CHAPTER II

MATERIALS AND METHODS

1. Isolation of yeasts from various foods

1.1 Materials

1.1.1 Samples from various foods

Fruits : White grape, Red grape, Jack-fruit, Lancet-fruit, Carambola, Sapota, Mango, Pineapple, Papaya, Coconut, Tamarind; Star-gooseberry.

Flowers : Jasmine, Jum-pee, Jum-pa, Rose, China-rose, Marigold, Doak Khem.

Juices : Cane juice, Palm juice, Coconut juice

Fermented foods : Kao-mark, Fermented soybean, Fermented bamboo shoot.

1.1.2 10 ml of sterile water in test tubes

1.1.3 YEPD slants (Appendix V 1.2)

1.1.4 YEPD plates containing 0.01 % chloramphenicol

1.2 Procedure

Yeasts were isolated from various food samples, i.e. fruits, flowers, juices and fermented foods. After collection, samples were diluted in sterile water and spreaded on YEPD plates containing 0.01 % chloramphenicol. The plates were incubated at 30 °C for 2 days. All different shape colonies of yeasts on the plates were treated as different species. These colonies were restreaked on new YEPD plates in order to obtain pure cultures. The isolates were maintained on YEPD slants.

2. Isolation of killer yeasts

2.1 Materials

2.1.1 Yeast strains

2.1.1.1 40 isolates from various foods (Table 1)

2.1.1.2 729 isolates from KU-NRIB joint research project.

2.1.1.3 109 strains from IFRPD stock culture

2.1.1.4 20 strains from Pradit collection

2.1.1.5 173 strains from Patoomporn collection

2.1.1.6 119 strains from Boontiem collection

2.1.1.7 Torulopsis glabrata IFO 0622 : The tester sensitive strain

2.1.2 Killer detect assay medium plates or YEPD-MB plates (Appendix V 1.13)

2.1.3 Sterile water

2.1.4 Sprayer

2.1.5 Haemacytometer

2.2 Procedure

Each strain was tested for killing activity by seeded agar phenotype (Woods and Bevan, 1968). Particular strains were streaked on YEPD-MB plates and incubated at 20 °C, 25-28 °C, 37 °C and 40 °C for 3 days. At that time, the tester sensitive strain was suspended in sterile water, to give a concentration of 1×10^6 cells/ml which counted by haemacytometer (see Appendix II). Those plates were sprayed with a cell suspension of the tester sensitive strain. After being incubated for 2 days at 20 °C, 25-28 °C, 37 °C and 40 °C respectively killer colonies were identified by the formation of a clear zone

(The difference of killing ability was observed by the width of clear zone around the colony.). The sensitive colonies were identified by themselves turn blue since methylene blue is a specific stain for dead yeast cells (Kirsop, Painting and Henry,1984). Neutral coloines showed as, no reaction on either killer or sensitive assay medium plates.

3. Identification of the isolates

3.1 Materials

3.1.1 Yeast strains

3.1.1.1 Sc.90 , the identified Saccharomyces cerevisiae

3.1.1.2 No 2, the best laboratory strains of wine yeast from Mr. Pradit Karuwanna IFRPD.

3.1.1.3 No 23, the selected strain from isolation of killer yeasts.

3.1.1.4 No 50 " " "

3.1.1.5 No 265 " " "

3.1.1.6 No 266 " " "

3.1.2 5 ml of YM broth in test tubes (Appendix V 1.1)

3.1.3 YM agar plates (Appendix V 1.1)

3.1.4 PDA plates (Appendix V 1.12)

3.1.5 Potassium acetate agar (Appendix V 1.3)

3.1.6 Gorodkowa's agar (Appendix V 1.4)

3.1.7 Fermentation medium (Appendix V 1.5)

3.1.8 Starch fermentation medium (Appendix V 1.6)

3.1.9 Nitrogen assimilation medium (Appendix V 1.7)

3.1.10 Carbon assimilation medium (Appendix V 1.8)

3.1.11 Vitamin free medium (Appendix V 1.9)

3.1.12 Cycloheximide medium (Appendix V 1.10)

3.1.13 Glucose chalk agar (Appendix V 1.11)

3.1.14 Sterile water

3.1.15 Sterile Pasteur pipette

3.1.16 Sterile cover slip

3.1.17 Microscope

3.1.18 Haemacytometer

3.2 Procedure

Morphological and physiological characteristics of the isolates were examined by methods mentioned by Lodder (Lodder et.al., 1970) and were also compared with the identified strain.

Preparation of inoculum

The yeast strains No. 2, 23, 50, 265, and 266 including Sc.90 to be identified were grown in YM broth for 3 days at 25 ^{o}C. The cells were centrifuged, washed with sterile water and used for the preparation of a standard suspension (approximately 10^{6} cells/ml).

Inoculation

All tests were inoculated with 1 drop of the standard yeast suspension delivered from a Pasteur pipette and incubated at 25 ^{o}C.

3.2.1 Morphological Tests

3.2.1.1 Vegetative cells and culture characteristics

Cells from the YM broth cultures were examined at 48 hours and 21 days for the presence of a pellicle or ring , and the type of deposit. Cells from the YM agar cultures were examined for the colour, texture and surface characteristics at the same time. In addition, cells from both cultures were examined microscopically at 48 hours for the method of budding and cells shape.

3.2.1.2 Mycelial growth, arthroconidia, endospores, chlamydoconidia.

PDA plates were inoculated with a streak and a spot of the inoculum. The spot was covered with a sterile cover slip before incubation. At 21 days the growth at the end of the streak (aerobic) and under the cover slip (anaerobic) were examined microscopically , using low power (10x, 40x) objectives initially. Further detail could be observed by covering the edge of the streak with a cover slip and examining this and the 'anaerobic' growth with the high power (100x) objective.

3.2.1.3 Ballistospores

PDA plates were inoculated with a cross and inverted over YM agar plates. The two plates were bound firmly together with sellotape and incubated with the inoculated plate uppermost.

The plates were examined at 7 ,14 and 21 days for the formation of a mirror image of the cross on the lower YM plates, caused by the ejection of ballistospores from the growth on the plates above.

3.2.1.4 Sexual reproduction

Potassium acetate slants and Gorodkowa's slants were inoculated. Cells from these media were examined microscopically for ascus and ascospore using 100x objective at intervals for up to 21 days.

3.2.2 Physiological tests

3.2.2.1 Fermentation of carbon compounds.

Screw capped tubes containing a durham tube and filled with fermentation medium or starch fermentation medium were inoculated with one drop of the standard suspension delivered with a Pasteur pipette. Tubes were examined for gas production at 1, 2, 7, 14 and 21 days. During examination the tubes were kept tightly sealed. Larger amounts of gas collected in the durham tube and were readily observed.

3.2.2.2 Aerobic utilisation of carbon compounds

Carbon assimilation medium or starch assimilation medium were dispensed in test tubes and inoculated with one drop of the standard yeast suspension delivered with a Pasteur pipette. Tubes were examined at 2, 7, 14 and 21 days for growth. The extent to which growth had taken place was recorded by placing well shaken tubes against a card on which black lines had been drawn and assessing the level of growth as follows.

0 No growth - no turbidity ; black lines clearly visible.

+1 Very weak growth - slight turbidity ; black lines distinct but edges slightly blurred.

+2 Weak growth - black lines appear as diffuse bands.

+3 Good growth - as black lines or diffuse bands invisible.

3.2.2.3 Aerobic utilization of nitrogen compounds

Nitrogen assimilation medium was inoculated with one drop of the standard suspension, delivered with a Pasteur pipette tubes were examined at 1, 2, 7, 14 and 21 days for growth as 3.2.2.2

3.2.2.4 Growth in vitamin-free medium

Tubes containing vitamin-free medium were inoculated with one drop of the standard suspension. Tubes were examined for growth as 3.2.2.2. If the growth occurred heavily a loopful of cell suspension was transferred to the second tube of vitamin-free medium and further incubation was carried out.

3.2.2.5 Cycloheximide resistance

Tubes containing cycloheximide medium were inoculated with one drop of the standard inoculum. Tubes were examined for growth as 3.2.2.2.

3.2.2.6 Urease activity

Urea agar slants were inoculated with a loopful of the standard inoculum, the slants were examined at 7, 14 and 21 days for the formation of a deep pink color, denoting urease activity. The test distinguishes anascosporogenous yeast (urease positive) from ascosporogenous yeast (urease negative).

3.2.2.7 Acid production

Glucose chalk agar plates were inoculated with a loopful of standard inoculum in a cross pattern. Plates were examined at 7, 14 and 21 days for the appearance of clear zones around the streaks, denoting the formation of acid.

4. Interaction between killer yeasts.

4.1 Materials

4.1.1 Killer yeast strains used were listed

Strain	Killer type	Origin
Saccharomyces cerevisiae KL-88	K-1	NRIB
Saccharomyces cerevisiae NCYC 738	K-2	NRIB
Saccharomyces capensis NCYC 761	K-3	NRIB
Hansenula mrakii NCYC 500	K-9	NRIB
Kluyveromyces drosophilarum NCYC 575	K-10	NRIB
Torulopsis glabrata ATCC 15126	K-11	NRIB
Torulopsis glabrata IFO 0622	sensitive	NRIB
Hansenula saturnus IFO 0117 (KY 78)	Killer selected strain	
No.23	"	
No.50	"	
No.265	"	
No.266	"	

4.1.2 YEPD-MB plates

4.1.3 10 ml of YEPD broth test tubes

4.1.4 Sterile water

4.1.5 Sprayer

4.1.6 Haemacytometor

4.2 Procedure

Each killer yeast was tested for its ability to kill or for its sensitivity to the killing activity of each other killer strains. Killing and resistance ability were determined by inoculating tested strains with a streak onto a petri-plate with YEPD-MB medium. There were 8 colonies per each plate and incubated at 20 °C for 3 days, afterwards spraying with a lawn of appropriate cell (Somers and Bevan 1969),i.e., a killer yeast strain, an unknown or a standard yeast strain at a concentration of $1x10^6$ cells/ml. Incubation was carried out at 20 °C for 2 days. Yeast which killed the seeded strain as shown by clear zones was designated killer .

5. Killing reaction of selected killer yeast against various yeasts

5.1 Materials

5.1.1 Yeast strains used were listed.

Strain	Origin
Saccharomyces cerevisiae Sc 90	IFRPD stock culture
Saccharomyces bailii OK 94	Patoomporn collection
Saccharomyces chevalieri J-6	Patoomporn collection
Saccharomyces fermentati TC-19	Patoomporn collection
Saccharomyces italicus KY 18	IFRPD stock culture
Saccharomyces marxianus TC-21	Patoomporn collection
Saccharomyces rosei KY 74	IFRPD stock culture
Saccharomyces rouxii KY 75	IFRPD stock culture
Saccharomyces uvarum KY 14	IFRPD stock culture
Candida krusei KY 73	IFRPD stock culture
Candida mycoderma	MIRCEN Bangkok

Strain	Origin
Candida valida	MIRCEN Bangkok
Candida lypolytica NRRL Y1095	IFRPD stock culture
Hansenula anomala KY 79	IFRPD stock culture
Hansenula saturnus KY 78	IFRPD stock culture
Pichia membranefaciens KY 81	IFRPD stock culture
Kleckera sp. KY 21	IFRPD stock culture
Kluyveromyces sp. KY 111	IFRPD stock culture
Saccharomyces cerevisiae KL-88 (K-1)	NRIB (standard type)
Saccharomyces cerevisiae NCYC 738 (K-2)	NRIB "
Saccharomyces capensis NCYC 761 (K-3)	NRIB "
No.23 ; one of the selected killer yeasts	
No.50 ; one of the selected killer yeasts	
No.265 ; one of the selected killer yeasts	
No.266 ; one of the selected killer yeasts	

5.1.2 YEPD-MB plates

5.1.3 10 ml of YEPD broth in test tubes

5.1.4 sterile water

5.1.5 sprayer

5.1.6 Haemacytometer

5.2 Procedure

Standard type killer yeasts and selected killer yeasts were tested for its ability to kill the various given strains. The killing ability was determined by inoculating tested strains with a streak onto YEPD-MB plates and incubated at 20 °C for 3 days, afterwards, spraying with lawn of appropriate cell (Somers and Bevan, 1969) of the given various strains. The sprayed strains were expected to be contaminated yeasts in wine fermentation. The suspension of various strains were diluted to $1x10^6$ cells/ml. Incubation was carried out at 20 °C for 3 days. Yeasts which killed the seeded strains as shown by clear zones were designated killers.

6. Extraction and analysis of ds RNA

6.1 Materials

6.1.1 Yeast strains

6.1.1.1 Saccharomyces cerevisiae KL-88 K-1

6.1.1.2 Saccharomyces cerevisiae NCYC 738 K-2

6.1.1.3 Saccharomyces capensis NCYC 761 K-3

6.1.1.4 Strain No 23 , 50, 265 and 266

6.1.2 DNA marker (人DNA digested with Hind III)

6.1.3 50 ml per flask of YEPD broth

6.1.4 50 mM EDTA ph 7.0 (Appendix V 2.1)

6.1.5 50 mM Tris-H_2SO_4 pH 9.0 (Appendix V 2.2)

6.1.6 2-mercaptoethanol

6.1.7 Sodium dodesyl sulfate (SDS) solution (Appendix V 2.3)

6.1.8 Redistilled phenol

6.1.9 Cold ethanol (-20 °c)

6.1.10 Electrophoresis buffer (Appendix V 2.4)

6.1.11 Ethidiumbromide

6.1.12 DNase solution

6.1.13 Agarose slab gel (Appendix III)

6.1.14 Loading buffer (Appendix V 2.5)

6.1.15 Vaccuum dessicator

6.1.16 Electrophoresis and power supply

6.1.17 Short wave UV light illuminator

6.1.18 Sterile tips and pipette

6.2 Procedure

Extraction was done by a modification of the method of Fried and Fink (1975). Cells of tested yeast grown in 50 ml of YEPD medium were harvested by centrifugation, washed with 30 ml solution of 50 mM EDTA (pH 7.0) and incubated 10 minutes at room temperature. After centrifugation the pellet was resuspended in 30 ml of 50 mM Tris-H_2SO_4 (pH 9.0) containing 0.5 ml of 2 mercaptoethanol. The mixture was shaken for 15 minutes at 30 °C and centrifuged. After being centrifuged, cells were incubated for 10 minutes at 30 °C with sodium dodesyl sulphate solution. Deproteinization was carried out with an equal volume of phenol for 20 minutes at 30 °C. After centrifugation, the clear aqueous phase was precipitated with two volume of cold ethanol overnight. After being centrifuged, the sediment was suspended in sterile were and added with DNase solution for 15 minutes at 30 °C. The suspension was precipitated again with two volume of cold ethanol overnight. The final pellet was dried in a vacuum dessicator for 10 minutes and resuspended in electrophoresis buffer. Samples of 20 ul were mixed with 20 µl of loading buffer and were loaded into the slots

of agarose slab gel. Run the electrophoresis at 10 °C for 6-8 hours, at a constant voltage of 50 V. The gels were stained by immersion in electrophoresis buffer containing 0.4 ug/ml of ethidiumbromide. Photographs were taken under short-wave UV light illuminator.

7. Curing of killer yeasts

7.1 Materials

7.1.1 Yeast strains

7.1.1.1 No.23 ; one of the selected killer yeasts

7.1.1.2 No.50

7.1.1.3 No.265

7.1.1.4 No.266

7.1.2 YEPD broth

7.1.3 YEPD plates

7.1.4 YEPD-MB plates

7.1.5 Cycloheximide

7.1.6 Sterile 0.45 um pore size membrane filters

7.1.7 Sterile velveteen pads

7.1.8 Sprayer

7.1.9 Incubator controled at 37 and 40 °C

7.2 Procedure

Treatment with cycloheximide (Fink and Styles , 1972) or incubation at elevated temperature (Wickner, 1974) was used to cure killer strains. Killer cultures were grown in YEPD broth for 24 hours at 20 °C, serially diluted in sterile water to give a concentration of 1×10^6 cells/ml and 0.1 ml samples were spreaded on YEPD plates.

For cycloheximide curing , media were prepared containing 2 concentration of cycloheximide (0.1 and 0.2 ppm) by the aseptic

addition of filter sterilization of cycloheximide to autoclaved media and plates were incubated at 20 °C.

For temperature curing, plates were incubated at 37 and 40 °C for 3 days.

Colonies of the killer yeast were spreaded and incubated on YEPD plates then replica-plated on YEPD-MB plates and sprayed with the sensitive tester strain. After 3 days of incubation, the cured strains which had lost their killing activity were detected by observing the clear zone.

8. Effect of pH on killer toxin production and the effect of temperature on the killer toxin stability of killer yeast

8.1 Materials

8.1.1 Yeast strains

8.1.1.1 Strain No. 266; the best selected killer strain

8.1.1.2 Torulposis glabrata IFO 0622; the tester sensitive strain

8.1.2 The 10 ml GY medium plates (Appendix V.1.16)

8.1.3 YEPD broth

8.1.4 0.1 M citrate-phosphate buffer, pH range 2.0-7.6

8.1.5 Sterile water

8.1.6 0.22 um pore-size membrane filters

8.1.7 Well cutter

8.1.8 Sterile tips and automatic pipette

8.1.9 Sprayer

8.1.10 Haemacytometer

8.1.11 Water bath

8.2 Procedure

Killing ability of cell-free culture filtrates of killer yeast was assayed by the Well test method (Wood and Bevan, 1968). The tested killer strain was grown in 50 ml of buffered YEPD broth at different pH values, in pHincrements of 0.2 units from 2.0-7.6 and incubated at 20°c for 3 days. The final cell concentration of each culture was counted by haemacytometer and diluted into the same count i.e. approximately 5-10 x 10^6 cells/ml). The culture was filtered by using 0.22 um pour-sige membrane filters and retained at 20o , 25-28o, 37° and 40oC for 1 day and 2 days and the 10 ml GY medium plates (9 cm diameter plate) were cut into 10 mm diameter-well by using a well cutter. There were four wells per each plate. Fifty microlitres of wash filtrate was added to each well and sprayed with the sensitive tester strain. Incubation was carried out at 20° c for 3 days.The diameter of the inhibition zone around each well was measured.

9. Estimations of SO_2 tolerance

9.1 Materials

9.1.1 Yeast strains

9.1.1.1 Strain No.2

9.1.1.2 Strain No.266

9.1.2 YEPD broth

9.1.3 Grape juice contain 100 ppm of SO_2

9.1.4 Haemacytometer

9.1.5 KMS

9.2 Procedure

The yeast strains preincubated in YEPD medium at 25°C for 48 hours were inoculated in grape juice containing 100 ppm of SO_2. Incubation was carried out at 25°c. The time needed for the beginning of fermentation was estimated the degree of SO_2 tolerance which was graded as follow ; + (under 30 hours), ± (30 to 48 hours) and - (over 48 hours).

10. Sporulation ability

10.1 Materials

10.1.1 Yeast strains

10.1.1.1 Strain No.2

10.1.1.2 Strain No.266

10.1.2 YEPD broth

10.1.3 Presporulation medium (Appendix V 1.15)

10.1.4 Sporulation medium

10.1.5 1 % Methylene blue

10.1.6 Haemacytometer

10.1.7 Microscope

10.2 Procedure

The yeast strains were streaked on the plates of pre-sporulation medium, incubated at 20°c for 48 hours then re-streaked thickly from the plates on slopes of sporulation agar. Wet-mount on haemacytometer with 1 % methylene blue were prepared from the slope cultures after 5 days and examined under the microscope for ascus formation. When four-spored asci were produced, they were employed to estimate the level of sporulation using comparison ascus strain with vegetative cells.

11. Determination of killing curve

11.1 Materials

11.1.1 Yeast strains

11.1.1.1 Strain No 2

11.1.1.2 Strain No 266

11.1.2 3 % Ethylmethane sulfonate (EMS)

11.1.3 10 ml YEPD broth per test tube

11.1.4 0.1 M Sodium phosphate buffer pH 8.0 (Appendix V 2.6)

11.1.5 sterile water

11.1.6 Haemacytometer

11.2 Procedure

The yeast cells were cultivated in 10 ml of liguid YEPD medium at 30°c for 16 hours (at exponential phase of yeast cells). The cultures were centrifuged at 3,000 rpm for 5 minutes and washed twice with sterile water. After the supernatant liquid was discarded, the cells were suspended in 10 ml of 0.1 M Sodium phosphate buffer pH 8.0 and 0.3 ml of EMS was added. The mixture was incubated at 30°C. At intervals, the viable cell numbers of each strains were measured by haemacytometer (Appendix II). The killing curve were examined by plotting the percentage of survival against incubation time of EMS treatment.

12. Induction and characterisation of mutants.

12.1 Materials.

12.1.1 Yeast strains

12.1.1.1 Strain No 2

12.1.1.2 Strain No 266

12.1.2 10 ml YEPD broth per test tube

12.1.3 Ethylmethanesulfonate (EMS)

12.1.4 0.1 M Sodium phosphate buffer pH 7.5 and 8 (Appendix V 2.6)

12.1.5 5 % sodium thiosulfate

12.1.6 Synthetic complete medium (Appendix V 1.14)

12.1.7 Synthetic minimum medium (Appendix V 1.14)

12.1.8 Sterile water

12.1.9 Sterile velveteen pads

12.1.10 Centrifuge

12.2 Procedure

The yeast cells were culturedin 10 ml of liquid YEPD medium at 30°C for 16 hours. The culture were contrifuged at 3,000 rpm for 5 minutes, and washed twice with sterile water. After supernatant liquid was discarded, the cells were suspended in 10 ml of 0.1 M sodium-phosphate buffer pH 8.0 and 0.3 ml of EMS was added. The mixture was incubated for 1.5-2 hours at 30°c. This treatment was stopped by 5 % sodium thiosulfate for 10 minutes. After that the cells were washed twice and resuspended in sterile water. The diluted suspension was spreaded on a complete medium plate and the plate was incubated at 30°c. Between 50 and 100 colonies should appear on each petriplate. The plates were marked on the bottom and the colonies were transfered by replica-plating to minimum medium plates. The original and newly replicated plates were incubated at 30°c overnight. The colonies, appeared on the complete medium plate but not on the minimum plate, were auxotrophic mutants. Each mutant was retested for the

growth requirment by omission test on the synthetic minimum medium using 14 amino acids and 2 nucleic acid. After mutagenesis, the mutants were plated on a complete medium plate unit. These cells had grown into colonies, they were then transfered to various types of synthetic media by replica-plating. The auxotrophic mutants were identified by lack of growth on the omitted specific requirement of synthetic medium.

13. Isolation of haploids

13.1 Materials

13.1.1 Yeast strains

13.1.1.1 No. 2-28 diploid lys [KIL-0]

13.1.1.2 No. 2-36 diploid his [KIL-0]

13.1.1.3 No. 266-1 diploid lys [KIL-k]

13.1.1.4 No. 266-2 diploid trp [KIL-k]

13.1.2 YEPD plates

13.1.3 YEPD agar slabs (Appendix IV)

13.1.4 Pre-sporulation medium

13.1.5 Sporulation medium

13.1.6 Zymolyase solution (Appendix V 2.7)

13.1.7 Sterile plates

13.1.8 Microscope

13.1.9 Micromanipulator ; Leitz Mot E Micromanipulator or Skerman's Micromanipulator

13.2 Procedure

The yeast strains were induced to sporulate on sporulation medium and incubated at 30 °C until asci were microscopically visible. From each sporulating culture, a suspension of cells including asci (10^{7} -10^{8} cells/ml) was prepared in Zymolyase solution and was

incubated at 37 ± 1 °C for 45-50 minutes. Dissection was performed on an agar slab with micromanipulator. The enzyme treated mixture of asci and vegetative cells were streaked on edge of agar slab. Rupture of selected asci and isolation of spores were carried out by using a microneedle attached to the micromanipulator and its level and position were adjusted until the tip was just touching the surface of the agar slab near the streaked line. Each asci was selected from the smear, pulled away and ruptured by rolling. Seperated spore was placed by marked position on the agar slab. Each agar slab was kept in a sterile plate and incubated overnight at 20 °C.

The three or four-single germinated spores of each asci were picked up into the section of complete medium plate (YEPD plate). The plates were incubated at 20 °C for 3 days and maintained to test for mating type and sporulating ability.

14. Determination of mating type

14.1 Materials

14.1.1 Yeast strains

14.1.1.1 3 spores of strain No.2-28

14.1.1.2 10 spores of strain No.2-36

14.1.1.3 36 spores of strain No.266-1

14.1.1.4 12 spores of strain No.266-2

14.1.1.5 AH-22 (cir$^+$) : a leu 8-3 leu 2-112 his 4 can 1; a mating type tester strain from AMBO

14.1.1.6 STX 166-17 c : α lys 9 ade 2; α mating type tester strain from Prof.H.Heslot, Institute National Agronomigue Paris

14.1.2 3 ml of YEPD broth per test tube

14.1.3 Sporulation medium

14.1.4 Microscope

14.2 Procedure

The spores were precultured in YEPD medium for 24 hours at 30°C. Then supernatant liquid of killer spores were discarded after centrifugation in order to dilute the concentration of killer factor and prevent killer action during crossing . The sediment of unknown cells were mixed with one of the mating type tester strains on YEPD medium and left overnight. The mixtures were subcultured in sporulation medium and observed until spores were formed.

15. Spore-to-cell mating

15.1 Materials

15.1.1 Yeast strains

15.1.1.1 strain No.266-1 ; diploid HO lys [KIL-k]; The parental killer cell

15.1.1.2 strain No.2-36-2.3 ; a ho his [KIL-0]; The best of the selected wine haploid.

15.1.2 YEPD agar slabs

15.1.3 YEPD plate

15.1.4 Pre-sporulation medium

15.1.5 sporulation medium

15.1.6 Minimum medium

15.1.7 Zymolyase solution

15.1.8 Micromanipulator

15.2 Procedure

Cell was induced to sporulate in sporulation medium at 30°c, These asci were treated by Zymolyase solution and incubated at 37± 1°C for 45-50 minutes. Dissection was performed on an agar slab with micromanipulator as experiment 12. The treated sample of sporulated killer yeast was streaked at one edge on the left side of the slide and the same edge on the other side had a streaked line of a cell (2-36-2.3). When the asci of killer were segregated, each ascospore was marked and then loaded on the agar slab. The a cell was pulled to the positions of ascospore. Each cell was directly contacted with one of the 4 ascospore of the killer ascus. After incubation, the viable zygotes were picked up and transfered into the minimum medium plates.

16. Determination of fermentation ability

16.1 Materials

16.1.1 Yeast strains

16.1.1.1 strain No.2 ; the properly parental wine yeast

16.1.1.2 strain No.266 ; the best of selected killer yeast

16.1.1.3 strain No.2-36-2.3 x 266-1 ; the hybrid obtained

16.1.2 YEPD medium

16.1.3 Grape juice

16.1.4 Sugar

16.1.5 Potassium metabisulfite

16.2 Procedure

The strains preincubated in YEPD medium at 20°C for 2 days were inoculated in grape juice containing 25 % sugar and 100 ppm of potassium metabisulfite. Incubation was performed at 20°C.

16.2.1 Ethanol concentration was measured by Gas chromatograph (Hitachi, Gaschromatograph 90A) One mg/ml of n-propanol was used as internal standard.

16.2.2 Residual sugar was determined by using Lane and Eynon method

16.2.3 pH was measured by pH meter

16.2.4 Total acid was measured by titration with sodium hydroxide

16.2.5 Volatile acid was measured by titration of the distillate with sodium hydroxide.