MATERIALS AND METHODS

1. Sample Collection

Samples were collected from various kinds of natural habitats such as flowers, insect frass, mosses, mushrooms and some others such as fruits and exudates, from several places of Thailand (Fig. 2).

Figure 2 Places where samples were collected (●).

2. Isolation of Yeasts

The yeasts were isolated by direct streaking of samples on YM agar plates supplemented with 100 μg/l chloramphenical and 0.2% sodium propionate (Appendix A). The enrichment technique was also used for isolation. The technique was carried out by addition of small amount of sample into YM broth supplemented with 100 μg/l chloramphenical and 0.2% sodium propionate (Appendix A) and incubated for several days until a film and / or sediment of yeasts are produced. The film of yeasts was streaked on YM agar supplemented with 100 μg/l chloramphenical and 0.2% sodium propionate. The sediment was also streaked on agar after removal of film and media by decantation. After incubation for several days, colonies appeared on agar media were picked up and purified by the conventional streaking technique on YM agar (Appendix A). The pure cultures were suspended in YM broth supplemented with 10% glycerol as a cryoprotectant and maintained in a deep freezer (-80°C).

3. Identification of Yeasts

Yeasts were identified by polyphasic taxonomic studies. The molecular, chemotaxonomic and conventional techniques were used for this purpose. The sequences of D1/D2 domain of 26S rDNA were compared with nucleotide database by BLASTn programs (http://www.ncbi.nlm.nih.gov/BLAST/ and http://www.ddbj.nig.ac.jp/E-mail/homology.html). The morphological, physiological, biochemical and chemotaxonomic characteristics were studied and compared with the standard descriptions in The Yeasts, A Taxonomic Study 4th ed. (Kurtzman and Robnett, 1998) and Yeasts: Characteristics and identification (Barnett et al., 2000). DNA-DNA reassociation experiment was used for confirmation of the correctness of identification if it was necessary.

3.1 Molecular techniques

3.1.1 Nucleotide sequencing

a. Isolation of DNA for Polymerase Chain Reaction (PCR). Isolation of DNA was carried out by boiling of cells with lysis buffer according to the methods of Manitis et al. (1982) with slight modification. A loopful of yeast cells was transferred to 1.5 ml Eppendorf tube. The 100 µl of lysis buffer was added. Cell suspensions were boiled in water bath or metal block bath for 15 min. After boiling, 100 µl of 2.5 M potassium acetate (pH 7.5) was added and placed on ice for 1 hr, and centrifuged at 14,000 rpm for 5 min. Supernatant was extracted twice with 100 µl of chloroform:isoamyl alcohol (24:1 v/v). DNA was precipitated with isopropanol, placed at 20°C for 10 min and centrifuged at 15,000 rpm for 15 min. DNA pellet was rinsed with 70% and 90% ethanol and then dried up (15-30 min at room temperature). The dried DNA was dissolved in 30 µl milli Q water.

b. Polymerase Chain Reaction (PCR) for D1/D2 domain of 26S RDNA. The divergent D1/D2 domain of 26S rDNA was amplified with primers NL-1 (5'- GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (Kurtzman and Robnett, 1998). Amplification was carried out in 100 µl reaction mixture conditioning 100 ng of genomic DNA, 2.5 U of Taq polymerase, 20 mM of each dNTP, 40 mM of each primer, 10 mM Tris-HCl and 1.5 mM $MgCl_2$. The reaction was pre-denatured at 94°C for 5 min, then repeated for 30 PCR cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2.5 min and then followed by the final extension at 72°C for 10 min. The amplified DNA was purified with QIAquick PCR Purification Kit according to the manufacturer's instruction. Visualization of the purified amplified DNA was performed by electrophoresis using 0.8% agarose gel in 1X TBE buffer and stained with ethidium bromide ($8x10^{-5}$ µg/ml) and observed under UV illuminator.

c. D1/D2 domain of 26S rDNA sequencing. The nucleotide sequences of D1/D2 domain of 26S rDNA were directly determined using PCR products according to Kurtzman and Robnett (1998) with slight modification. Cycle

sequencing of the D1/D2 domain was employed with forward primer NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3'), and reverse primer, NL4 (5'-GGT CCG TGT TTC AAG ACG G-3'), by ABI Prism™ BigDye™ Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems, Stafford, USA) according to the manufacturer's instruction.

3.1.2 BLAST analysis

The sequences of D1/D2 domain of 26S rDNA were compared by BLASTn Homology Search (http://www.ncbi.nlm.nih.gov/blast).

3.1.3 Phylogenetic analysis

Generated sequences were aligned with related species by using the CLUSTAL X ver. 1.8 computer programs (Thompson et al., 1997). The phylogenetic trees were constructed from the evolutionary distance data according to Kimura (1980) by the neighbor-joining method (Saitou and Nei, 1987). Sites where gaps existed in any sequences were excluded. Bootstrap analysis (Felsenstien, 1985) is performed from 1,000 random re-samplings.

3.1.4 DNA-DNA reassociation

DNA-DNA reassociation was carried out by photobiotin microplate hybridization method (Ezaki et al., 1989).

a. Isolation and purification of DNA. Two methods were used for isolation and purification of DNA.

Method I: This method was modified from Holm et al. (1986). A loopful of cells was inoculated into 50 ml of YPD in a 200 ml flask and incubated overnight at 25° C with shaking. Cells were harvested by centrifugation in a sterile 25 ml centrifuge tube at 3,000 rpm for 5 min. The cell pellet was resuspended in 20 ml of ice-cooled 50mM EDTA (pH 7.5). The pellets were collected and resuspended in 4 ml SCE (Appendix A) after centrifuge at 6,000 rpm for 5 min. Suspended cells were lysed by incubation with 0.1 ml Zymolyase solution (Appendix A) at 37°C for 1-1.5 hr. The supernatant was removed completely after centrifugation at 15,000 rpm for 3 min. Two volume of ice cooled of 99% ethanol was added and mixed gently after the

solution reached to room temperature. The supernatant was removed completely after centrifugation at 12,000 rpm for 3 min. The DNA pellet was dissolved completely in 1 ml of 10X TE (Appendix A) with a spatula. The DNA solution was incubated with 30 µl RNase solution (Appendix A) at 37°C for 1 hr. Then the DNA solution was incubated with 30 µl Proteinase K solution (Appendix A) at 65°C for 30 min. After the DNA solution reached to room temperature, 3 ml Tris saturated phenol was added and mixed. Aqueous layer was removed to a clean centrifuge tube after centrifugation at 15,000 rpm for 5 min. DNA was precipitated with 1/10 volume of sodium acetate and 2 volumes of ice cooled 99% ethanol with gently shaking to mix. Precipitated DNA was washed by mixed with 500 µl of 80% ethanol. Purified DNA was dried at 65°C for 15-30 min. Dried DNA was incubated with 500 µl TE at 50°C to dissolve. DNA solution was purified again by 300 µl Tris saturated phenol and mixed. Aqueous layer was transferred to a clean centrifuge tube after centrifugation at 12,000 rpm for 5 min. Aqueous layer was incubated with 10 µl RNase at 37°C for 1 hr. The solution was incubated with 10 µl Proteinase K solution at 65°C for 30 min. After the solution reached to room temperature, 300 µl Tris saturated phenol was added and mixed by vortex. Aqueous layer was transferred to a clean centrifuge tube after centrifugation at 12, 000 rpm for 5 min. DNA was precipitated with 1/10 volume of sodium acetate and 2 volumes of ice cooled 99% ethanol with gently shaking to mix. Precipitated DNA was transfered to a microtube containing 500 µl of 80% ethanol. Purified DNA was dried at 65°C for 15-30 min. Dried DNA was dissolved in 100-500 µl of sterilized distilled water. Protein and the DNA concentration were measured by spectrometer. Unpurified DNA was precipitated and purified again to clean up DNA further.

Method II (Modification of Mamar's Methods): Cells were grown in 500 ml Erlenmeyer flask containing 250 ml of YM broth on a rotary-shaker at 150 rpm at 25°C and were harvested in the logarithmic growth phase. The cells were washed twice with 1/15 M phosphate buffer with 0.1 M EDTA (pH 7.5). Cells (5-10 g) were suspended in 1.5 vol. of 1/15M phosphate buffer with 0.1 M EDTA and lysed by incubation with 5 mg of 100T Zymolyase and 0.2 ml of mercaptoethanol for 30 min under gentle shaking. The suspension was incubated with 0.5% of SDS at 35°C for

15min. The reaction mixture was vigorously shaken with equal volume of chloroform:isoamyl alcohol (24:1) and 5% of sodium perchlorate to denature proteins. The supernatant was removed after centrifugation. DNA was precipitated by addition of 2 vol. of chilled ethanol, and then spooled around a glass rod. Crude DNA was dissolved in 4.5 ml of 0.1 XSSC, then 0.5 ml of 10 XSSC was added. The remaining protein was digested with Proteinase K treatment at 37°C for 30 min. The solution was repeatedly by treated with 1 volume of chloroform:isoamyl alcohol (24:1) with vigorous shaking, and then centrifuged to get thin denatured protein layer. RNA was removed by incubation with 0.2 mg of RNase A at 37°C for 2 hr. DNA solution was treated with chloroform:isoamyl alcohol (24:1) to remove RNase. After centrifugation, DNA was precipitated with ethanol, and then spooled around a glass rod. DNA was dissolved in 4.5 ml 0.1X SSC and 0.5 ml of 10X SSC (Appendix A) was added. After addition of 0.5 ml of acetate-EDTA, 0.54 vol. of chilled isopropanol was added and DNA was spooled around a glass rod. Purified DNA was washed stepwise with 70%, 80%, 90% and 99.5% ethanol.

b. Immobilization of DNA. DNA solution was heated in boiling water bath for 5 min, and then quickly transferred to ice water bath. Heat denatured DNA solution was diluted to 4 ng/μl with PBSM1 (Appendix A). 100 μl of diluted DNA solution was dispensed to respective wells of microplate (Immunoplate MaxiSorp, Nunc), and then left at 28°C for 16 hr. The solution was discarded from wells, and washed twice with 200 μl of PBS, then the plate was dried at 60°C for 2 hr. Dried plate was stored at room temperature until use.

c. Labeling of DNA with photobiotin. Amount of 0.5-1 mg DNA was dissolved in 1 ml sterilized milli Q water. DNA solution was mixed with two volumes of photobiotin solution (Appendix A), with vigorous shaking in the dark room (the amount of solution should be 50 μl). Eppendorf tube (without cap) was stored on ice for 20 min under murcury lamp (distance ca. 10 cm). TE buffer, pH 9.0 was added in Eppendorf tube to get the final volume of 100 μl. After that 100 μl of isobutanol was added and mixed well by shaking. Upper layer (isobutanol layer) was

removed after centrifugation at 12,000 rpm for 10 sec. This step was repeated again. The pellet was dissolved in 200 μl of water, and then 5 μl of 3M sodium acetate solution and 100 μl of ethanol were added to the Eppendorf tube. The Eppendorf tube was stored in -20°C freezer over night and centrifuged at 12,000 rpm to obtain brown colored precipitate. The DNA pellet was washed with 70% ethanol and dried. DNA pellet was dissolved in approximate amount of TE and stored at -20°C until use.

d. Hybridization. Amount of 200 μl of pre-hybridization solution was added to respective wells with immobilized DNA, and then incubated at 37°C for 30 min. Microplate was turned upside down and liquid was removed carefully. Amount of 100 μl of hybridization solution containing 0.5 μg of heat-denatured photobiotin labeled DNA was added to respective wells and cover with plate seal, then left at 42°C for 24 hr. The solution was removed and wells were washed two times with 0.2X SSC.

e. Binding of streptavidin-β-galactosidase complex (SABG) to DNA. Amount of 100 μl of PBS-BSA-Triton solution (Appendix A) was added into wells and left at room temperature for 10 min. The solution was removed, and 100 μl of SABG solution (Appendix A) was added and left at 37°C for 30 min then wells were washed two times with 200 μl of PBS (Appendix A).

f. Measurement of β-galactosidase activity (measurement of 4-MUF). Amount of 200 μl of MUF-Gal solution (Appendix A) was added into wells. After that, 1 μl/ml MUF-Gal solution was added in A01 well for primary setting and checking of the microplate reader, and MUF-Gal solution was used as a blank by adding to A02 well, left at room temperature. The results were checked by measuring the released 4-methylumbelluferon by microplate reader, after 0 min, 10 min, 20 min and 30 min.

g. Calculation of% DNA similarity

% Similarity of DNA=100X(X-N) / (P-N)

X: measured value of DNA to be tested

P: measured value of DNA as probe

N: measured value of DNA that has no relationship to DNA to be tested

(P/N should be more than 8-10)

3.1.5 Determination of DNA base composition

DNA base composition was analyzed according to the method of Nakase and Suzuki (1986b). Amount of 20 µl (300-500 mg) of DNA solution (from 14.1) in 0.1XSSC was boiled for 10 min and rapidly cooled in ice water. Amount of 20 µl of 2 unit/ml Nuclease P1 was added and incubated at 50°C for 60 min. Amount of 20 µl of 2.4 unit/ml alkaline phosphatase was added and incubated at 37°C for 60 min. The DNA base composition was determined by HPLC using Cosmosil column (Waters, 5C18, 4.6 mm x 250 mm). The elution system was 0.02 M $NH_4H_2PO_4$: acetonitrile (20:1 v/v) at a flow rate of 1 ml/min. The nucleosides were detected at 270 nm. DNA-GC Kit (Yamasa Co., Tokyo) was used as the quantitative standard.

3.2. Morphological characteristics

Most of methods used to examine the morphological, physiological and biochemical characteristics are those described in The Yeasts, a Taxonomic Study, 4th ed. (Yarrow, 1998) except for several experiments mentioned later.

3.2.1 Morphology of vegetative cells

Yeast culture was inoculated in YM broth and incubated at 25°C for 3-5 days. The morphology of cells was examined under microscope. The shape, arrangement and the size of cells were recorded.

3.2.2 Cultural characteristics

Yeast culture was inoculated on YM agar and YM broth and incubated at 25°C until 4 weeks. The characteristics of colony on YM agar such as

color, size, surface, margin, and texture were recorded. The characteristics of culture observed in YM broth in the formation of sediment, coherent, flocculent or mucous, a ring, islets or a pellicle.

3.2.3 Pseudomycelium and true mycelium formation

The formation of pseudomycelium and true mycelium were examined using slide culture method. Glass slide in Petri dish with U-shaped glass rod (on a filter paper) and a tweezers wrapped with aluminium foil are sterilized at 160-170°C for 30-60 min. Sterilized glass slide was removed from Petri dish by sterilized tweeezer and was dipped into molten 50-55°C PDA (Difco), then placed on U-shaped glass rod in the Petri dish and allowed agar to solidify. After drying of agar surface, yeast was inoculated with a tiny loop (light inoculum), a sterilized cover slip was placed on the line and incubated at 25°C. The formation of pseudomycelium and true mycelium were examined under microscope at a few day's intervals until 2 weeks.

3.2.4 Morphology of ascospores

The active young culture grown on YM agar was streaked on YM agar, Acetate-GSH agar (Appendix A) and 5% malt extract agar (Appendix A). Mode of ascus formation and shape, surface structure, number per ascus and size of ascospores were examined under microscope after incubation for 7, 14, 21 and 28 days at 25°C.

3.2.5 Morphological characteristics of vegetative cells and ascospores under scanning electron microscope

Yeasts were grown in 1.5 ml YM broth for at 25°C 48 hr. The 0.5 ml of 1-2% glutaraldehide in 1/15 M phosphate buffer pH 7.0 was added to yeast culture. Yeast cells were harvested by centrifugation. Yeast cells were fixed by 0.5 ml 1-2% glutaraldehide in 1/15 M phosphate buffer pH 7.0 at room temperature for 1 hr 30 min, then centrifuged and washed with phosphate buffer for 3 times. The 0.5 ml of 2% osmium was added and left at the room temperature for 1-2 hr, then washed with phosphate buffer for 3 times. Fixed cells were washed stepwise with 0.5 ml of

increase alcohol concentration from 30%, 50%, 70%, 80%, 90%, 95% for 30 min and 100% for 30 min for 2 times. Fixed cells were dried with 0.5 ml of alcohol:acetone (1:1) for 30 min. After centifugation, fixed cells were dried twice with acetone for 30 min then centrifuged and 0.5 ml of isoamyl acetate:acetone (1:1) was added and left for 30 min, then centrifuged. Isoamyl acetate was added and left for 1 hr, then centrifuged. This step was repeated. Cells were moved to critical point dryer then coat by platinum. Cells were observed by scanning electron microscope.

3.3 Physiological and biochemical characteristics

3.3.1 Assimilation of carbon compounds

Assimilation of carbon compounds was investigated in liquid media according to the method described by van der Walt and Yarrow (1984) and Yarrow (1998). In the present study 42 carbon compounds were employed in the description of each species (Table 8). Ten folds medium stock solution (Appendix A) was prepared by filter sterilization and stored in a freezer at -20°C until use. Amount of 0.2 ml of thawed stock solution was added to 1.8 ml of sterilized water in a cotton plugged test tube (13x100 mm). Media for inulin, soluble starch, ethanol, galactitol, 2-ketogluconic acid and 5-ketogluconic acid were prepared at every experiment. In the case of ethanol and 5-ketogluconic acid, 3% and 0.3% solution were employed, respectively. The young culture grown on YM agar was used as the inoculums. A very light suspension in Yeast Nitrogen Base (YNB) medium was prepared and incubated at 25°C for 1 week to exhaust the carbohydrate pool in the cells.

A drop of inoculums was inoculated to the carbon assimilation test medium by sterilized Pasteur pipette and incubated at 25°C for 4 weeks. Basal medium without carbon source was employed as a negative control. The growth was observed and recorded every week up to 4 weeks. After dispersed the cells by shaking, the degree of growth was assessed by eye by placing tubes against a white card on which lines of 0.75 mm thick were drown 5 mm apart with Indian Ink. The result was scored as +++ if the lines are completely obscured; as ++ if the lines appears as diffuse bands; as + if the lines are distinguishable as such but have blurred edges; as – if the lines were distinct and sharp edged.

The results are presented in the descriptions as follows:

+, positive, either a ++ or +++ reading after 1 week, or 2 weeks

l, delayed positive (latent), a ++ or +++ reading develops rapidly, but after 2 weeks or longer

s, slow positive, a ++ or +++ reading develops slowly over a period exceeding 2 weeks

w, weakly positive, a + reading

-, negative

v, variable: some strains are positive, others negative

+/w, positive or weak: all strains grow, but some of them grow slowly

w/-, weak or negative

Table 8 Carbon compounds used in the description of each species

Group	Carbon compounds
Hexoses	Glucose, galactose and sorbose
Disaccharides	Cellobiose, lactose, maltose, melibiose, sucrose and trehalose
Trisaccharides	Melizitose and raffinose
Polysaccharides	Inulin and soluble starch
Pentose	D-arabinose, L-arabinose, D-ribose, L-rhamnose and D-xylose
Alcohols	Galactitol, meso-erythritol, D-glucitol, glycerol, myo-inositol, D-mannitol, ribitol, ethanol and methanol
Organic acids	Citric acid, DL-lactic acid, succinic acid and D-gluconic acid
Glycosides	α-methyl-D-glucoside and salicin
Other compounds	D-glucosamine hydrochloride, *N*-acetyl-D-glucosamine and hexadecane
Additional compounds	2-keto-D-gluconate, 5-keto-D-gluconate, saccharate, xylitol, D-glucuronate and L-arabinitol

3.3.2 Assimialtion of nitrogen compounds

Assimilation of nitrogen compounds was investigated on solid media using starved inoculum according to the method of Nakase and Suzuki (1986a). Six kinds of nitrogen compounds including amonium sulfate ($(NH_4)_2SO_4$), potassium nitrate (KNO_3), sodium nitrite ($NaNO_2$), ethylamine, L-lysine and cadaverine were employed. The young culture grown on YM agar was used as the inoculums. A very light suspension in YCB medium (Appendix A) was prepared and incubated at 25°C for 1 week to exhaust the carbohydrate pool in the cell. A half-drop of starved culture was inoculated on nitrogen agar plate with sterilized Pasteur pipette and incubated at 25°C. The YCB agar without nitrogen source was used as a negative control. The growth of yeast was observed every 2-4 days up to 14 days.

3.3.3 Production of starch-like substances

The production of starch-like substance was determined according to the mothod described by Wicherham (1951). After assimilation test was finished, Lugol's solution (Appendix A) was added to glucose assimilation medium and anmonium sulfate assimilation medium. A positive result was indicated by the development in the culture of a color varying from dark blue to green.

3.3.4 Fermentation of sugar

Fermentation test was examined according to the method described by Wickerham (1951). A loop full of young culture grown on YM agar was inoculated into fermentation medium (Appendix A) and incubated at 25°C. Carbondioxide gas production was observed and recorded every day until 7 days and then at 14, 21 and 28 days of cultivation.

3.3.5 Vitamin requirements

Vitamin requirements were determined according to Komagata and Nakase (1967). Young culture grown on YM agar for 2-4 days was suspended in the basal medium (Appendix A) and incubated for 5-7 days at 25°C to deplete the vitamins carried from the pre-culture medium and vitamin pool of cells. A drop of

starved cell suspension was inoculated into the test medium then incubated at 25°C for 3 weeks. The growth of yeast was observed at 3, 5, 7, 10, 14 and 21 days.

3.3.6 Miscellaneous tests

a. Gelatin liquefaction

The ability to liquefy gelatin was examined according to the method described by Wickerham (1951). The yeast was inoculated by a needle into the gelatin medium (Appendix A) tube and incubated at 25°C. The cultures were examined regularly up to 3 weeks for signs of liquefaction.

b. Lipase test

Lipase activity was determined according to Eijkman (1901). Yeast cells was inoculated by streaking on the lipase test medium (Appendix A) and incubated at 25°C for 1 week. The result was recorded as positive if the strain produced an opaque zone.

c. Acid formation from glucose

The production of acid from glucose was determined according to van der Walt (1970). The yeast was streaked onto slants or plate of Custer's chalk medium (Appendix A) and incubated at 25°C for 2 weeks for clearing of the medium around the streaks. The result was recorded as positive if the chalk dissolved.

d. Cycloheximide resistant

Cycloheximide resistant was determined according to Whiffen (1948). This experiment was examined in liquid Bacto Yeast Nitrogen Base with D-glucose (basal medium), with cycloheximide added to give a final concentration of either 100 ppm or 1000 ppm. The inoculation and detection was done in the same way as for the carbon assimilation test.

e. Growth at 50% glucose medium

The ability to at high sugar concentration was examined according to the method described by Wickerham (1951). Young culture of yeasts was inoculated on a slant of 50% glucose agar medium (Appendix A). The growth of yeast was observed after 7 days.

f. Maximum growth temperature

The maximum growth temperature was examined according to the method described by Wickerham (1951). Young culture of yeasts was inoculated into YM broth medium. The growth of yeast was observed every week until 3 weeks. The result was detected as same as the assimilation of carbon compounds.

3.4 Chemotaxonomic Characteristics

3.4.1 Ubiquinone analysis

The analysis of biquinone system was carried out following the method of Nakase and Suzuki, 1986b). Cells grown in YPD medium (1% yeast extract, 2% peptone and 2% glucose) at 25°C for 16-30 hr with shaking were harvested by centrifugation at 6,000 rpm for 5 min. Harvested cells from 400 ml of medium were washed with distilled water and freeze-dried. Dried cells are suspended in 50 ml chloroform/methanol (2:1) and kept at room temperature for one night. Cells were removed by paper filtration. Filtrate was evaporated to dryness using a rotary evaporator and then residues were dissolved with 0.5 ml of acetone. Ubiquinone was purified by using the preparative thin-layer chromatography (0.5 mm silica gel, 60F254 layers on 20 x 20 cm glass plate, Merck, with hexane:diethyl ether (85:15) as developer). A band of ubiquinone detected under short wave UV light was scrapped off. Yeast having respective ubiquinones, Q-6 (*Saccharomyces cerevisiae,* NBRC 10515), Q-7 (*Pichia anomala* NBRC 10213), Q-8 (*Saccharomycopsis vini* NBRC 1749), Q-9 (*Debaryomyces hansenii* var. *hansenii* NBRC 1751) and Q-10 (*Schizosaccharomyces pombe* NBRC 1608*)* were used as references. Scrapped powder was transferred to a tube and extracted with 1 ml of acetone. The solution was filtered with a 0.2 μm membrane filter and concentrated by the blow of N_2 gas.

Ubiquinone homologues were identified by HPLC (Cosmosil column (Waters, 5C18, 4.6 mm x 250 mm), using methanol:isopropyl alcohol (2:1) as mobile phase at flow rate of 1 ml/min, detected at 275 nm, and identified by comparing with known ubiquinones as standards).

3.4.2 Characterization of cell wall polysaccharides

Yeasts were grown on YM agar plates supplemented with 2% glucose for 3 days at 25°C and harvested with by centrifugation after filtration of cell suspensions by defatted cotton cloth, then was washed twice with purified water. Mannose-containing polysaccharides were extracted and purified according to the methods of Gorin and Spencer (1970). The polysaccharides were extracted by boiling of ca. 5 g of packed cells with 25 ml of 2% potassium hydroxide solution for 2 hr. After cooling the suspension was neutralized with acetic acid to pH 6-7 and centrifuged at 15,000 rpm for 7 min. The supernatant was evaporated to approximately 10 ml, then 200 ml of methanol was added and mixed and placed overnight at 5°C. After centrifugation at 15,000 rpm for 7 min, the precipitate was washed twice with 40 ml of methanol and 3 times with 40 ml of acetone, then air-dried at room temperature. Fine powder of crude polysaccharide (0.2 g) was dissolved with 10 ml of water and boiled for 2 hr. The insoluble materials were removed by centrifugation at 15,000 rpm for 7 min, then 10 ml of Fehling solution was added to the supernatant and mixed, then left overnight at 5°C. The copper complex was washed twice with 20 ml of 2% potassium hydroxide and 3 times with 20 ml of methanol. The copper complex was decomposed by shaking in purified water with Amberite IR 120. The resins were washed twice with small amount purified water. The polysaccharide solution was evaporated to approximately 2 ml, then 20 ml of methanol and one drop of conc. hydrochloric acid were added. After centrifugation, the precipitated was washed 3 times with 2 ml of methanol and dried by washing 3 times with 2 ml of acetone.

The purified polysaccharide was hydrolyzed with 1N H_2SO_4 (10 mg/ml) at 100°C for 18 hr. The hydrolyzate was neutralized by adding barium hydroxide ($Ba(OH)_2$) to pH 6-7 and centrifuged at 15,000 rpm for 7 min. The supernatant was applied to TLC plate with ethyl acetate:isopropanol:water, 65:22:11 (v/v), as developer. A band of polysaccharide was detected by spraying with *p*-anisidine solution (Appendix A). D-glucose, D-mannose and D-galactose were used as standard sugar. The proton NMR spectra of purified polysaccharides were determined in D_2O at 70°C with tetramethylsilane as external standard by JEOL 500 MHz NMR spectrometer.