APPENDICES

APPENDIX A

Media

Media

A1. Yeast-extract malt-extract (YM) medium

Yeast extract (HiMedia laboratory, India)	3	g
Malt extract (HiMedia laboratory, India)	3	g
Peptone (HiMedia laboratory, India)	5	g
Glucose (Ajax, New Zealand)	10	g
Distilled water	1000	ml

The medium was autoclave at 121°C for 15 min.

A2. Inulin medium (Castro et al., 1995)

K_2HPO_4 (Merck, Switzerland)	14	g
Inulin (Fluka, Switzerland)	10	g
KH_2PO_4 (Merck, Switzerland)	6	g
$(NH_4)_2PO_4$ (BDH, England)	2	g
Trisodium citrate (Merck, Switzerland)	1	g
$MgSO_4.7H_2O$ (Fluka, Switzerland)	0.2	g
Distilled water	1000	ml

The medium was autoclave at 121°C for 15 min.

APPENDIX B

Reagents for DNA isolation

Reagents for DNA isolation

B1. Lysis buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTD (pH 8.0)]

Triton X-100	2	ml
SDS	1	g
1 M NaCl	10	ml
1 M Tris-HCl	1	ml
0.5 M EDTA	0.2	ml

Deionized water (dH_2O) was added to a final volume of 100 ml. The compositions were autoclaved at 121ºC for 15 min and stored at 4ºC before use.

B2. 1 M NaCl

NaCl	5.84	g
dH_2O	100	ml

The solution was autoclaved at 121ºC for 15 min and stored at 4ºC before use.

B3. 1 M Tris-HCl (pH 8.0)

Tris base	12.11	g

The solution was adjusted pH to 8.0 with cHCl and dH_2O was added to a final volume of 100 ml. The solution was autoclaved at 121ºC for 15 min and stored at 4ºC before use.

B4. 0.5 M EDTA (pH 8.0)

EDTA	18.6	g

The solution was adjusted pH to 8.0 with NaOH and dH_2O was added to a final volume of 100 ml. The solution was autoclaved at 121ºC for 15 min and stored at 4ºC before use.

B5. TE (pH 8.0) (10 mM Tris-HCl, 1 mM EDTA)

1 M Tris-HCl	1	ml
0.5 M EDTA	0.2	ml
dH_2O	98.8	ml

The compositions were autoclaved at 121ºC for 15 min and stored at 4ºC before use.

B6. 10 mg/ml RNase A in TE (pH 8.0)

RNase A	100	mg
TE (pH 8.0)	10	ml

The solution was boiled at 100ºC for 10 min and stored at -20ºC before use.

APPENDIX C

Reagents for electrophoresis

Reagents for agarose gel electrophoresis

C1. 50X TAE buffer

Tris base	121.0	g
Acetic acid	28.55	ml
0.5 M EDTA (pH 8.0)	50	ml

Distilled water was added to final volume of 500 ml. The solution was autoclaved at 121ºC for 15 min and stored at 4ºC before use.

C2. 1X TAE buffer

50X TAE	10	ml
dH2O	490	ml

The solution was autoclaved at 121ºC for 15 min and stored at 4ºC before use.

C3. Gel-loading buffer

Bromophenol blue	25	mg
Xylene cyanol	25	mg
Sucrose	4	g

Distilled water was added to final volume of 10 ml. The solution was autoclaved at 121ºC for 15 min and stored at 4ºC before use.

C4. Ethidium bromide (10 mg/ml)

Ethidium bromide	1	g

Distilled water was added to final volume of 100 ml.

APPENDIX D

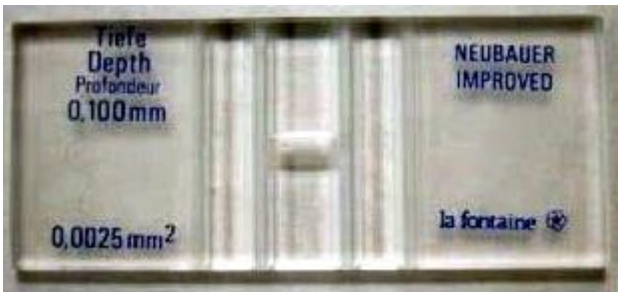
Analytical methods

Analytical methods

D1. Determination of viable yeast cell concentration by haemacytometer using straining technique (Zoecklein et al., 1995)

The most commonly used method for determination of viable yeast cells is dye reduction. The basis for this viable straining technique is that there is a change in the color of a reducible dye (usually methylene blue) when it is used as a hydrogen acceptor in lieu of oxygen. In order to quantitate cell numbers, it is necessary to use a cell counting slide, haemacytometer (Figure A1). It is specially ruled so that the volume present in the counting area is constant. Counting grid of haemacytometer is shown in Figure A2.

(A)



(B)

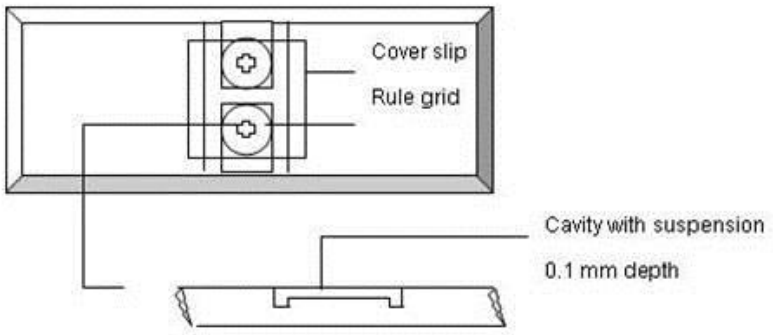


Figure A1 (A) Haemacytometer, (B) Chamber of haemacytometer (top view and side view) (Lobban, 1988)

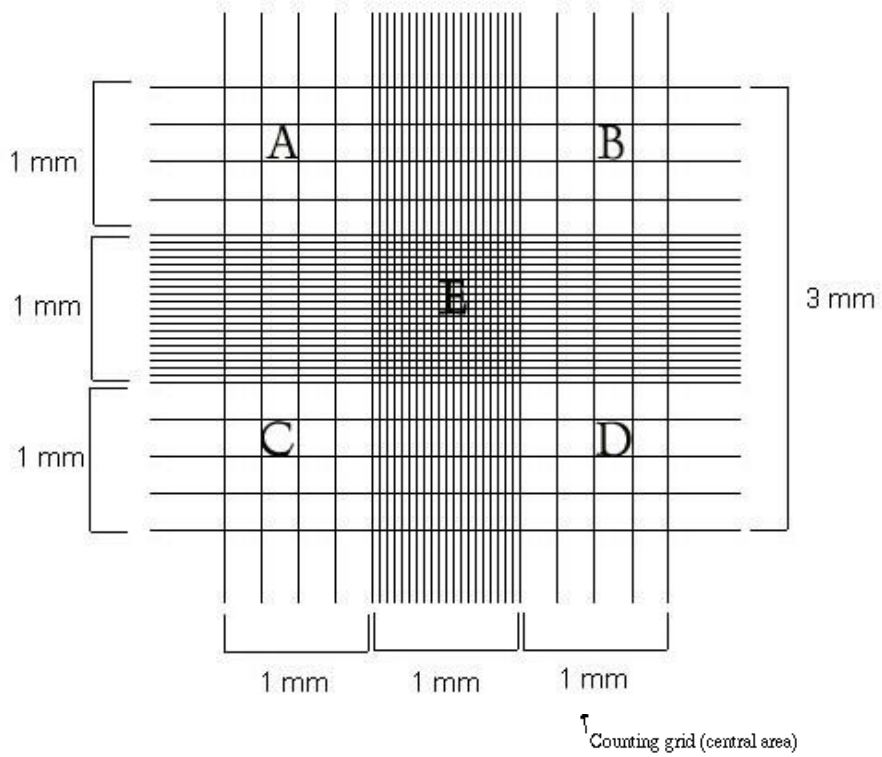


Figure A2 Counting grid of haemacytometer (Lobban, 1988)

Equipments

1. Cell counting slide or haemacytometer and cover glass (Neubauer Improved bright line, Boeco, Germany)
2. Pipette
3. Dilution bottles
4. Test tubes
5. Vortex mixer (VS-1300V, Vision Scientific Co., Ltd., Korea)
6. Microscope (Olympus Optical Co., Ltd., Japan)

Reagents

1. Methylene blue: Dissolved 0.01 g of methylene blue (Unilab, Australia) and 2 g of Na_3-citrate (Univar, Australia) in 10 ml of distilled water and bring to a final volume of 100 ml with distilled water. Before use, the dye should be membrane-filtered and examined for biological activity.
2. Dilution water

Procedure

1. Depending on the stage of yeast growth, prepare the appropriate dilution from the collected sample in dilution water.

2. Vortex each diluted sample before going to the next dilution in the series.

3. After vortexing the final dilution in the series, transfer a small volume from the center of the dilution tube (bottle) to the counting slide.

4. Using the cover glass provided with the kit, carefully position it over the counting grid. Using a pipette, transfer well-mixed sample to counting slide. Allow diluent and yeast to flow under the cover glass and into the counting area via the groove provided.

5. Count all the cells with the bounded, area of the grid (400 squares) using microscope at magnification of 40X. By using methylene blue in the final diluent one may also score viable/nonviable cells as part of the total cell count. In this case, viable cells will be colorless (i.e. they reduce the blue dye to its colorless or leuco-form). Nonviable cells, by comparison, appear blue or have concentrated areas of blue strain within the boundaries of the cell wall.

Tally nonviable and viable as well as percent budding cells present in each field. Each counting slide is provided with two fields for duplication purposes. Count the second and average the results.

6. When one count all the cells present within the grid network the expression of results (in cells/ml) may be calculated using the following equation:

$$\text{Cells/ml} = (\text{number cells counted})\ (10^4) \times \text{Dilution factor}$$

Supplemental notes

1. Although statically approved procedures exist for counting portions of the grid field, they apply to measurement of blood cells. In case where uniform distribution of cells is difficult to achieve (e.g. wine yeast) it is recommended that the entire field of 400 squares be counted. For reproducible results it is necessary that each laboratory establish whether cells lying on grid boundaries be included in the count or rejected. Depending on the initial dilution created, a difference of five or more cells may have a significant effect on the final calculation of cell number.

2. Methylene blue, itself, eventually will poison living cells. Thus it is imperative that once of the dye is mixed with the final dilution, that cell numbers, and especially viability, be determined as soon as possible.

D2. Total sugar determination by phenol-sulfuric method (Zoecklein et al., 1995)

Equipments

1. Spectrophotometer (UV-1601, Shimadzu, Japan)
2. Hood (PNP, Thailand)
3. Auto pipette (P 5000 ,P 1000, Pipetman® P, Gilson, France))
4. Test tube
5. Vortex mixer (VS-1300V, Vision Scientific Co., Ltd., Korea)

Reagents

1. 5% aqueous solution of phenol (5 g of phenol (BDH, England) in 100 ml of H_2O)
2. 95.5% of H_2SO_4 (analytical grade, BDH, England)
3. Standard glucose (anhydrous glucose, Fluka, Switzerland) solution containing 0, 20, 40, 60, 80 and 100 mg/l

Procedure

1. Pipette 1.0 ml of standard glucose solution or a sample into test tubes.
2. Add 1.0 ml of the phenol solution and 5 ml of the H_2SO_4.
3. Carefully vortexed mix and allow the tube to stand for 15 min.
4. Measure the absorbance at 490 nm by spectrophotometer.
5. Prepare the calibration curve of glucose against absorbance.
6. Calculate total sugar concentration in the test solution by reference to the calibration curve.

D3. Reducing sugar determination by DNS method (Zoecklein et al., 1995)

Equipments

1. Spectrophotometer (UV-1601, Shimadzu, Japan)
2. Boiling water bath
3. Auto pipette (P 5000 , P 1000, Pipetman® P, Gilson, France))
4. Test tube
5. Vortex mixer (VS-1300V, Vision Scientific Co., Ltd., Korea)
6. Ice water bath
7. Pipette tip (5.0-ml. and 1.0-ml.)

Reagents

1. 3, 5-Dinitrosalicylic acid (Fluka, Switzerland)
2. NaOH (BDH, England)
3. Sodium potassium tartrate (BDH, England)
4. DNS solution: Dissolve 10.0 g of 3, 5-dinitrosalicylic acid in 2 M of sodium hydroxide solution. A separate solution of 300 g sodium potassium tartrate solution was prepared in 300 ml of distilled water. The hot alkaline 3, 5-dinitrosalicylate solution was added to sodium potassium tartrate solution. The final volume of DNS solution was made up to 1000 ml with distilled water.
5. Standard glucose (anhydrous glucose) solution containing 0, 100, 200, 300, 400 and 500 mg/l.

Procedure

1. Pipette 1.0 ml of standard glucose solution or a sample into test tubes and add 1.0 ml of DNS solution.
2. Vortex mix and boil for 20 min.
3. Immerge the tubes into ice water bath and add 10.0 ml of distilled water.
4. Vortex mix and measure the absorbance at 520 nm by spectrophotometer.
5. Prepare a calibration curve of glucose against absorbance.
6. Calculate reducing sugar concentration in the test solution by reference to the calibration curve.

D4. Total soluble solid measurement (Zoecklien et al., 1995)

As the concentration of soluble solids increases, so too does the refractive index of the sample. The principle here is that the molecules there are in the sample, the more the light passing through the sample is bent (refracted). Most refractometers have a °Brix (°Bx) scale (calibrated against sucrose solutions) along with the refractive index scale; so one may read °Bx directly in samples. Because all soluble solids (especially ethanol) in the sample affect the measured refractive index, fermenting samples give °Bx measurements that are erroneously high.

Equipment

2. Hand-held refractometer (N1, ATAGO, Germany)
3. Distilled water
4. Len paper
5. Cotton swab

Procedure

1. On instruments using water-cooled prisms, connect the laboratory water line to the intake nipple on the instrument and return water to the drain via the exit port.
2. Circulate water to constant temperature through the illuminating prism.
3. Follow the manufacturer's instructions for setup and operation of the instrument used.
4. Field-type refractometers must have their reading temperature corrected to 20°C (68°F).
5. With the blotter and len paper provided, dry the prisms.
6. Using a cotton swab, apply several drops of sample or appropriately diluted concentrate to the lens, and repeat the operations.
7. Should the refractometer not be calibrated in °Bx, refractive index reading can be converted to equivalent % sucrose values.

Steps of the measurement of total soluble solids by hand-held refractometer are summarized in Figure A3.

Supplemental notes

1. The refractive index is critically dependent on the temperature of the solution measured. Unless corrected, small differences from the reference temperature should be expected to result in significant error.

2. Samples for analysis must be representative, homogenous, and free of particulate matter that could damage prisms or impair accuracy.

3. In addition to temperature, alcohol is a major interference in accurate measurements of the refractive index. Therefore, refractometer should not be used in soluble solids determinations of fermenting must or wine.

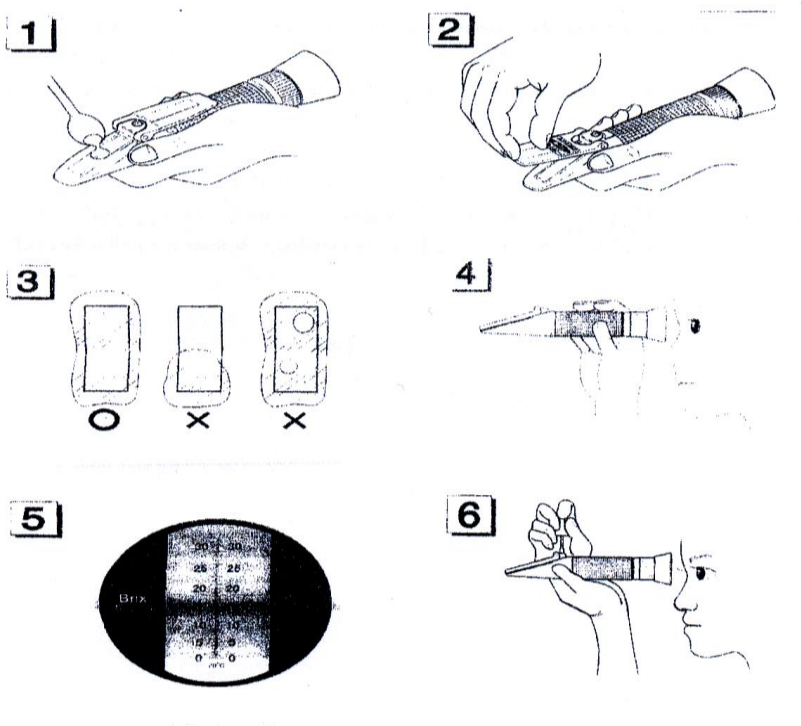


Figure A3 Steps of the measurement of total soluble solids by hand-held refractometer (ATAGO catalogue, 2003)

D5. Ethanol concentration measurement by gas chromatography (Sirisuntimethacom, 2006)

Equipments

1. Gas chromatography (GC) equipped with flame ionization detection (FID) (GC 14B, Shimadzu, Japan)
2. Electronic integrator (data processor) (C-R7 Ae plus Chromatopac, Shimadzu, Japan)
3. Auto pipette (P1000 and P200, Pipetman® P, Gilson, France)
4. 1.5-ml micro centrifuge tube
5. 10-µl GC syringe (ITO corporation, Japan)

Reagents

1. Absolute ethanol (HPLC grade, AnalaR®, BDH, England,)
2. 2-propanol (HPLC grade, Fisher Scientific, United Kingdom)
3. Deionized water

Procedure

1. Prepare ethanol standard solutions to give the final ethanol concentrations of 2.5, 5, 10, 15 and 20% (v/v).
2. Prepare internal standard solution of 2-propanol (10%, v/v).
3. Mix the ethanol standard solution with the internal standard solution at the ratio of 1:1 (v/v).
4. Inject 1.0 µl of the mixed ethanol and internal standard solution into the GC and record the resulting chromatograms.
5. Construct the calibration curve or standard curve between the ethanol concentrations and the ratios of peak area of the ethanol standard solutions and the peak area of the internal standard solution.
6. Mix the sample with the internal standard solution at the ratio of 1:1 and inject 1.0 µl of the mixture into GC.
7. Calculate the ethanol concentration in the sample by using the calibration curve.

Operational condition for analysis

1. Carrier gas: Nitrogen gas
2. Column pressure: 200 kPa

3. Column: 20% PEG 20M (Shimadzu, Japan) 3 m length.
4. Injector temperature: 180℃
5. Detector temperature: 250℃
6. Oven temperature program: Initial temperature at 150℃ for 11 min, increase the temperature to 220℃ at the rate of 20℃/min and hold for 15 min.

Retention time

1. Ethanol: 5.5 min
2. 2-propanol: 7.5 min

APPENDIX E

Standard curves

Standard curves

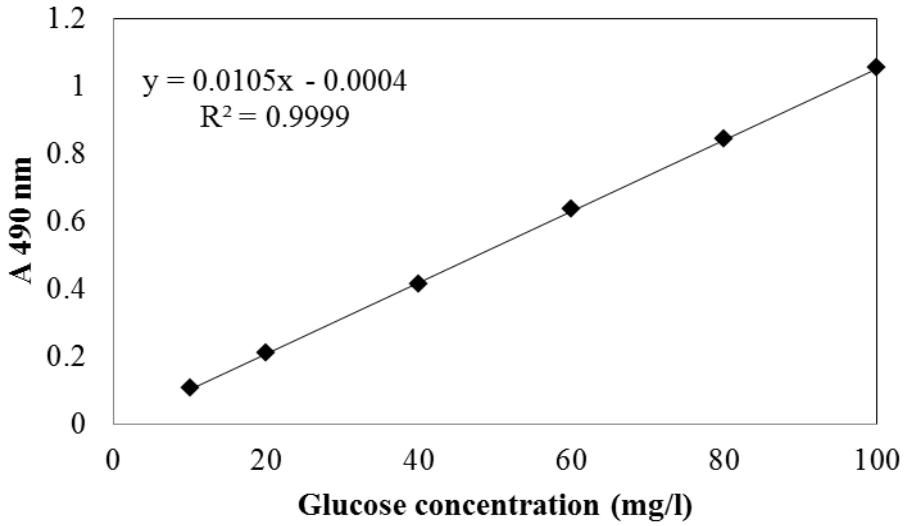


Figure A4 A standard curve of total sugar by phenol-sulfuric method

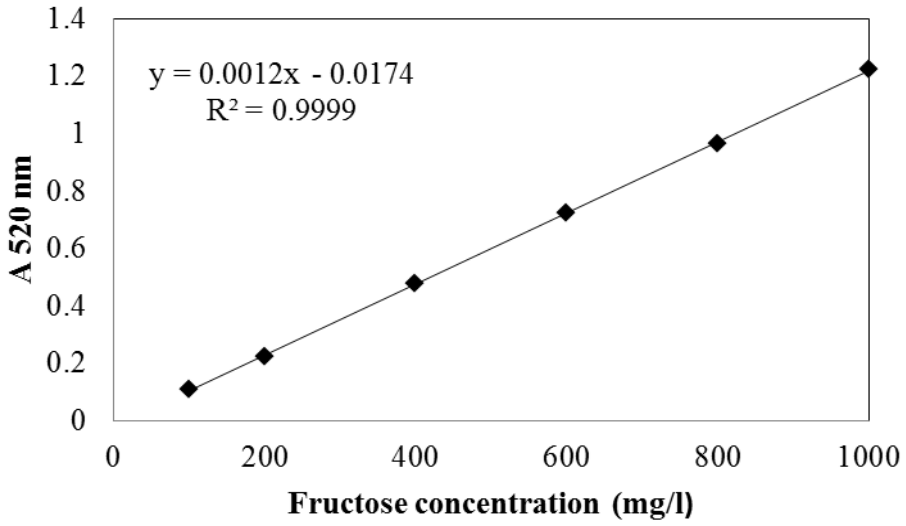


Figure A5 A standard curve of reducing sugar by DNS method

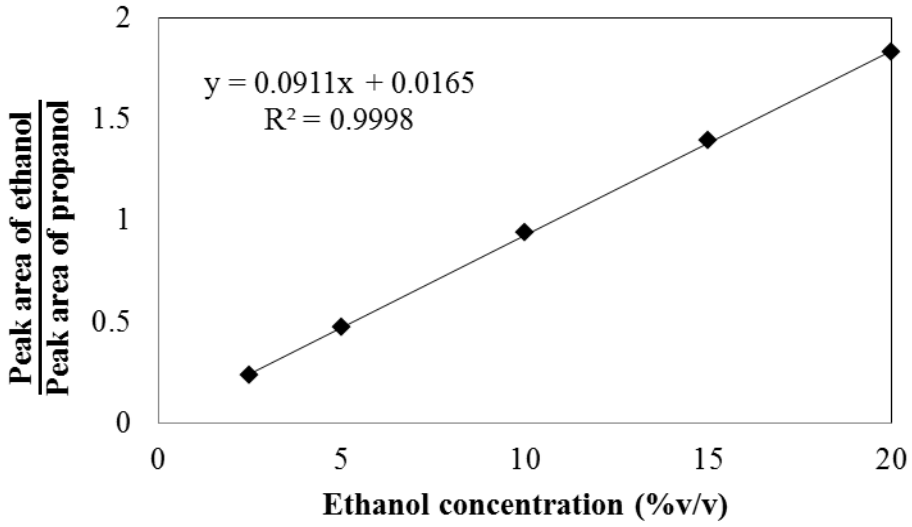


Figure A6 A standard curve of ethanol concentration by gas chromatography

APPENDIX F

Nucleotide sequences of the selected thermotolerant yeasts

Nucleotide sequences of the selected thermotolerant yeasts

TAGCAGCATC CTTGAAAAAG TCGCAATCCT CAGTCCCAGC
TGGCTGTATT CCCACGGGCT ATAACACTCT ACCGAAGCAG
AGCCACATTC CCGAGGATTT ATCCAACCGC TAAAACTGAT
GCTGGCCCAG CGAAAGCCGA AGCAAACGCC ATGTCTGATC
AAATGCCCTT CCCTTTCAAC AATTTCACGT ACTTTTTCAC
TCTCTTTTCA AAGTTCTTTT CATCTTTCCA TCACTGTACT
TGTTCGCTAT CGGTCTCTCG CCAATATTTA GCTTTAGATG
GAATTTACCA CCCACTTAGA GCTGCATTCC CAAACAACTC
GACTCGTCGA AAGCACTTTA CAAATAACTG GGATCCTCGC
CACACGGGAT TCTCACCCTC TATGACGTCC TGTTCCAAGG
AACATAGACA AGGACCAGCT ACAAAGTCGC CTTCTTCAAA
TTACAACTCG GACGTCGAAG ACGCCAGATT TCAAATTTGA
GCTTTTGCCG CTTCACTCGC CGTTACTAAG GCAATCCCGG
TTGGTTTCTT TTCCTCCGCT TAATGATATG CAAAA

Figure A7 26S rDNA sequence of the *K. marxianus* DBKKU Y-102

CCTTTGAAAA AGTCGCAATC CTCAGTCCCA GCTGGCTGGT
ATTCCCACGG GGCTATAACA CTCTACCGAA GCAGAGCCAC
ATTCCCGAGG ATTTATCCAA CCGCTAAAAC TGATGCTGGC
CCAGCGAAAG CCGAAGCAAA CGCCATGTCT GATCAAATGC
CCTTCCCTTT CAACAATTTC ACGTACTTTT TCACTCTCTT
TTCAAAGTTC TTTTCATCTT TCCATCACTG TACTTGTTCG
CTATCGGTCT CTCGCCAATA TTTAGCTTTA GATGGAATTT
ACCACCCACT TAGAGCTGCA TTCCCAAACA ACTCGACTCG
TCGAAAGCAC TTTACAAATA ACTGGGATCC TCGCCACACG
GGATTCTCAC CCTCTATGAC GTCCTGTTCC AAGGAACATA
GACAAGGACC AGCTACAAAG TCGCCTTCTT CAAATTACAA
CTCGGACGTC GAAGACGCCA GATTTCAAAT TTGAGCTTTT
GCCGCTTCAC TCGCCGTTAC TAAGGCAATC CCGGTTGGTT
TCTTTTCCTC CGCTTAATGA TATGCAAAAA GCT

Figure A8 26S rDNA sequence of the *K. marxianus* DBKKU Y-103

```
ATAGCAGCAT      CCTTGAAAAG      TCGCAATCCT      CAGTCCCAGC
TGGCTGTATT      CCCACGGGCT      ATAACACTCT      ACCGAAGCAG
AGCCACATTC      CCGAGGATTT      ATCCAACCGC      TAAAACTGAT
GCTGGCCCAG      CGAAAGCCGA      AGCAAACGCC      ATGTCTGATC
AAATGCCCTT      CCCTTTCAAC      AATTTCACGT      ACTTTTTCAC
TCTCTTTTCA      AAGTTCTTTT      CATCTTTCCA      TCACTGTACT
TGTTCGCTAT      CGGTCTCTCG      CCAATATTTA      GCTTTAGATG
GAATTTACCA      CCCACTTAGA      GCTGCATTCC      CAAACAACTC
GACTCGTCGA      AAGCACTTTA      CAAATAACTG      GGATCCTCGC
CACACGGGAT      TCTCACCCTC      TATGACGTCC      TGTTCCAAGG
AACATAGACA      AGGACCAGCT      ACAAAGTCGC      CTTCTTCAAA
TTACAACTCG      GACGTCGAAG      ACGCCAGATT      TCAAATTTGA
GCTTTTGCCG      CTTCACTCGC      CGTTACTAAG      GCAATCCCGG
TTGGTTTCTT      TTCCTCCGCT      TATTGATATG      CA
```

Figure A9 26S rDNA sequence of the *K. marxianus* DBKKU Y-104

```
ATAGCAGCAT      CCTTGAAAAG      TCGCAATCCT      CAGTCCCAGC
TGGCTGTATT      CCCACGGGCT      ATAACACTCT      ACCGAAGCAG
AGCCACATTC      CCGAGGATTT      ATCCAACCGC      TAAAACTGAT
GCTGGCCCAG      CGAAAGCCGA      AGCAAACGCC      ATGTCTGATC
AAATGCCCTT      CCCTTTCAAC      AATTTCACGT      ACTTTTTCAC
TCTCTTTTCA      AAGTTCTTTT      CATCTTTCCA      TCACTGTACT
TGTTCGCTAT      CGGTCTCTCG      CCAATATTTA      GCTTTAGATG
GAATTTACCA      CCCACTTAGA      GCTGCATTCC      CAAACAACTC
GACTCGTCGA      AAGCACTTTA      CAAATAACTG      GGATCCTCGC
CACACGGGAT      TCTCACCCTC      TATGACGTCC      TGTTCCAAGG
AACATAGACA      AGGACCAGCT      ACAAAGTCGC      CTTCTTCAAA
TTACAACTCG      GACGTCGAAG      ACGCCAGATT      TCAAATTTGA
GCTTTTGCCG      CTTCACTCGC      CGTTACTAAG      GCAATCCCGG
TTGGTTTCTT      TTCCTCCGCT      TTTGATATGC      A
```

Figure A10 26S rDNA sequence of the *K. marxianus* DBKKU Y-105

TAGCCAGCAT CCTTGGACAA AAGTCGCAAT CCTCAGTCCC
AGCTGGCTGT ATTCCCACGG GCTATAACAC TCTACCGAAG
CAGAGCCACA TTCCCGAGGA TTTATCCAAC CGCTAAAACT
GATGCTGGCC CAGCGAAAGC CGAAGCAAAC GCCATGTCTG
ATCAAATGCC CTTCCCTTTC AACAATTTCA CGTACTTTTT
CACTCTCTTT TCAAAGTTCT TTTCATCTTT CCATCACTGT
ACTTGTTCGC TATCGGTCTC TCGCCAATAT TTAGCTTTAG
ATGGAATTTA CCACCCACTT AGAGCTGCAT TCCCAAACAA
CTCGACTCGT CGAAAGCACT TTACAAATAA CTGGGATCCT
CGCCACACGG GATTCTCACC CTCTATGACG TCCTGTTCCA
AGGAACATAG ACAAGGACCA GCTACAAAGT CGCCTTCTTC
AAATTACAAC TCGGACGTCG AAGACGCCAG ATTTCAAATT
TGAGCTTTTG CCGCTTCACT CGCCGTTACT AAGGCAATCC
CGGTTGGTTT CTTTTCCTCC GCTT

Figure A11 26S rDNA sequence of the *K. marxianus* DBKKU Y-106

TCGCAGCATC CTTGAAAAGT CGCAATCCTC AGTCCCAGCT
GGCTGTATTC CCACGGGCTA TAACACTCTA CCGAAGCAGA
GCCACATTCC CGAGGATTTA TCCAACCGCT AAAACTGATG
CTGGCCCAGC GAAAGCCGAA GCAAACGCCA TGTCTGATCA
AATGCCCTTC CCTTTCAACA ATTTCACGTA CTTTTTCACT
CTCTTTTCAA AGTTCTTTTC ATCTTTCCAT CACTGTACTT
GTTCGCTATC GGTCTCTCGC CAATATTTAG CTTTAGATGG
AATTTACCAC CCACTTAGAG CTGCATTCCC AAACAACTCG
ACTCGTCGAA AGCACTTTAC AAATAACTGG GATCCTCGCC
ACACGGGATT CTCACCCTCT ATGACGTCCT GTTCCAAGGA
ACATAGACAA GGACCAGCTA CAAAGTCGCC TTCTTCAAAT
TACAACTCGG ACGTCGAAGA CGCCAGATTT CAAATTTGAG
CTTTTGCCGC TTCACTCGCC GTTACTAAGG CAATCCCGGT
TGGTTTCTTT TCCTCCGCTT ATTGATATGC A

Figure A12 26S rDNA sequence of the *K. marxianus* DBKKU Y-107

APPENDIX G

Research publications

APPENDIX G1

An abstract published in the proceeding of Commission on Education Congress II: University Staff Development Consortium, 27th -29th August 2009, Dusit Thani Pattaya, Pattaya, Thailand.

ISOLATION OF THERMOTOLERANT YEASTS FOR ETHANOL PRODUCTION FROM JERUSALEM ARTICHOKE

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Walai Rukhavej Botanical Research Institute, Mahasarakham University, Mahasarakham 44150, Thailand[2]

Objective

To isolate and screen for the thermotolerant yeasts capable of producing ethanol from Jerusalem artichoke as well as to identify the selected yeast strain by using molecular technique.

Methods

Isolation of the thermotolerant yeast was performed by enrichment culture technique as described by Limtong et al. (2007). Pure cultures were maintained on YM agar plate and morphology of the cells was observed under microscope. Inulin medium was used to screen for the inulin-utilizing yeasts. Ethanol and high temperature tolerance ability were tested by culturing yeast strains in YM medium containing various concentrations of ethanol or by incubating at temperature ranged from 30-50^{o}C. Identification of the selected yeast was performed using molecular technique.

Results

A total of 50 isolates of yeast were obtained after enrichment culture technique. Among them, 6 isolates were able to grow in the inulin medium. All these 6 isolates

exhibited the ability to grow in medium containing 4% ethanol and at temperature up to 46^{o}C.

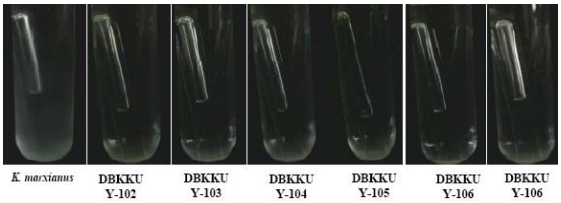

Fig. 1 Cultures growing in inulin medium. The bubble in the Durham tube indicates gas production.

Number	**Ethanol concentration (%, v/v)**				**Temperature (°C)**			
	4	6	8	10	40	42	44	46
Control	√	X	X	X	+	+	+	+
DBKKU Y-102	√	X	X	X	+	+	+	+
DBKKU Y-103	√	X	X	X	+	+	+	+
DBKKU Y-104	√	X	X	X	+	+	+	+
DBKKU Y-105	√	X	X	X	+	+	+	+
DBKKU Y-106	√	X	X	X	+	+	+	+
DBKKU Y-107	√	X	X	X	+	+	+	+

Table 1 The ability of yeast to grow in medium containing various ethanol concentrations or at different temperatures.

Discussion

Based on our finding, at least 6 isolates were able to utilize inulin, a major carbohydrate compound in Jerusalem artichoke, as carbon and energy sources. The ability of growing at high temperature up to 46^{o}C suggested that these are thermotolerant yeast strains.

APPENDIX G2

An abstract published in the proceeding of The 14th International Biotechnology Symposium and Exhibition, Biotechnology for the Sustainability of Human Society, 14th -18th September 2010, Palacongressi, Rimini, Italy.

ISOLATION AND CHARACTERIZATION OF THERMOTOLERANT YEASTS FOR BIOETHANOL PRODUCTION FROM JERUSALEM ARTICHOKE

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Abstract

Utilization of thermotolerant microorganisms enables fermentation to be performed at high temperatures, which have several advantages such as reduce cost for cooling system, reduce contamination of mesophilic microorganisms and increase the speed of catalytic reactions related to fermentation. In this work, isolation and characterization of thermotolerant yeasts capable of producing ethanol from Jerusalem artichoke (*Helianthus tuberosus* L.), one of the most suitable materials for bioethanol production as it contains nearly 20% of carbohydrates, were investigated. Soils and plant materials collected from Jerusalem artichoke plantation were subjected to isolation of the thermotolerant yeast using enrichment culture technique. As the results, fifty isolates of yeast were obtained and they were maintained on YM agar. Among these isolates, only six isolates were able to use inulin, a major carbohydrate compound contained in Jerusalem artichoke tuber, as carbon and energy source for growth. The tolerance ability of these six isolated yeasts to high ethanol concentration and high temperature were tested by culturing them in YM medium containing

various concentrations of ethanol (0-10% v/v) or by incubating at different temperatures (30-50^{o}C). The results showed that all six isolated yeasts were able to grow in medium containing up to 4% ethanol at initial concentration and up to 46^{o}C of incubation temperature. Their ability to grow at high temperature indicating that these isolated yeasts are thermotolerant yeasts. Identification of the yeast strains was carried out using morphological and D1/D2 domain of 26S rDNA sequencing analysis and the results revealed that these isolated yeasts were *Kluyveromyces marxianus*. Ethanol fermentation ability of the six isolated yeasts was compared using Jerusalem artichoke juice without acid or enzymatic pre-treatment as a raw material. The results showed that all isolates were produced relatively high ethanol concentration after 4 days of fermentation, however the highest ethanol concentration (8.0% v/v) was obtained from strain A102 and A103. These results suggested that the newly thermotolerant yeasts, *K. marxianus* strain A102 and A103, have high potential for ethanol production from Jerusalem artichoke at high temperature.

Key Words: Thermotolerant yeast, Jerusalem artichoke, Ethanol production, *Kluyveromyces marxianus*

APPENDIX G3

An abstract published in the proceeding of Abstract of The 4th International Conference on Fermentation Technology for Value Added Agriculture Products, 29th -31st August 2011, Kosa Hotel, Khon Kaen, Thailand.

SELECTION OF THERMOTOLERANT YEASTS FOR BIOETHANOL PRODUCTION FROM JERUSALEM ARTICHOKE JUICE

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The isolation and selection of thermotolerant yeasts capable of producing ethanol from Jerusalem artichoke (*Helianthus tuberosus* L.) juice were investigated. Yeasts were isolated from soils and plant materials collected from Jerusalem artichoke plantations using an enrichment culture technique. Totally fifty isolates of yeast were obtained. Among these isolates, only six isolates were able to use inulin as carbon and energy source for growth. The tolerance ability of the six isolates to high temperatures was tested by incubating at temperatures ranging from 30-50°C. The results showed that all six isolates were able to grow at temperature up to 46°C. Ethanol fermentation ability of the six isolates under static and shaking conditions in batch mode was compared using Jerusalem artichoke juice without acid or enzymatic pre-treatment as a raw material. The results showed that all six isolates produced relatively high ethanol concentration at both 37°C and 40°C, however the highest

ethanol concentration (60.86 g/l) and theoretical ethanol yield (88%) were obtained from strain DBKKU Y-102 under shaking condition at 40°C. These results suggest that the newly thermotolerant yeast, DBKKU Y-102, has high potential for ethanol production from Jerusalem artichoke juice at high temperatures. Identification of the six isolated yeasts was carried out using morphological and D1/D2 domain of 26S rDNA sequencing analysis and the results revealed that all these isolated yeasts were *Kluyveromyces marxianus*.

Keywords: Thermotolerant yeast, ethanol, Jerusalem artichoke, *Kluyveromyces marxianus*

APPENDIX G4

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BIOETHANOL PRODUCTION FROM JERUSALEM ARTICHOKE TUBERS JUICE BY THERMOTOLERANT YEAST *KLUYVEROMYCES MARXIANUS* DBKKU Y-102

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Abstract

Ethanol fermentation at high temperature with thermotolerant yeasts have several advantages such as reduce cost for cooling system, reduce risk of contamination, and increase the speed of catalytic reactions related to fermentation. Jerusalem artichoke (*Helianthus tuberosus* L.) is one of the most suitable materials for bioethanol production as it contains nearly 20% of carbohydrates, 70-90% of which is inulin. In this work, the batch ethanol fermentation of Jerusalem artichoke juice without acid or enzymatic pre-treatment by isolated thermotolerant yeast *Kluyveromyces marxianus* DBKKU Y-102 was investigated. Effect of incubation temperature, initial pH of ethanol production medium, initial sugar concentrations, and initial cell number on ethanol production was studied. The results showed that *K. marxianus* DBKKU Y-102 produced relatively high ethanol concentration at 37°C and 40°C. The highest ethanol concentration (99.29 g/l), ethanol productivity (2.76 g/l/h), and theoretical ethanol yield (92.03%) were obtained at 40 °C under the following optimal conditions: pH 5.5, 250 g/l initial sugar concentrations, and 1×10^8

cells/ml initial yeast cell. These results suggested that the thermotolerant yeast, *K. marxianus* DBKKU Y-102, has high potential for ethanol production at high temperature from Jerusalem artichoke juice without pre-treatment.

Key Words: Bioethanol, Jerusalem artichoke, *Kluyveromyces marxianus*, Thermotolerant yeast

APPENDIX G5

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ISOLATION AND CHARACTERIZATION OF THERMOTOLERANT YEAST CAPABLE OF PRODUCING BIOETHANOL FROM JERUSALEM ARTICHOKE JUICE (*HELIANTHUS TUBEROSUS* L.)

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Objectives

To isolate and select for the thermotolerant yeast capable of producing ethanol from Jerusalem artichoke tubers juice.

To investigate the influence of some fermentation parameters on ethanol production by selected yeast.

Methods

Isolation of yeasts from various samples is carried out at 30°C by an enrichment culture technique as describes by Limtong et al. (2007). Identification of the yeast strains was carried out using morphological and D1/D2 domain of 26S rDNA sequencing analysis. Ethanol fermentations ability of the isolated yeasts in flask scale under shaking condition at 30, 37, 40 and 45^{o}C was compared using Jerusalem artichoke juice as a raw material. The effect of pH on ethanol fermentation was carried out by adjusting the initial pH of medium to 4.0, 4.5, 5.0 5.5 and 6.0. The effect sugar concentrations (230, 250 and 270 g/l) initial cell number (1×10^6, 1×10^7 and 1×10^8 cells/ml) and nitrogen sources ($(NH_4)_2SO_4$, $(NH_4)_2PO_4$, yeast extract and corn steep liquor) were also examined. Total sugar was analyzed by phenol sulfuric

method (Dubois et al., 1956). pH was monitor with pH meter. Cell number was count using haemacytometer (Zoecklien et al., 1995) and ethanol was analyzed using Gas chromatography (Laopaiboon et al., 2009).

Results and Discussion

A total of 50 isolates of yeast were obtained after enrichment culture technique. Among them, six isolates (DBKKU Y-102, DBKKU Y-103, DBKKU Y-104, DBKKU Y-105, DBKKU Y-106 and DBKKU Y-107) were able to grow in the inulin medium. All these six isolates exhibited the ability to grow in medium containing 4% ethanol and at temperature up to 46^{o}C. Identification of the selected yeast strains was performed by morphological and sequencing of the D1/D2 domain of 26S rDNA and the results revealed that all six isolated yeasts were *K. marxianus*. Batch ethanol fermentation of six isolated were compared at different temperatures. Among the strains tested, *K. marxianus* DBKKU Y-102 gave the relatively high ethanol concentration at 37 and 40°C, as compared to the others. Therefore, this strain was selected for the next experiments. The influence of some fermentation factors was studied. The results showed that *K. marxianus* DBKKU Y-102 gave the highest ethanol concentration (106.62±1.08 g/l), ethanol productivity (2.96±0.03 g/l/h), and theoretical ethanol yield (96.77%) at 37°C under the following optimal conditions: pH 5.5, 250 g/l initial sugar concentration, 1×10^{8} cells/ml initial yeast cell without additional of nitrogen source.

Conclusion

The six isolated yeasts in this study are classified as *K. marxianus* based on D1/D2 domain sequencing. The *K. marxianus* DBKKU Y-102 was an effective strain that could be employed for ethanol production directly from Jerusalem artichoke juice at both 37 and 40°C. Its ability to produce ethanol from Jerusalem artichoke was determined and the maximum ethanol concentration produced by this strain under the optimal conditions was 106.62±1.08 g/l, with 96.77% of the theoretical ethanol yield. The experiments reveal that Jerusalem artichoke juice can served as an ethanol

production medium for yeast growth and ethanol fermentation without nitrogen supplementation.

Publication Outputs:

1. Kanlayani Charoensopharat, Sudarat Thanonkeo and Pornthap Thanonkeo. 2010. Isolation and characterization of thermotolerant yeasts for bioethanol production from Jerusalem artichoke**.** The 14th International Biotechnology Symposium and Exhibition: Biotechnology for the Sustainability of Human Society. September 14th -18th 2010, Rimini, Italy. (Poster).
2. Kanlayani Charoensopharat, Sudarat Thanonkeo, Mamoru Yamada and Pornthap Thanonkeo. 2011. Selection of thermotolerant yeasts for bioethanol production from Jerusalem artichoke juice. The 4th International conference FerVAAP 2011. August 29th-31st 2011, Khon Kaen, Thailand. (Poster).
3. Kanlayani Charoensopharat, Sudarat Thanonkeo and Pornthap Thanonkeo. 2012. Bioethanol production from Jerusalem artichoke tubers juice by thermotolerant yeast *Kluyveromyces marxianus* DBKKU Y-102. The 15th International Biotechnology Symposium and Exhibition. September 16th-21st 2012, Daegu, Replublic of Korea. (Poster).

Keywords:

Ethanol, Jerusalem artichoke, *Kluyveromyces marxianus*, Thermotolerant yeast

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APPENDIX G6

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THE BATCH ETHANOL PRODUCTION FROM JERUSALEM ARTICHOKE JUICE BY THERMOTOLERANT YEAST *KLUYVEROMYCES MARXIANUS* DBKKU Y-102

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In this study, selection and characterization of thermotolerant yeasts *Kluyveromyces marxianus* capable of producing ethanol from Jerusalem artichoke juice (ARJ) (*Helianthus tuberosus* L.) were investigated. Batch ethanol fermentation of six isolated *K. marxianus* strains, that is, DBKKU Y-102, DBKKU Y-103, DBKKU Y-104, DBKKU Y-105, DBKKU Y-106 and DBKKU Y-107, were compared at different temperatures. Among the strains tested, DBKKU Y-102 gave the relatively high ethanol concentration at 37°C and 40°C, as compared to the others. The effect of initial pH, sugar concentration, cell concentration, nitrogen source and

concentration of magnesium sulfate on ethanol production by *K. marxianus* DBKKU Y-102 was determined. The results showed that the highest ethanol productivity (4.24±0.02 g/l.h), ethanol concentration (101.74±0.47 g/l), and theoretical ethanol yield (90.24%) were obtained at 37°C under the following optimal conditions: pH 5.5, 250 g/l initial sugar concentration, 1×10^8 cells/ml initial yeast cell, 0.5 g/l diammonium phosphate as nitrogen source. The batch ethanol fermentation was conducted in a 2-L jar fermenter under the optimal conditions with an agitation speed of 100 rpm. *K. marxianus* DBKKU Y-102 yielded the final ethanol concentration of 94.62±0.86 g/l, a productivity of 2.63±0.02 g/l.h, and 90.26% of the theoretical ethanol yield. These results suggested that the thermotolerant yeast, *K. marxianus* DBKKU Y-102, has high potential for ethanol production at high temperature from ARJ.

Keywords: Ethanol, Jerusalem artichoke, *Kluyveromyces marxianus*, Thermotolerant yeast