# Summary

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United Graduate School of Agricultural Sciences
Course：Biological Science and Technology
Name：Ahangangoda Arachchige Maduka Subodinee

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| **Title** | Studies on yeast strains from Coconut (*Cocos nucifera*) Toddy in Sri Lanka: the tolerance to high temperature, salt, and inhibitors derived from lignocellulosic hydrolysates and mechanism of thermotolerance |

Key word（**Yeast**） （**Tolerance**） （**Fermentation**）

**“Introduction and Purpose”**

Coconut toddy is a kind of alcoholic beverage, and it acquires about 3% - 4.5% (w/v) alcohol within 24 h with a considerable quantity of live microorganisms. Since the samples collected from the coastal area of the Sri Lanka which has a tropical climate, I assume that the isolated yeast strains should show greater salt tolerance and thermotolerance. Most previous investigations of yeasts isolated from fermenting coconut sap have been limited only to the identification of the microorganisms and the detailed research on the physiological characterization of these toddy yeasts had not been completed previously.

Lignocellulosic biomass from industrial and agricultural residues is considered to be an ideal substrate for the production of bioethanol because it is the most abundant renewable feedstock on the planet and does not compete with foods for human beings, and the use of the biomass supports an environmentally sustainable process. However, although lignocellulosic materials contain 70% carbohydrates (cellulose and hemicellulose), several pretreatments are necessary in order to convert carbohydrates to fermentable sugars. With high temperature, numerous byproducts are generated: such as phenolic compounds including vanillin and 4-hydroxybenzoic acid (PHBA), furan derivatives, like5-hydroxymethyl-2-furaldehyde (5-HMF) and furfural, and weak acids (acetic acid and formic acid). These byproducts are toxic to yeast cells and eventually affect alcohol production. Klinke et al. reported that among those inhibitors, vanillin is found to be the most potent inhibitor, produced by the degradation of lignin from lignocellulosic materials, and it inhibits ethanol production at low concentrations. To produce alcohol from the hydrolysates of lignocellulosic materials in the presence of such inhibitors, *S. cerevisiae* strains with high tolerance to those inhibitors are required. Thermotolerance yeast strains are highly beneficial to the bioethanol industry, and the mechanism underlying the thermotolerance of these strains is still not known. Caspeta et al. have found that the nonsense mutation at position 185 in *ERG3* was seen in most of thermotolerant strains obtained by adaptation in the laboratory, and increased expression of genes involved in sterol biosynthesis, and then substantially changed the sterol composition in the membrane and were responsible for most of the thermotolerance. Several previous studies found that thermotolerance yeast possess high ergosterol content, and the *ERG3* gene is positively responsible for

ergosterol synthesis. Thus, the present research study was undertaken to isolate the yeast strains from coconut toddy at first and then the examined the physiological and biochemical characteristics of the isolated yeast strains following by observing the growth and fermentation ability of strains under different stress conditions. Finally, the mechanisms of thermotolerance was investigated.

**"Materials and method"**

Isolation of microorganisms was carried out by an enrichment technique. After inoculation, cultures were incubated for 48 h in a rotary shaker (200 rpm) at 30°C. The colonies with distinct morphological characteristics were selected and purified by repeatedly streaking on YPD agar medium. Stock cultures of the isolated strains were prepared by mixing with 30% (v/v) sterile glycerol and were frozen at −80°C until use. To identify the isolates, the PCR amplification of the 26S rDNA D1/D2 gene region was performed using the primers NL1: 5′-GCATATCAATAAGCGGAGGAAAAG - 3′ and NL4: 5′- GCTCCGTGTTTCAAGACGG- 3′. The purified DNA samples (<20 ng/µL) were sequenced with NL1 and NL4 primers and then the obtained DNA sequences were analyzed using BLAST (NCBI).

Pre-cultured yeasts were prepared on yeast extract-peptone-dextrose (YPD) (1.0 g of yeast extract, 2.0 g of polypeptone, 2.0 g of D-glucose per 100 mL distilled water) medium agar plate was transferred into 5 mL YPD broth and incubated at 30°C with agitation (200 rpm) for 22–24 h (McFarland units = 16.6 or $1x10^7$ CFU· $mL^{-1}$). A widely used laboratory yeast strain, S288C and Awamori yeast 101-18 strains were used as reference strains. All experiments were independently performed three times and expressed as means with standard deviations.

The growth of yeast strains on YPD media supplemented with different salt concentrations (5%, 7.5%, 10%, 11%, 12.5%, 13%, and 13.5%) or incubated at high temperatures (30°C, 37°C, 40°C, 41°C, 42°C, and 43°C) was measured by turbidity changes over time using McFarland units using a densitometer DEN–IB (Waken Btech Co. Ltd, Kyoto, Japan).

Because all the strains grew well at 30°C in the presence of 7.5% NaCl, I examined the yeast tolerance in the presence of 7.5% NaCl at 37°C or 40°C. Five milliliters of YPD liquid medium supplemented with 7.5% NaCl (w/v) were inoculated with 50 µL of pre-culture and then incubated at 37°C or 40°C with shaking at 200 rpm for 4 – 5 days. Cell growth was monitored by measuring culture turbidity as McFarland units.

Alcohol production by all *S. cerevisiae* isolated strains was investigated on batch-fermented media containing 1.0 g of yeast extract, 2.0 g of polypeptone, 10.0 g of glucose per 100 mL distilled water under static conditions. Alcohol production was examined for all strains at different temperatures: 30°C, 37°C or 40°C. Yeast strains exhibiting the highest alcohol production at 40°C were used to further examine their fermentation abilities at 41°C, 42°C, 43°C and 45°C; cultures were incubated under static conditions for 4 - 5 days. I also attempted to examine the fermentation ability of selected yeast strains using 160 g· $L^{-1}$glucose at 30°C, 40°C and 45°C. Collected 1 mL samples in 12-h intervals were centrifuged at 6,000 rpm for 5 min (4°C) to obtain the supernatant. Alcohol quantification from collected supernatants was carried out using the enzyme alcohol dehydrogenase (ADH) extracted from membrane fraction of acetic acid bacteria.

The 18 *S. cerevisiae* strains (SLY-1 to SLY-18) isolated from coconut toddy were used to examine the inhibitor tolerance in the presence of 3–24 mM vanillin (Wako, Japan). Selected yeast strains (SLY-3, SLY-4, SLY-8, SLY-9 and SLY-10) were used to examine their tolerance in the presence of up to 24 mM PHBA (Alfa Aesar, USA), up to 36 mM 5-HMF (SIGMA-ALDRICH, USA), up to 30 mM furfural (TCI, Tokyo, Japan), up to 75 mM acetic acid (NACALAI TESQUE, Kyoto, Japan) and up to 75 mM formic acid (Cica, KANTO CHEMICAL CO., INC., Tokyo, Japan). Prepared medium with inhibitor solution was subsequently inoculated with pre-cultured yeast (50 µL) and incubated at 30°C with shaking at 200 rpm. Cell growth was monitored by measuring culture turbidity.

To determine the remained vanillin in the culture during the lag or exponential phase of yeast growth, culture supernatants supplemented with 3–21 mM vanillin from the five strains (SLY-3, SLY-4, SLY-8, SLY-9 and SLY-10) were analyzed by high-performance liquid chromatography (HPLC). The remained vanillin concentrations were determined using HPLC (Waters$^{TM}$ LC Module 1 Plus, Japan) equipped with a Shodex RS pak DE-413L (4.6 mm I.D. x 250 mm) column (Showa Denko k. k, Tokyo, Japan). Prepared samples (50 µL) were separated with a linear gradient (0–100%) of acetonitrile (HPLC grade, Wako, Japan) to the MiliQ water with the flow rate of 1.0 mL· min$^{-1}$. Peaks were detected at 280 nm. Vanillin, vanillyl alcohol and vanillic acid concentrations in the samples were quantified using the corresponding standard curve prepared with the standard solutions.

To determine the residual furfural and 5-HMF during the lag, exponential and stationary phases of yeast growth, I analyzed the culture supernatants supplemented with 15-30 mM furfural and 30 mM 5-HMF from five strains (SLY-3. SLY-4, SLY-8, SLY-9 and SLY-10) by HPLC. Sampling was done by short intervals, and the culture supernatants were analyzed using HPLC with a linear gradient (5–95%) of acetonitrile (HPLC grade, Wako, Japan) to the 0.01% Trifluoroacetic acid (TFA) in MiliQ water with the flow rate of 1.0 mL min$^{-1}$. Furfural and 5-HMF were detected at 280 nm.

The fermentation abilities of the selected *S. cerevisiae* strains (SLY-3, SLY-4, SLY-8, SLY-9 and SLY-10) in the presence of 2.7–24 mM vanillin were examined under static conditions. The effect of incubation temperature (30°C or 40°C) on fermentation in the presence of vanillin on yeast extract-peptone (YP) medium containing 100 or 160 g· L$^{-1}$ glucose was investigated.

In addition to the vanillin, the alcohol production of all the five strains with other inhibitor alone in YP medium contained 100 g· L$^{-1}$ glucose: furfural up to 30 mM, 5-HMF up to 30 mM, acetic acid up to 75 mM, or formic acid up to 75 mM was also examined in the same way.

To examine yeast fermentation of selected *S. cerevisiae* strains (SLY-3, SLY-4, SLY-8, SLY-9 and SLY-10) in the presence of inhibitors, I prepared the YP medium containing 100 g· L$^{-1}$ glucose supplemented with the inhibitor mixture, 2.7 mM (0.41 g· L$^{-1}$) vanillin, 30 mM (3.8 g· L$^{-1}$) 5-HMF, 30 mM (2.9 g· L$^{-1}$) furfural, 75 mM (4.5 g· L$^{-1}$) acetic acid, and 75 mM (3.5 g· L$^{-1}$) formic acid to achieve initial concentrations of 100%. Then the medium with 100% inhibitor mixture was diluted to achieve 70%, 60%, 40%, 35%, 30% and 20% (v/v). Alcohol contents in the collected supernatants were quantified enzymatically by using ADH.

The DNA fragments containing the *ERG3* gene from all the five yeast strains (SLY-3, SLY-4, SLY-8, SLY-9, and SLY-10) were obtained by colony PCR and the amplification was performed using the primers 1st_seq_ERG3_fw and 1st_seq_ERG3_rev. Because of the DNA fragments of several yeast strains were not successfully amplified by colony PCR, the genomic DNA was extracted following by Hereford method and used for PCR. The purified DNA (~50 ng/µL) were sequenced with set of sequenced primers by several rounds and the obtained sequences were analyzed using BLAST (NCBI).

Selected five yeast strains (SLY-3, SLY-4, SLY-8, SLY-9, and SLY-10) that showed thermotolerance, inhibitor tolerance and produced substantial amounts of alcohol at high temperatures were used to determine the ergosterol contents at different temperatures. Also, three of the reference strains; two laboratory yeast strain, S288C (haploid), BY 4741 (diploid), and Awamori yeast strain 101-18, were used for this experiment. Freeze-dried yeast cells with methanol: propanol (1:1) were homogenized using the Ultra-sonic homogenizer (Smurt NR-50M). Extracted sterols were separated using HPLC (Waters™ LC Module 1 Plus, Japan) equipped with a COSMOSIL packed 5C18 – PAQ (4.6 mm I.D. x 250 mm) column (WATERS). Methanol at 0.8 mL/min was used as its mobile phase and the corresponding peaks for the ergosterol was detected at 282 nm.

**"Results"**

I isolated 27 yeast strains from coconut toddy in Sri Lanka. Sequences of the 26S rDNA gene D1/D2 region from 18 strains showed 100% identity to *Saccharomyces cerevisiae*, eight strains showed 99% identity to *Pichia manshurica* and a single isolate showed 99% identity to *Saccharomycodes ludwigii*. Regarding salt tolerance, all isolated strains of *S. cerevisiae* were found to grow in the presence of 10.0% (w/v) NaCl in yeast extract-peptone-dextrose (YPD), one of the isolates (SLY-10) grew even with 13.0% (w/v) NaCl. All the isolates grew well at 40°C, and the 18 *S. cerevisiae* strains showed significant growth even at 42°C. All the 18 *S. cerevisiae* strains still showed growth better than reference strains did in a medium with 7.5% (w/v) NaCl at 40°C. All *S. cerevisiae* isolates produced relatively high alcohol concentrations than reference strains did when growing on batch-fermented media with 100 $g \cdot L^{-1}$glucose in at 40°C. The five strains (SLY-3, SLY-4, SLY-8, SLY-9 and SLY-10) produced significant amounts of alcohol even at 45°C: alcohol productivity over 24 h at 40°C and 45°C was similar in two different glucose concentrations, and the amount of produced alcohol was higher with 160 $g \cdot L^{-1}$ glucose than with 100 $g \cdot L^{-1}$glucose, significantly at 45°C.

All the 18 strains (SLY-1 to SLY-18) could grow aerobically up to 18 mM vanillin in yeast extract-peptone-dextrose (YPD) medium, and the SLY-10 strain showed the highest vanillin tolerance (up to 21 mM). Among these 18 strains, even in the presence of 24 mM vanillin, five strains (SLY-3, SLY-4, SLY-8, SLY-9 and SLY-10) produced alcohol at 72 h in the range of 11.4–33.1 $g \cdot L^{-1}$ and the SLY-10 strain showed highest alcohol production. The five strains showed the conversion of vanillin to vanillyl alcohol. They also tolerated other strong inhibitors: 4-hydroxybenzoic acid (PHBA, 24 mM), furfural (30 mM), 5-hydroxymethyl-2-furaldehyde (5-HMF, 36 mM) and acetic acid (75 mM). All these five strains showed no growth and no alcohol production when cultured in the medium with inhibitor mixture of 2.7 mM vanillin, 30 mM furfural,

30 mM 5-HMF, 75 mM acetic acid and 75 mM formic acid but showed significant growth and alcohol production 16.2–32.7 g· $L^{-1}$ with inhibitor mixture of 30% concentration (0.81 mM vanillin, 9 mM furfural, 9 mM5-HMF, 22.5 mM acetic acid and 22.5 mM formic acid). The four strains (SLY-3, SLY-4, SLY-8 and SLY-9) were shown to tolerate the inhibitor mixture to a similar extent and better than SLY-10, while SLY-10 was the best to vanillin alone.

Ergosterol is a principle sterol in plasma membrane of the yeast cells which mainly regulates the membrane permeability and structure fluidity. Caspeta et al. showed that mutation in *ERG3* gene substantially changed the sterol composition in the membrane and were responsible for most of the thermotolerance. I analyzed the sequences of *ERG3* gene and the ergosterol contents in the cells grown at 30°C, 37°C, and 40°C. I found that in all the five strains, the same two single nucleotide variant (SNVs) in the *ERG3* gene, one is from C to T at the position at 279, and the other is from T to C at the position 461. The former SNV lead to no amino acid substitution, and the latter lead the change of the amino acid residue from valine to alanine, therefore *ERG3* gene in the five strains is normally functional as in the other strain. According to the sequence analyses, ergosterol contents in the five strains are comparable to the reference strains. The ergosterol contents of the five strains were higher at 37 or 40°C than at 30°C, and in fact, three strains (SLY-3, SLY-9, and SLY-10) showed significant amounts of ergosterol at 40°C than that of at 37°C.

**“Conclusion and Consideration”**

All the isolated strains of *S. cerevisiae* showed the tolerance in the presence of 10% (w/v) NaCl. Interestingly, the only one strain among all isolates, SLY-10 strain showed the tolerance up to 13.0% (w/v) NaCl. All the isolates grew well at 40°C and all the *S. cerevisiae* strains grew even at 42°C. Moreover, I found that all the isolated strains of *S. cerevisiae* still grew at 40°C in the presence of 7.5% (w/v) NaCl on YPD. All the strains of *S. cerevisiae* produced substantial amounts of alcohol with 100 g· $L^{-1}$ glucose at 40°C and five strains (SLY-3, SLY-4, SLY-8, SLY-9 and SLY-10) showed the significant ethanol production with 160 g· $L^{-1}$ glucose even at 45°C. The strains obtained in this study would be useful in bioethanol industry because of their robust properties.

Throughout this work, I obtained five yeast strains which tolerated vanillin and other strong inhibitors profoundly appeared in lignocellulosic materials. All the five strains showed the tolerance to high concentrations of vanillin by converting toxic vanillin into vanillyl alcohol. Interestingly, the SLY-10 strain showed much higher alcohol production in the presence of vanillin (18 mM) at 40°C with 160 g· $L^{-1}$ glucose than with 100 g· $L^{-1}$ glucose. All the five strains produced considerable amounts of alcohol with each inhibitor alone and still produced alcohol with inhibitor mixture contained relatively high concentrations of inhibitors. Therefore, I suggest that these strains examined in this study might be not only useful for the bioethanol industry using lignocellulosic biomass but also useful for study of response by combining inhibitors.

I found that in all the five *S. cerevisiae* strains, the same two single nucleotide variant (SNVs) in the *ERG3* gene were observed but *ERG3* gene in the five strains is normally functional as in the other strain. According to the sequence analyses, ergosterol contents in the five strains are comparable to the

reference strains. The ergosterol contents of the five strains were higher at 37 or 40°C than at 30°C, and in fact, three strains (SLY-3, SLY-9, and SLY-10) showed significantly higher amounts of ergosterol at 40°C than those of at 37°C. Although I observed that two different peaks (unknown compounds) from the five strains in addition to the ergosterol at high temperatures, however it is difficult to say the relationship between the contents of these unknown sterols and thermotolerance. Therefore, the mechanism of thermotolerance of the five strains seems different from the mechanism involved in the biosynthesis of ergosterol and/or any other sterol compound/s. Thus, the mechanism of thermotolerance was not yet concluded and further analyses will be required.