The research objective was to determine the occurrence of indigenous yeasts from napa cabbage (Brassica rapa subsp. pekinensis) wastes that potential in ethanol fermenting activity at stress condition such high concentration of glucose and ethanol. Indigenous yeasts were isolated from 1 g napa cabbage wastes and identified with RapID Yeasts Plus System. Glucose and ethanol tolerance was assessed by growing yeasts in modified Nutrient Broth/NB (Oxoid Ltd.) added with 10%, 20%, and 30% of glucose monohydrates or 10%, 15%, and 20% of ethanol concentration and measuring the optical density (OD) every 24 h until 72 h. The best isolates tested to ferment napa cabbage wastes were mixed with water (1:1.5) then the ethanol contents analyzed by dichromate oxidation method. Results showed that there are two potential ethanol-fermenting indigenous yeasts isolated from napa cabbage wastes which identified as Hanseniaspora guilliermondii and Candida krusei. Both yeasts have the ability to survive at 30% of glucose concentration until 72 h incubation, though the highest growth was reached at 10% of glucose concentrations for 24 h incubation. The yeasts growth in high ethanol concentration tends to decrease after 48 h for C. krusei and 24 h for H. guilliermondii. The highest ethanol production from napa cabbage fermentation showed that mixed culture (1:1) of C. krusei and H. guilliermondii with 1.43% ethanol contents for 24 h incubation.

Key Words: C. krusei, Ethanol, Glucose, H. guilliermondii, Napa Cabbage
condition such as high sugar and ethanol. Both stress condition could affect yeasts growth and fermentation yield by either altering osmotic stability or membrane fluidity. Therefore, high sugar and ethanol concentration tolerance of the indigenous yeasts still needs to be improved. Aims of the study were to determine the potential of indigenous yeasts from napa cabbage wastes that could ferment ethanol in stress condition.

2. Experimental

2.1 Yeast isolation and identification

The modified medium of Potato Dextrose Agar/PDA (Oxoid Ltd.) with the addition of 3% Yeasts Extract/YE (Kraft Foods) and 10 ppm amoxicillin has been used to isolate indigenous yeasts from 1 g of napa cabbage wastes by pour plate method. The emerging colonies were observed macroscopically and isolate having the macromorphological characteristic of yeasts were further tested with RapID Yeasts Plus System and analyzed by ERIC (Electronic Code Compendium) http://www.remel.com/eric\(^6\).

2.2 Determination of sugar and ethanol tolerance yeasts

The determination of sugar and ethanol tolerance was done by monitoring yeasts growth, expressed as increase or decrease in optical density (OD), under stress condition throughout the incubation period. Yeasts were grown in modified Nutrient Broth/NB (Oxoid Ltd.) with 3% Yeasts Extract/YE (Kraft Foods) and 10 ppm amoxicillin added with glucose monohydrates (10%, 20%, 30%) or ethanol (10%, 15%, 20%) and incubated at room temperature (23-28\(\degree C\)) for 72 h\(^7\). Yeasts growth was measured by quantifying the OD every 24 h until 72 h using UV-Vis spectrophotometer with \(\lambda\) 600 nm.

2.3 Ethanol fermentation testing

Yeasts isolate with the ability to tolerate stress condition were further tested to ferment napa cabbage waste to produce ethanol. Napa cabbage wastes were mixed with water (1:1.5) and added with 3% of yeasts isolates previously grown in modified Nutrient Broth. The mixture was incubated at room temperature (23-28\(\degree C\)) for 72 h. The ethanol content of the fermented mixture was measured by dichromate oxidation method, for every 24 h until 72 h\(^8\).

3. Results and Discussion

3.1 Indigenous Yeast Isolation and Identification

Isolation of indigenous yeast has found two yeast-like organisms which shown on Fig. 1. The identification results at Table 1 have shown the isolates having an identical characteristic with *Hanseniaspora guilliermondii* (NC1) and *Candida krusei* (NC2).

*Candida krusei* (NC2).

*Hanseniaspora guilliermondii* is apiculate yeast that usually found in spontaneous fermentation that can grow up to \(10^6-10^8\) cells/ml at the first fermentation week\(^9\). *Hanseniaspora* mainly isolated in soil, plant and also spoiled vegetables, the isolates have similar physiology such as ferment glucose, assimilate few carbon compounds (arbutin, cellobiose, glucose, glucono-\(\delta\)-lactone and salicin)\(^10\)-\(^11\). *H. guilliermondii* has multiple oenological traits such as low H2S production with high proteolytic and \(\beta\)-glycoside activities\(^12\).

*Candida krusei* is the imperfect form of *Issatchenkia orientalis* and usually found in natural food products, spontaneous fermentation and also natural alcoholic beverages fermentation\(^13\)-\(^14\). *C. krusei* are able to utilize N-acetylglucosamine, DL-lactate, glycerol, and glucose, with some strain, also have the ability to assimilate galactose, sorbose and inositol\(^15\)-\(^16\).
3.2 Tolerance ability towards glucose

Indigenous yeasts ability in tolerating osmotic stress from glucose contents shows that the increase of glucose concentrations leads to the decrease of indigenous yeasts OD, nevertheless both indigenous yeasts have survived at 30% of glucose concentration for 72 h incubation with *H. guilliermondii* shows OD of 0.129 and *C. krusei* with OD of 0.150 (Fig. 2).

Osmotolerant yeasts have the ability to consume glucose and synthesis glycerol with low acid production at the beginning of fermentation 7) 16). Efficient glycerol transport into yeasts cell is an essential mechanism in combating osmotic stress resulted from high glucose, concentrations 27) 18). *Non-Saccharomyces* yeasts such as *Candida* and *Hanseniaspora* could survive high glucose stress because of the ability to assimilate succinic and acetic acid that resulted from osmotic stress condition 16) 18).

3.3 Tolerance ability towards Ethanol

The tolerance of indigenous yeasts towards ethanol contents shows that increase of ethanol concentrations results in the decrease of *H. guilliermondii* OD, meanwhile, *C. krusei* shows increasing OD until 15% ethanol concentration for 48 h then decreased (Fig. 3).

The results show that at the beginning of fermentation, *H. guilliermondii* was unable to survive and tend to adapt high concentration of ethanol. The OD decreases at 24 h for 20% ethanol and 48 h at 15% ethanol concentration but then there is an increase. Meanwhile, at 10% ethanol concentration yeasts, OD still decreased until 72 h. Moreover, the increase of OD of *C. krusei* happened at 24 h for 10-15% ethanol concentrations, suggesting exponential phase. The OD then remained constant until 48 h, suggesting the yeast to have entered stationary phase. Yeasts cell entered death phase after 48 h to 72 h.

Meanwhile, at the 20% ethanol concentration treatment, the stationary phase is shown from 0-24 h followed by a decrease at 48 h, and increase at 72 h. *C. krusei* survived in 20% ethanol concentration for 24 h with OD of 0.268, however, *C. krusei* shown better endurance until 48 h with OD of 0.268 at 15% ethanol concentration. The increase of OD means that there is more yeasts cell, suggesting yeasts growing in number. Meanwhile, the decrease of OD shows less cell number as the result of lysis of dying cell.

The increased ethanol concentration affects the cell membrane integrity, membrane permeability to numerous ionic species will damage and plasma membrane fluidity also will decrease, which perturbs protein conformation of glycolytic enzymes (pyruvate kinase and hexokinase) then affects the uptake of glucose, maltose, ammonium, amino acids, and also causes cell leakage of nucleotides, amino acids, and potassium ions that shown by the disturbance of yeasts growth 29). Yeasts cell has developed in adapting the increase of ethanol concentration through the change of
membrane composition against membrane fluidization and plasma membrane stabilization\(^{21}\).

Non-Saccharomyces yeasts such as *Hanseniaspora* and *Candida* generally not tolerant to 4-6% ethanol concentration, however recent reports show the ethanol-tolerance ability similar to *S. cerevisiae* \(^{22} - 24\). *H. guilliermondii* and *C. krusei* are able to produce fatty acids in the plasma membrane as the mechanisms to adapt the presence of ethanol stress \(^{21}\). The increase in the proportion of ergosterol or oleic acid inside *H. guilliermondii* cells provides great adaptation to high ethanol concentration \(^{25}\). *C. krusei* in the form of *I. orientalis* could increase caprylic acid, stearic acid concomitant with the decreasing of oleic acid and palmitoleic acids to tolerate high ethanol concentration \(^{21}\).

### 3.4 Ethanol fermentation

Fig. 4 showed that the ethanol results from napa cabbage fermentation with the inoculation of indigenous yeasts. Ethanol contents of 1.43% result in highest ethanol fermentation from napa cabbage with the inoculation of indigenous *C. krusei* and *H. guilliermondii* (1:1) for 24 h incubation.

*Hanseniaspora* predominates the early days of fermentation with the production of organic acids, esters, and ethanol which involves the enzyme production i.e. \(\beta\)-glucosidase, \(\beta\)-xylosidase and some proteases \(^{11}\). *C. krusei* could assimilate organic acid such succinic acid into Krebs cycle to produce ethanol with low acid production in semi-aerobic condition through succinic dehydrogenase activity \(^{19} - 26\). The used of mixed culture has widened the sugar type hydrolyze ability, organic acid assimilation, glycerol synthesis and fatty acid production so that increases the stress tolerance ability with the potential in producing high ethanol contents \(^{21}\).

Despite the higher ethanol concentration produced by mixed culture, ethanol concentration of all culture showed a decline after 24 hours of fermentation. It is possible that both *Candida* and *Hanseniaspora* utilized the ethanol produced as a carbon source, due to lack of sugar that has all been used at the earlier stage of fermentation \(^{27}\).

### 4. Conclusion

*Hanseniaspora guilliermondii* and *Candida krusei* identified as two indigenous yeasts isolated from napa cabbage that has the potential for ethanol fermentation. The ability in tolerate 30% of glucose concentration and 20% ethanol has shown by both yeasts until 24 h and then the growth was decreased. Napa cabbage fermentation by mixed culture (1:1) of *C. krusei* and *H. guilliermondii* resulted in 1.43% ethanol contents for 24 h incubation.

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