Korean *nuruk*s. We then screened 24 strongly cerulenin-resistant wild yeasts using a cerulenin medium. As a second screening, the cerulenin-resistant yeasts were subjected to an olfactory evaluation to eliminate those which, despite being strongly cerulenin-resistant, emitted an unpleasant odor. We finally isolated 8 strains of aromatic wild yeasts, which also exhibited strong cerulenin resistance. The isolates were 4 strains of *Pichia anomala* (AY1, AY4, AY41, AY56), 2 strains of *Pichia fabianii* (AY42, AY47), *Pichia farinosa* AY5 and *Geotrichum candidum* AY6. *Pichia fabianii* AY47 was seen to produce a large amount of ethyl caproate in this study. In research with the traditional Japanese drink of *sake*, the highest ethyl caproate producing curulenin-resistant mutant of *Saccharomyces cerevisiae* produced levels 5 times (7.4 ppm) that of the parental strain [6]. By applying these non-*Saccharomyces* yeasts to fermentation processes, we expect the quality of traditional Korean alcoholic beverages to be notably improved.

2. MATERIALS AND METHODS

2.1. Application of Aromatic Yeasts for Brewing

For the fermentation of *yakju*, each 1 kg of non-glutinous rice was washed 10 times and soaked in clean, drinkable tap water for 12 hours and then hourhalf-dried for 2 hours. Subsequently, the rice was finely powdered using a roll-mill (Dong-Gwang Inc., Daegu, Gyeongbuk, Korea). 1.2 L of boiling water was added to the rice flour (120% water to raw starch material, v/w) and gently stirred until the mixture cooled down to approximately 30℃. Then 2 g of fermentation agent (sp 15,000, Chungmubalhyo, Ulsan, Gyeongnam, Korea) was added for the hydrolysis of the rice flour and the 8 isolated and 3 commercial yeasts, which were adjusted to 10^6/mL, inoculated into the mixtures in quantities of 2 mL, respectively. The mixture was fermented at 20℃ for 7 days. All fermentations were performed in triplicate.

2.2. Flavor Compound Analyses of Fermented Alcoholic Beverages

The flavor compounds of each brewed sample were analyzed by GC (GC 2010, Shimadzu Co., Kyoto, Japan). The flavor compounds of each 1 μL cultured sample were analyzed by GC (GC 2010, Shimadzu Co., Kyoto, Japan). The column employed was a HP-INNOWAX capillary column (60 m × 0.25 mm id × 0.25 μM film thickness, J & W Scientific, Folsom, CA, USA). The carrier gas was nitrogen and the flow rate was 22.0 cm/sec (linear velocity). The column's oven temperature was held at 45℃ for 5 min and then set to rise to 100℃ at a rate of 5℃ /min and sustained at 100℃ for 5 min. Subsequently the column's oven temperature was increased to 200˚C at a rate of 10℃ /min and sustained for 10 min. Each sample injection volume was 1 μL and the split ratio was 50 : 1. The injector temperature and detector (FID) temperature were sustained at 250℃.

2.3. Physico-Chemical Analyses of Fermented Alcoholic Beverages

The pH was measured with a pH meter (Metrohm 691, Metrohm, Harisau, Switzerland). Total acid content was measured as lactic acid concentration and was determined by adding two to three drops of mixed indicator (0.2 g of bromothymol blue and 0.1 g of neutral red in 300 mL of 95% ethanol) to each 10 mL sample and then titrated with 0.1 N NaOH until the solution turned light green. Phthalic acid (Sigma-Aldrich, St. Louis, Mo, USA) was used as the standard [12]. Amino acidity was determined by adding two to three drops of phenolphthalein to each 10 mL sample and then neutralized with 0.1 N NaOH until the solution turned light red. Amino acidity was measured by adding 5 mL of neutral formalin to the sample and then measuring the amount of 0.1 N NaOH consumed to turn the solution light red [12]. The concentration of soluble solids was measured with a hand refractometer (PR101; ATAGO Co. Ltd., Tokyo, Japan) and expressed in Brix units (% sucrose). Reducing sugar concentration was analyzed by the DNS (dinitrosalicylic acid) method with a UV/VIS spectrophotometer (JP/U-2000, Hitachi, Ltd., Tokyo, Japan), with the glucose standard procured from Sigma-Aldrich (St. Louis, MO, USA) [11]. Alcohol content was measured using a gravimeter after the 100 mL of alcoholic beverage sample was distilled and then adjusted to 15℃ [13].

2.4. Sensory Evaluation of Fermented Alcoholic Beverages

7 experienced panelists from our Fermented Food Science Division evaluated the 11 resulting alcoholic beverages for taste on the basis of nine-point hedonic scales (1 = dislike extremely, or extremely weak; 9 = like extremely, or extremely strong). The experiment was conducted in triplicate and the mean values and standard deviation were calculated from the data obtained from the three separate experiments. These data were then compared using Duncan's multiple range method.

3. RESULTS AND DISCUSSION

3.1. Application of Aromatic Yeasts for Brewing

The 8 isolated yeasts and 3 commercial yeasts, which were adjusted to 10^6/ml and inoculated at 2 ml into the *yakju*-making mixtures (approximately 2.2 kg respectively). The adding ratio of water to this mixture was 120% of the quantity of rice (v/w). The mixture was fermented at 20℃ for 7 days. The physicochemical characteristics of the *yakju* after fermentation with the 8 isolates is shown in table 5. The overall pH range of the *yakju* was between 3.1 - 3.4. This latter result is seen to be quite low when compared to *makgeolli*'s pH when fermented using *S. cerevisiae* (the range was 3.5 - 4.2) and similar to *makgeolli* fermented with *P. anomala* (the range was 3.3 - 3.4) in Kim *et al*'s study [9]. In terms of alcohol production capabilities, the commercial yeasts of *Saccharomyces cerevisiae* (AY36, AY38 and AY39) produced 14.97% - 15.87% alcohol (v/v), while *Pichia anomala* (AY1 and AY4) produced 4.57% - 5.00% (v/v), and other isolated yeasts produced between 0.43%-2.17% (v/v). From Kim *et al*'s study [9] it does seem possible that *P. anomala*, well as *S. cerevisiae*, can produce high concen- trations of alcohol. In the latter study when using *P.anomala* Y197-13 and *koji*, the alcohol content of the resulting *makgeolli* was found to be 11.1% (the water adding ratio was 186% of the raw starch material used). Thus it may well be possible to raise the alcohol levels produced by *P. anomala* (AY1 and AY4) by controlling the fermentation environment. Alternatively, *Pichia anomala* (AY1 and AY4) could be employed in the production of alcoholic beverages with lower alcohol contents. In relation to levels of soluble solids, it was observed that, for the most part, the isolated yeasts had higher levels of soluble solids (26.43 - 21.17 brix) than the commercial yeasts (14.30 - 13.13 brix). Due to the fact that the isolated yeasts' alcohol production ability is lower than the commercial yeasts', the remaining sugar is likely to have brought about the higher levels of soluble solid content. Likewise, in relation to reducing sugar, the isolated yeasts were mostly seen to have higher levels (2.22% - 9.98%, w/v) than the commercial yeasts (1.34% - 1.93%, w/v). The probable reason for this is also likely to be the same as with the case of soluble solids. It is possible that the sugar-acid ratio will have a great effect on the overall taste of the *yakju*. In champagne wine, the sweetness contributes to a good perception of its taste. This sweetness results from high concentrations of sugar in the fermentation mixture used (20.5 g/l sugar - 4.22 g/l tartaric acid) [20]. Further study is needed to discover an appropriate sugar-acid ratio in order to provide for tastier *yakju*.

3.2. Flavor Compound Analyses of Fermented Alcoholic Beverages

For decades, so-called non-*Saccharomyces* yeasts (such as *Pichia*, *Candida*, *Hanseniaspora*, *Hansenula* etc.) have been reported to be producers of high concentrations of fermentation compounds, such as acetic acid, esters and acetoin. The effects of these latter compounds on the sensory qualities of wines have been similar to the role of the AATase of *S. cerevisiae* in the production of acetate esters, such as ethyl acetate. Ethyl acetate exhibits a solvent like-odor and AY42 possessed the lowest amount of this compound, 49.9 ± 8.6 mg/l – well below the 160 mg/l odor threshold in case of wine [1]. In the Japanese drink *sake*, *Saccharomyces cerevisiae* produced ethyl acetate in the range of 50 - 100 ppm (160% of water to raw starch material v/w) as analyzed by quantitative traits loci (QTL) [8]. In this study the *yakju* which used AY4 was seen to be within the latter range even though the water ratio differed (120% of water to raw starch material v/w, **Table 2**). Isoamyl acetate (i-AmOAc), which emits a banana-like aroma, is synthesized from isoamyl alcohol and acetyl coenzyme A and is catalyzed by alcohol acetyltransferase (AATase, EC 2.3.1.84) [4]. i-AmOH and i-AmOAc are usually within the ranges of 190 - 350 ppm and 8.0 - 19.3 ppm, respectively, in *sake* (160% of water to raw starch material v/w) [2,8]. Further studies in relation to the E/A ratio of *yakju* are needed. Ethyl caproate, with an apple like aroma, is synthesized by the enzymes esterase and alcohol acyltransferase in yeast, with the substrates being ethanol and caproic acid or caproyl-CoA [6]. In this study, in most of the *yakjus* ethyl caproate levels were higher than in *sake* (55.0 - 176.6 ppm) and wine. AY47 (176.6 ppm) and AY56 (170.1 ppm) were seen to be higher than those for the other treatments, and the commercial yeasts AY36, AY38 and AY39 were significantly lower (11.4 -19.1 ppm). There have been several reports where non-*Saccharomyces* yeasts have been described as producers of high concentrations of some fermentation compounds such as esters and acetic acid, which influence the sensory quality of wines [14]. In this study, the results further support these reports. Generally in this study, the isolated yeasts produced between 3.4 - 24.3 ppm of 2-phenylethyl alcohol. Of the commercial yeasts, AY38 produced the highest concentration of the compound at 39.4 ppm. In wines 2-phenylethyl alcohol's concentration is generally between 53 - 82 ppm [1,14] and in *Agave tequilana* it is 3.3 - 26.6 ppm [3]. *Pichia* sp. produced more 2-phenyl alcohol (up to 18%) when sucrose was used as a carbon source, and when phenylalanine (up to 0.1%) was added to the medium. Hence, further study is required to see how production levels are influenced by modifying the compositions of the media.

3.3. Sensory Evaluation of the Brewed Yakju

The 8 isolated wild yeasts and 3 commercial yeasts were used for the brewing of *yakju*. Following the completion of the brewing process the resulting concoctions were subjected to sensory evaluations (**Table 3**). AY6 *yakju* was strongest among the strains in terms of sweetness at 6.2 ± 0.9, and AY6 *yakju's* soluble solid and reducing sugar content also belonged to the highest group as seen with Duncan's multiple range test in **Table 5**. AY42 *yakju* was rated to be the strongest in terms of sourness among the strains, at 6.5 ± 0.8. In color, AY36 *yakju* received the highest score, at 7.4 ± 0.9, with a clean green-yellow color. For flavor, AY6 and AY42 *yakjus* surpassed other treatments in terms of possessing a fruity aroma. It is certain that the quantity of the aromatic compounds is not important. Instead what is important is having the right proportions of the compounds (ethyl caproate, isoamyl alcohol, ethyl acetate, 2-phenyl alcohol etc.) so as to produce a pleasant flavor [10].

AY42 *yakju*, we surmise, had the best overall flavor out of all the *yakjus* tested, when considering GC data (**Table 2**). In terms of taste and overall preference, the *yakjus* with the highest soluble solid and reducing sugar content received the highest scores. However, AY42, which scored the highest in overall preference, had a high concentration of soluble solids, but a comparatively low concentration of total acid content (**Table 1**). Commercial yeasts. AY36, AY38 and AY39 received relatively low scores in overall preference because of the comparatively higher concentrations of ethanol, and the panels gave their opinion that the high ethanol gave the *yakju* a bitter taste. Meanwhile, AY4 has high potential

Table 1. Physicochemical characteristics of the low alcoholic beverages using isolated yeasts.

Yeasts	**pH**	**Total acid (lactic acid, %, w/v)**	**Amino acidity (mL)**	**Alcohol (%, v/v)**	**Soluble solids (% sucrose)**	**Reducing sugar (%, w/v)**
AY1	3.12 ± 0.20^{f}	1.20 ± 0.10^{ab}	5.27 ± 0.84^{c}	4.57 ± 1.97^{b}	22.27 ± 2.31^{bc}	7.99 ± 0.96^{b}
AY4	3.32 ± 0.01^{abc}	1.20 ± 0.05^{ab}	5.34 ± 0.31^{c}	5.00 ± 1.23^{b}	21.17 ± 1.45^{c}	8.41 ± 1.34^{b}
AY5	3.28 ± 0.08^{bcd}	1.17 ± 0.05^{abc}	6.16 ± 0.46^{ab}	2.17 ± 1.59^{c}	24.37 ± 1.72^{ab}	8.48 ± 0.22^{b}
AY6	3.37 ± 0.03^{abc}	1.09 ± 0.03^{bcde}	6.21 ± 0.42^{ab}	0.97 ± 0.74^{c}	25.03 ± 0.72^{a}	8.83 ± 0.27^{ab}
AY36	3.40 ± 0.05^{ab}	0.77 ± 0.03^{g}	4.34 ± 0.07^{d}	15.87 ± 0.06^{a}	13.50 ± 0.26^{d}	1.36 ± 0.11^{c}
AY38	3.45 ± 0.02^{a}	0.75 ± 0.04^{g}	3.43 ± 0.09^{e}	15.40 ± 0.20^{a}	14.30 ± 0.40^{d}	1.93 ± 0.27^{c}
AY39	3.32 ± 0.04^{abc}	0.91 ± 0.04^{f}	3.63 ± 0.04^{e}	14.97 ± 0.40^{a}	13.13 ± 0.65^{d}	1.34 ± 0.50^{c}
AY41	3.23 ± 0.06^{cde}	1.14 ± 0.08^{abcd}	5.67 ± 0.69^{bc}	1.00 ± 0.36^{c}	24.40 ± 2.94^{ab}	8.25 ± 0.45^{b}
AY42	3.24 ± 0.13^{cde}	1.06 ± 0.08^{cde}	6.10 ± 0.13^{ab}	0.47 ± 0.42^{c}	25.53 ± 0.97^{a}	8.11 ± 0.66^{b}
AY47	3.30 ±0.08^{abcd}	1.00 ± 0.11^{ef}	6.41 ± 0.09^{a}	0.43 ± 0.06^{c}	26.43 ± 0.31^{a}	7.85 ± 1.31^{b}
AY56	3.28 ± 0.10^{bcd}	1.04 ± 0.05^{de}	6.40 ± 0.30^{a}	0.77 ± 0.15^{c}	25.30 ± 1.20^{a}	9.98 ± 0.39^{a}

Values are mean ±SD. $^{a-d}$ in a column by different superscripts are significantly different at the $p < 0.05$ by Duncan's multiple range test (n = 3). Total acid was converted to lactic acid (consumed ml of 0.1N NaoH × 0.09). AY 36, AY38 and AY39 are commercial yeasts.

Table 2. Flavor compounds analysis of the fermented alcoholic beverages expressed as mg/l.

Yeast No.	Acet aldehyde	EtOAc	Iso butanol	i-AmOH	Ethyl caprylate	Acetic acid	Furfral	Linalool	Butyrate	Ethyl caproate	2-Phenyl alcohol
AY1	1458.7±54.4^{abcd}	293.6±32.6^{a}	-	8.5±1.8^{cd}	74.7±38.6^{bc}	4801.6±298.0^{a}	342.2±60.7^{a}	148.3±18.2^{ab}	11.7±4.0^{cde}	55.0±5.8^{c}	6.6±2.1^{cde}
AY4	1250.1±196.6^{cde}	130.3±53.3^{b}	-	7.4±3.0^{cd}	90.6±2.5^{b}	1213.2±114.2^{g}	79.2±23.3^{d}	131.2±22.9^{ab}	8.8±1.7^{cde}	66.0 ± 2.6^{c}	9.4±7.2^{cde}
AY5	1567.0±157.2^{ab}	-	-	12.2±2.1^{bcd}	132.4±30.2^{a}	4028.4±161.5^{b}	68.9±5.6	148.4±23.8^{ab}	15.7±11.7^{bcde}	61.4 ±17.1^{c}	24.3±11.6^{b}
AY6	1633.4±94.6^{a}	-	-	6.97±1.3^{d}	31.5±3.8^{de}	3404.6±230.6^{bcd}	232.3±79.3^{b}	143.7±49.0^{ab}	6.1±0.8^{e}	69.3±12.6^{c}	4.7±1.5^{de}
AY36	1281.2±82.9b^{cde}	-	-	-	76.5±10.4^{bc}	3180.4±124.8^{cd}	40.5±4.6^{d}	30.7±2.4^{cd}	7.3±0.9d^{e}	12.3±1.1^{d}	4.9±0.4^{de}
AY38	1541.9±146.1^{abc}	-	-	15.8±2.3^{ab}	25.1±2.54^{de}	2722.7±212.8^{de}	37.2±3.8^{d}	22.5±2.2^{d}	7.2±0.8d^{e}	11.4±0.6^{d}	39.4±6.9^{a}
AY39	1257.1±157.8^{cde}	3.5±1.2^{d}	5.3±0.3^{a}	11.2±2.9^{bcd}	-	2351.5±132.0^{ef}	-	2.2±0.2^{a}	24.3±2.2^{b}	19.1±3.4^{d}	6.4±0.3^{cde}
AY41	1444.2±83.7^{abcd}	67.4±13.5^{c}	3.3±0.9^{ab}	14.2±6.2^{bc}	-	3718.7±478.7	196.7±10.3^{bc}	146.8±22.8^{ab}	24.7±5.6^{b}	59.0±7.6^{c}	14.2±3.0^{c}
AY42	1504.8±182.1^{abc}	49.9±8.6^{c}	-	7.7±1.7^{cd}	65.3±9.7^{bc}	3026.6±373.4^{cde}	149.3±19.5^{c}	67.5±17.4^{c}	17.0±7.8^{bcd}	99.4±16.5^{b}	5.6±0.7^{de}
AY47	1386.6±97.4^{abcd}	82.5 ± 6.^{6c}	-	6.7±1.7^{d}	55.4±3.1^{cd}	3452.1±192.8^{bcd}	62.0 ± 19.4^{d}	152.6±25.1^{a}	7.6±2.4^{de}	176.6±17.4^{a}	6.9±1.7^{cde}
AY56	1398.7±38.4^{abcd}	-	-	8.1±1.5^{cd}	1.5±0.3^{e}	1851.1±173.2^{fg}	68.7 ± 12.0^{d}	125.1±17.8^{ab}	53.4±5.1^{a}	170.1±11.6^{a}	3.4±0.5^{e}

Values are mean ±SD. $^{a-e}$ in a column by different superscripts are significantly different at the $p < 0.05$ by Duncan's multiple range test (n = 3). AY36, AY38 and AY39 are commercial yeasts.

Table 3. Sensory evaluation of the brewed low alcoholic beverages.

Strain	Sweetness	Sourness	Color	Flavor	Taste	Overall preference
AY1	4.7±1.0^{cde}	6.3±1.9^{ab}	4.5±1.4^{de}	4.0±2.3^{c}	4.1±1.8^{cd}	3.9±1.9^{de}
AY4	5.0±1.1^{bcd}	6.1±1.8^{ab}	6.2±2.1^{abc}	5.4±1.5^{ab}	4.9±1.6^{abcd}	5.2±1.6^{abc}
AY5	5.0±1.6^{bcd}	5.6±1.9^{ab}	4.5±1.5^{de}	4.0±2.5^{c}	3.9±1.9^{d}	3.4±2.1^{e}
AY6	6.2±0.9^{a}	6.3±1.3^{ab}	5.3±2.0^{cde}	6.0±1.9^{a}	5.6±1.6^{ab}	5.7±1.7^{ab}
AY36	4.3±2.2^{de}	5.2±2.1^{b}	7.4±0.9^{a}	5.3±1.9^{abc}	4.5±2.1^{bcd}	4.5±2.2^{bcde}
AY38	4.3±2.0^{de}	5.5±1.9^{ab}	6.7±1.4^{ab}	5.7±1.8^{ab}	4.7±2.1^{abcd}	4.8±2.2^{bcd}
AY39	3.9±1.6^{e}	5.4±1.9^{ab}	6.7±1.1^{ab}	5.1±1.8^{abc}	4.0±1.9^{cd}	4.0±1.8^{cde}
AY41	5.4±1.4^{abc}	6.2±1.4^{ab}	5.9±2.2^{bc}	5.4±1.4^{ab}	5.3±1.6^{abc}	5.4±1.6^{ab}
AY42	5.9±1.1^{ab}	6.5±0.8^{a}	5.5±2.0^{bcd}	6.4±1.4^{a}	5.9±1.5^{a}	6.1±1.5^{a}
AY47	5.2±1.0^{bcd}	5.7±1.6^{ab}	4.2±1.3^{e}	4.4±2.1^{bc}	5.1±1.8^{abcd}	4.7±1.9^{bcde}
AY56	5.7±1.0^{abc}	6.2±1.5^{ab}	5.3±1.8^{cde}	5.3±1.7^{abc}	5.4±1.4^{ab}	5.5±1.2^{ab}

Values are mean ±SD. $^{a-e}$ in a column by different superscripts are significantly different at the $p<0.05$ by Duncan's multiple range test (n = 3). AY36, AY38 and AY39 are commercial yeasts.

for use in a sweet-*yakju* because of its alcohol production ability and AY6, AY41 and AY56 have also great potential for use to create fruity beverages. AY42, we suggest, have the best flavor composition among the isolates for the production of good quality of *yakju*, when considering the GC analyses and sensory evaluations.

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