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Yeast from *Pyrus ussuriensis* Maxim Fruit Peel

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From *Pyrus ussuriensis* Maxim fruit peel, we obtained a yeast strain (number 2014103005) with high alcohol tolerance. The strain was determined as *Pichia kudriavzevii* (primitive name: *Issatchenkia orientalis*,can be generating typicality fragrance) and showed the following properties: optimum pH, 5~7; glucose tolerance, >10%; and alcohol resistance, >10%. This strain can resist more than 240 mg/L SO_2. Its logarithmic growth phase starts at 4 h, whereas its stable growth phase starts at16 h. The fermentation ability is superior to that of Angel yeast (*Saccharomyces cerevisiae*). *P. kudriavzevii* could be used for fruit wine fermentation.

Keywords: *Pyrus ussuriensis* Maxim; yeast; fermentation.

Pyrus ussuriensis Maxim is known as Persian pear, a unique kind of pear. The fruit is bright yellow and tastes slightly sour in autumn. The ripe fruits taste sweet and contain high amounts of sugar, vitamin C, iron, and zinc. When harvested at mature stage, the fruits must be consumed immediately. During winter, the fruit is cryopreserved. When defrosted, the flesh appears brown in color contains a high volume of juice, is nutrient rich, and is eaten similar to gambier. This fruit is beneficial to the lungs as it exhibits anti-tussive and anti-alcohol properties. The process of removing ice during defrosting is called "sweating." *P. ussuriensis* Maxim cold storage is done in the autumn and winter, after which repeated ice removal induces changes in the fruit's component. The change in the taste of *P. ussuriensis* Maxim fruit from sour to sweet involves a complicated physiological process[1]. Nowadays, this fruit is one of the commodities in the market. When *P. ussuriensis* Maxim fruit matures, it should be transported in a timely manner; if not, the fruit should be kept in frozen storage. The fruit is still edible after freezing, but the frozen pulp appears brown. This discoloration would greatly reduce its visual appeal and limit the market sales. Therefore, developments in the industry and utilization of *P. ussuriensis* Maxim products are absolutely necessary.

In this experiment, we isolated yeast strains from the mature *P. ussuriensis* Maxim fruit. These yeast strains are excellent for fermenting fruit wine. The fermentation ability of these strains was analyzed, and the parameters for screening the strains suitable for were optimized. This research aims to establish *P. ussuriensis* Maxim fruit wine as a special germplasm of *Saccharomyces cerevisiae* and to optimize the parameters for fermentation, thereby providing technical reference for further studies on fruit wine fermentation.

MATERIALS AND METHODS

Experimental organisms

P. ussuriensis Maxim was obtained from a pear plantation in Guanqiao Township in Zhongnin City in Ningxia. A commercial yeast strain (Angel strain) was used in wine fermentation.

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Media

The PDA[2] medium consists of the filtrate obtained from 200 g peeled potatoes boiled for 0.5 h, 20 g of glucose, and 20 g AGAR. These components were dissolved to a final volume of 1000 ml; The natural pH of the mixture was maintained.

The YEPD[3] medium consists of 20 g of peptone, 20 g of glucose, yeast extract, 10 g the capacity 1000 ml, and distilled water; The natural pH of the mixture was maintained.

The double resistance enrichment medium[3] consists of 1 ml of 100 mg/ml double resistance solution (penicillin and streptomycin) per 100 ml YEPD medium.

The composition of the TTC media[4] is as follows: The upper medium contains 0.05 g TTC (2,3,5-triphenyl four nitrogen chloride), 1.5 g AGAR, and 0.5 g of glucose.

The lower medium consists of 10.0 g of glucose, 1.5 g yeast extract, 1.5 g peptone, 0.4 g magnesium sulfate, 0.27 g citric acid, 1.0 g acid potassium phosphate, and 20.0 g agar.

The domesticated culture medium: YEPD medium with 10%012%014%016%(w/v) ethanol respectively; The natural pH of the mixture was maintained.

Equipment

The following devices were used in the experiment: LDZX-50KBS vertical pressure steam sterilizer, LRB-150 biochemical incubator, UV1000 ultraviolet spectrophotometer, FA2204B electronic balance, THZ-9511K double thermostatic oscillator, 5415D centrifuge, VORTEX-5 vortex, FE20 pH meter, and SW-CJ-1F clean bench.

METHODS

Yeast separation

In a sterile environment, the peelings of *P. ussuriensis* Maxim fruit were cut using a sterile knife and placed in the double resistance enrichment medium. The peels were cultured at 28 °C with shaking at 100 r/min for 24 h. A 1 ml volume of the fermented liquid (diluted to 10^{-5} concentration) was coated in PDA medium and then cultivated at 28 °C for 48 h. According to the yeast preliminary isolated from yeast cells morphological characteristics, Vaccination cant in PDA medium, 28 °C cultivate colony generated 48 h, back up at 4 °C refrigerator[5].

Yeast screening

TTC method

The yeast was isolated using the TTC chromogenic method. Isolated yeasts were screened for their strong ability to produce alcohol during primary screening. The isolated yeast was coated onto the lower TTC plate. The plate was inverted, and the strain was cultivated at 28 °C for 48 h. The upper TTC medium (12 ml) was added, thereby covering and preventing the original colonies from receiving light for 2–3 h at 28 °C. According to the color of colony, a preliminary judgment of the yeast's capacity to produce alcohol was performed, thereby isolating bright red yeast strains[4].

Duchenne tube method

Ethanol (8%, 10%, 12%, 14%, 16%, and 18% (v/v)) was added into a test tube containing YEPD liquid medium. SO_2 (60, 120, 180, and 240 mg/L) was also separately added into the YEPD liquid medium with the Duchenne tubules. High temperature sterilization and cooling to 30 °C were performed in an ultra-clean bench in anhydrous ethanol. According to the amount of yeast, the inoculation concentration was 107 cfu/ml, and culture was done at 28 °C for 48 h. We observed the Duchenne tubules for the generation of air bubbles. Through the bubble volume, we obtained the yeast strains for ethanol generation and the degree of SO_2 tolerance. Further studies revealed suitable strains for fruit wine brewing[6,7]. Three parallel experimental setup.

Determination of CO_2 weightlessness

In a 100 ml fermentation bottle (dried and weighed), we added 50 ml YEPD medium at 121 °C. Sterilization was performed for 20 min. After cooling to 30 °C, we inoculated the strains after the activation of the screening. We selected three bottles of each species (Gasser and weighed). In the process of fermentation and timely weighing, we recorded the data, calculated the release rate of CO_2. CO_2 weightlessness value was used to judge the yeast's fermenting power[8].

The ITS sequence determination strain

In the strains genomic DNA preparation of reference method, 26 rDNA areas were PCR amplified, and sequence determination was adopted by using fungus ribosome universal primer NL1 rDNA (5'-GCA TAT CAA TAA GCG

GAG GAA AAG-3') and NL44(5'-GGT CCG TGT TTC AAG ACG G-3'). Primers synthesized from Shanghai Invitrogen Biotechnology Co. The PCR reaction system with reference method was used. The procedure was as follows: 94 °C pre-modified for 4 min and then into the circulation; 94 °C modified for 1 min; 55 °C annealing for 40 s; and 72 °C extension for 72 s. A total of 35 cycles were performed, and these cycles were maintained in 72 °C for 10 min. Amplification products were separated by 1% agarose gel electrophoresis. Target fragment was obtained after DNA gel recovery kit recycling and purification and was cloned into pMD19-T carrier. Positive clone sequencing was performed by Invitrogen biological engineering technology co., Ltd. Guangzhou branch.

Assessment of yeast performance

Determination of tolerance to sugar

The yeast strains were cultured at 28 °C for 48 h in YEPD liquid medium containing 6%, 8%, 10%, 12%, 14%, 16%, 18%, and 16% glucose. The amount of gas produced in the Duchenne tubes represents the sugar concentration. Three parallel experiments were performed[9].

Determination of the optimum pH for growth

The activated test bacterial culture solution (107 cfu/ml) was added into 50 ml YEPD liquid media with initial pH values of 4, 5, 6, 7, 8, and 9. After inoculation, the mixture was gently shaken at 150 r/min at 28 °C for 24 h. The OD value at 560 nm was obtained spectrophotometrically. Using the pH as the abscissa and the OD value of bacterial suspension as the ordinate, we obtained the growth curves of the test bacteria at various pH values[10].

Determination of alcohol resistance

Ethanol at concentration ratios of 8%, 10%, 12%, 14%, 16%, and 18% (v/v) was added into YEPD liquid medium in each test tube and then added into Duchenne tubules. High temperature sterilization and cooling to 30 °C was done in an ultraclean bench. Anhydrous ethanol was added. The amount of yeast (inoculation concentration) was 107cfu/ml. Culturing was conducted at 28 °C for 48 h. We observed the Duchenne tubules for the generation of air bubbles; we assessed the yeast's alcohol tolerance[6].

Determination of SO_2 resistance

Sulfurous acid (60, 120, 180, and 240 mg/L) was added to YEPD liquid medium contained in 10 ml test tubes, after which the culture was added into Duchenne tubes. The setup was sterilized at 121 °C for 20 min. Yeast (107 cfu/ml) was inoculated at 28 °C for 48 h. The volume of air in the Duchenne tubes represents the tolerance of the yeast strains to SO_2. Three parallel experiments were performed[7].

Determination of yeast growth curve

The selected yeast was inoculated in 50 ml YEPD liquid medium at optimum temperature and initial pH. The culture was shaken at 150 r/min at constant temperature, and samples were obtained every 2 h. The OD value was measured at 560 nm. With time as the abscissa and absorbance as the ordinate, the growth curve of the yeast was determined. Three parallel experiments were performed[11].

Comparison with the commercial yeast

P. ussuriensis Maxim juice (50 ml) was placed in an Erlenmeyer flask and sterilized at 121 °C for 20 min and then cooled to 30 °C. The commercial and the isolated yeasts were inoculated at 28 °C. Data on the weight of CO_2 were obtained at a 12 h interval. The fermentation power of the two strains was compared based on CO_2 weight loss. Three parallel experiments were performed.

RESULTS

Yeast screening and yeast strain isolation

A total of 88 strains exhibiting the characteristics of yeast were isolated from *P. ussuriensis* Maxim peel, from the orchard soil, and from naturally fermented pear wine. These strains were coated in PDA medium, cultivated for 48 h, and then stored at 4 °C.

TTC screening results

The TTC method revealed 6 scarlet-colored yeast strains, 6 deep red strains, 36 red strains, 26 pink strains, and 24 colorless strains (Table 1).

Duchenne tube method screening results

Six dark red yeast strains isolated through the TTC screening were used to determine alcohol resistance. Results show that strains 2014103005, 2014103003, and 2014100107 exhibited higher resistance to alcohol than other strains (Fig. 1). These bacterial strains are also resistant to SO_2, with 2014103005 being the most resistant (Fig. 2).

CO_2 weightlessness method screening results

In addition to Duchenne tube screening, the CO_2 weightlessness of the three yeast strains exhibiting high alcohol resistance was determined. The flask containing the culture was shaken until the CO_2 weighed less than 0.2 g. The results show that 2014103005 exhibited the least value for CO_2 weight (Fig. 3).

Sequence analysis

Strain 2014103005 was obtained with PCR amplification of the original peak Fig.. We cut off both ends after the sequence of poor quality. Using SeqMan joining together and use the BLAST analysis program in NCBI nucleic acid database online, we compared this strain's 26 srDNA sequences with that of the model strain *Pichia kudriavzevii* CBS5147 (Gen Bank U76347). Results of comparison showed that these strains are completely the same.

Yeast properties

Through the above methods and after comprehensive screening, we finally selected yeast strain 2014103005, which comprehensively had strong properties, for performance measurement.

Determination of tolerance to sugar

The Duchenne tube method was used to determine the resistance of the isolated yeast to sugar. The bubble volume revealed that the yeast tolerated a maximum of 10% glucose concentration. This sugar-tolerant yeast strain is thus suitable

Table 1. TTC test results

	Oxblood red	Red	Light red	Colorless
bacterial strain(a)	6	36	26	24

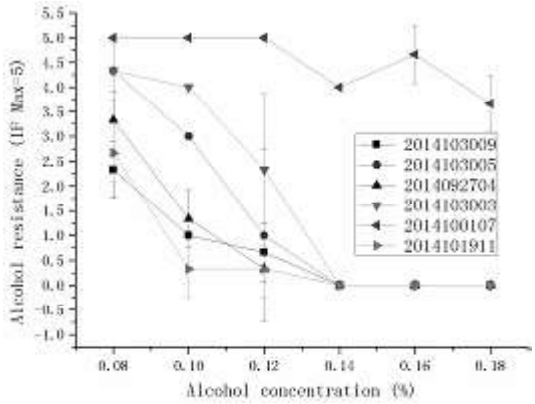

Fig. 1. Determination of alcohol alcohol resistance concentration

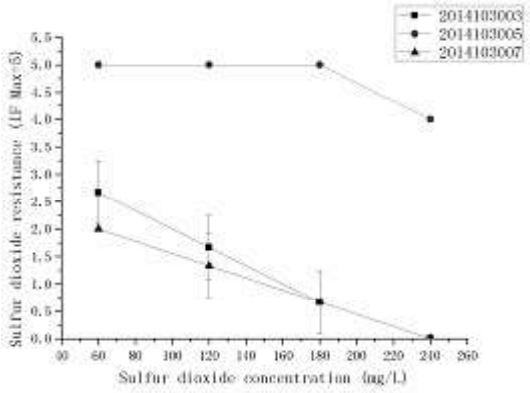

Fig. 2. Determination of sulfur dioxide resistance concentration

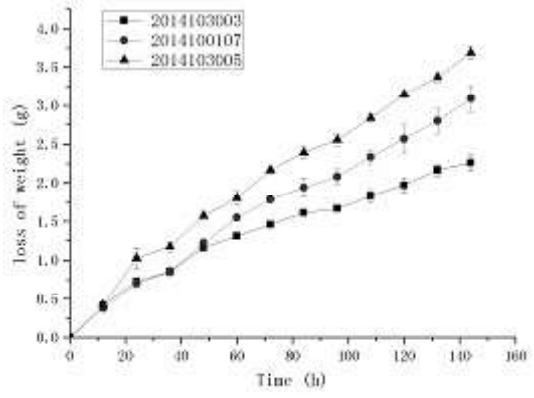

Fig. 3. Comparison of fermentation forces in weight loss

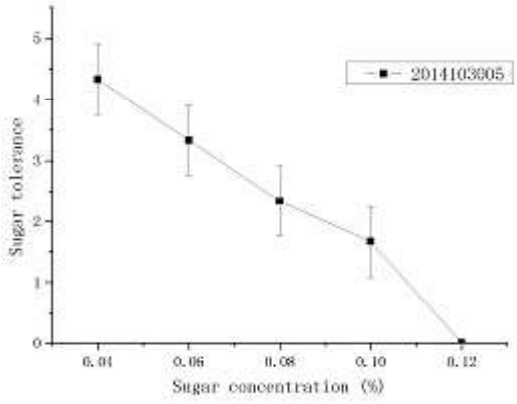

Fig. 4. Sugar tolerance test

for the fermentation of high sugar-containing fruits (Fig. 4).

Determination of the optimum pH for growth

The absorbance of strain 2014103005 at pH values of 5, 6, and 7 was not significantly different. Thus, the optimum growth of the yeast occurred at pH 5–7 (Fig. 5).

Determination of alcohol resistance

The 2014103005 strain can grow very well in YEPD medium containing 10% alcohol. However, this strain cannot grow at 12% alcohol concentration. Thus, maximum alcohol tolerance of this strain is approximately 10% (Fig. 6).

Determination of yeast SO_2 resistance

The bubble volume in the Duchenne tube revealed that the yeast can tolerate SO_2 concentrations of higher than 240 mg/L. The SO_2 content of fermented wine is commonly 100–200 mg/L (Fig. 7).

Determination of growth curve

Absorption photometry revealed that the density of bacteria rapidly increased within 4 h and then entered the logarithmic phase. After 16 h, the growth curve reached a plateau (Fig. 8). Considering that bacterial death affects spectrophotometry results, eliminating the interference was done at around 16 h into the stable growth phase.

The comparison of yeast with the product

Fig. 9 shows the 48 h culture of *P. ussuriensis* Maxim juice. Data showed that isolated yeast is superior over the commercial yeast in terms of cell vitality and the weight of evolved CO_2. The aroma of the wine produced by the isolated yeast fragrance is stronger than that produced by the commercial yeast. However, the wine produced by the latter is softer.

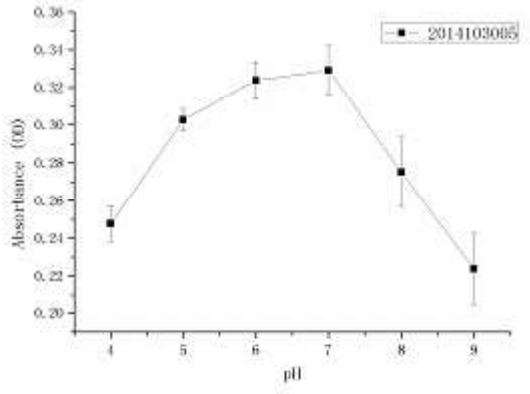

Fig. 5. pH determination

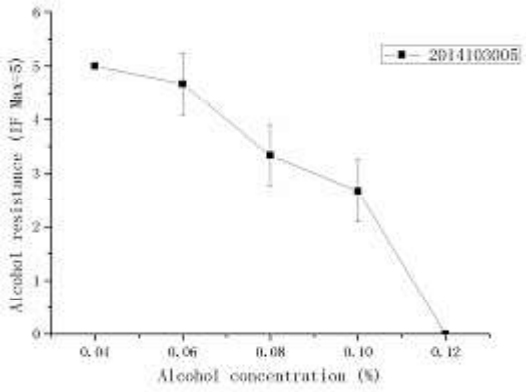

Fig. 6. Determination of resistance to alcohol

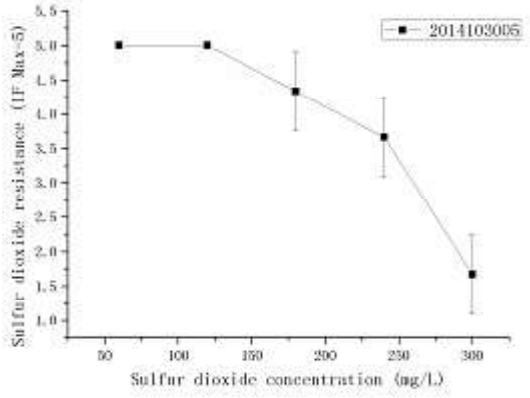

Fig. 7. Sulfur dioxide concentration

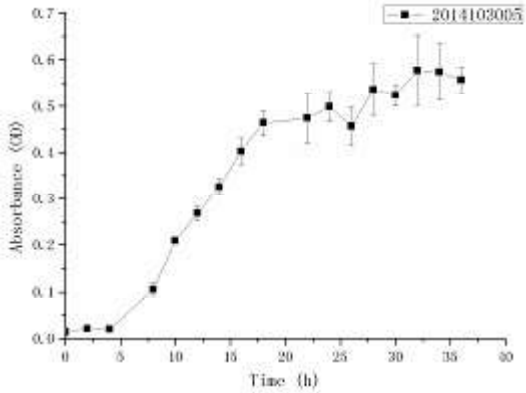

Fig. 8. Strain growth curve

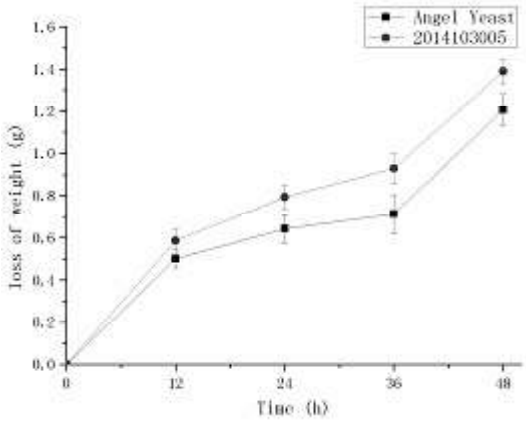

Fig. 9. Comparison of fermentation forces in CO_2 weight loss

DISCUSSION

In this experiment, yeast can be cultured from pear fruit. We obtained 6 yeast strains. By acid, alcohol, SO_2, and fermenting power selection, we obtained one yeast strain (2014103005) for fruit wine brewing. By sequencing, we identified strain 2014103005 as *P. kudriavzevii*. Through the experiment, we determined strain 2014103005's maximum glucose tolerance was >10%. Maximum alcohol tolerance was around 10%. Maximum SO_2 tolerance was >240 mg/L. The optimum growth pH was between 4 and 7. After 4 h, strain 2014103005 showed slow growth, accelerated upon approaching the logarithmic phase. After 16 h, the strain's growth started leveling off. Under natural fermentation conditions, the fermenting power of 2014103005 was slightly higher than that of the strain currently used for industrial production (Angel yeast). This study focused on screening the isolated yeast for fermentation ability. The presence of *P. kudriavzevii* is responsible for the rapid fermentation of wine made from *P. ussuriensis* Maxim fruit. In the fermentation process of *P. ussuriensis* Maxim typicality fragrance. We aimed to improve the quality of *P. ussuriensis* Maxim fruit wine.

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