4. DISCUSSIONS

4.1. Production of Volatile Compounds in Beer

Esters and total higher alcohols are secondary metabolites produced by the brewer's yeast. These substances are produced in low concentrations and they have tremendous impact on the sensory qualities of beer. The concentration of esters and total higher alcohol should be maintained on moderate level, where they impart pleasant and full-bodied character to beer flavour. If they present in excess, they give overly fruity flavour, which is undesirable to most consumers (Gee & Ramirez, 1994).

Heineken®, a world-famous lager beer has special specification for esters concentration in their beer. The specifications are divided in two categories *i.e.* control range and tolerance range. Control range is a stricter specification with narrower range. The production of Heineken® beer strived for esters concentration in control range. The major esters found in Heineken® global recipe specifications are ethyl acetate and isoamyl acetate, as they are the source of Heineken® beer's balanced fruity note. The esters concentrations are strictly kept at 19-26 mg/L for ethyl acetate in tolerance range and 3.25-4.25 mg/L for isoamyl acetate in tolerance range. The optimal concentration would be 22.5 mg/L for ethyl acetate and 3.75 mg/L for isoamyl acetate (Heineken, 2018). There are several factors that affect esters production, which are yeast strain, wort composition, fermentation temperature, fermenter design, pitching rate, and genetic modification (Verstrepen *et al.*, 2003).

4.2. The Effect of Fermentation Temperature

According to Saerens *et al* (2010), there are two important factors of esters production *i.e.* the concentration of the two substrates of ester (acetyl CoA and total higher alcohol) and the activity of enzymes responsible for esters formation.

Fermentation temperature largely influences the fermentation rate. An increase in temperature can result in decrease of pH, increase in yeast activity, and deterioration of foam

stability and beer color (Kucharczyk & Tuszyński, 2018). This means that temperature control is one of the most effective way to modify the fermentation speed, which influence the sensorial properties of beer (Solgajova *et al*., 2013).

Titica *et al*., (2000) reported that the concentration of esters is proportional to temperature. This report was consistent with previous study that an increase in fermentation temperature in the range of 10-25°C lead to increased esters production. Based on Table 2., it can be observed that beer fermented in higher temperature produced higher concentration of esters and total higher alcohol. Mason & Dufour (2000) and Peddie (1990) suggested that fermentation temperature affect the availability and activity of alcohol acetyl transferase (AATase), the enzyme that's involved in the formation of acetate esters. Esters are synthesized from the reaction of acetyl CoA and total higher alcohols (THA), catalyzed by alcohol acetyl transferase (AATase). An increase of THA as a substrate of ester will result in an increased esters production, *i.e.* isoamyl alcohol, obtained from total higher alcohol, plays a major role in the production of isoamyl acetate (Inoue *et al*., 1997). This is proven with a strong correlation between total higher alcohols concentration, ethyl acetate, and isoamyl acetate shown in Table 3.

Higher alcohols are synthetized during fermentation through catabolic pathway (otherwise known as the Erlich pathway) and anabolic pathway (amino acid metabolism) (Stewart, 2017). In Erlich pathway, the amino acids in wort are used by yeast to produce α-keto acid. Then, remaining α-keto acid is decarboxylated into aldehydes, which then reduced by alcohol-dehydrogenase into higher alcohols. In anabolic pathway, higher alcohols are synthetized from α-keto acids during the synthesis of amino acids from the carbohydrate source (Chen, 1978). The production of higher alcohols is influenced by fermentation conditions. According to Landaud *et al* (2001), higher alcohols production is increased by conditions that promote yeast cell growth, *e.g.* high levels of nutrients, lower fermentation vessel pressure, and increased temperature. Yoshioka & Hashimoto (1984) suggested that a lack of alcohol acetyl transferase activity and total higher alcohols concentration result in restricted acetate esters production. Dufour *et al*. (2008), indicated that increased temperature

stimulates the activity of AATase, which leads to increase in acetate esters production. A study by Peddie (1990) reported that higher fermentation temperature results in an increase in membrane fluidity. This allows more esters to diffuse into the medium.

Calderbank *et al*. (1994), confirmed that the production of total higher alcohols is suppressed in fermentation with lower temperatures. This is linked to the theory of Pronk *et al*., (1996), that stated that esters formation occurs via reaction between higher alcohols and acetyl CoA, thus lower fermentation temperature will decrease the substance of esters formation. Verstrepen *et al*., (2003) and Saerens *et al*. (2010) stated that the formation of esters is dependent on enzyme activity involved. This being said, temperature, one of the factors affecting enzyme activity, plays a great role in esters production.

4.3. The Effect of Yeast Pitching Rate

The effect of yeast pitching rate variation to the concentration of ethyl acetate, isoamyl acetate, and total higher alcohol can be observed (Table 1). There is no significant difference between yeast pitching rates in the production of ethyl acetate and total higher alcohol. However, a significant difference is apparent in isoamyl acetate concentration. A significant rise of isoamyl acetate can be seen when the yeast pitching rate increased from 2 g/L to 3 g/L. Beer with 1 g/L pitch rate had no significant difference with beer fermented using 2 and 3 g/L pitch rate. The isoamyl acetate concentration decreased when the yeast pitching rate increased to 2 g/L but increased again when the yeast pitching rate increased to 3 g/L.

There has been an interest to improve volumetric productivity of breweries. A strategic approach is to enhance the yeast cells in fermentors (increasing yeast pitching rate), but increasing the pitching rate could also damage the final sensory quality of beer (Verbelen *et al*., 2009). Flavour compounds such as ethyl acetate and isoamyl acetate are closely related to the growth and physiological aspects of the brewer's yeast. Alteration of process parameters (temperature, wort composition, yeast pitching rate) can largely influence beer flavour characteristics (Saerens *et al*., 2008).

The activity and physiology of yeast play a vital role in fermentation. In the early stage of fermentation, the yeast breaks down glycogen in order to form fatty acids and sterols. These lipids are essential for growth stage of yeast (David & Kirsop, 1973). This means, glycogen availability is crucial in yeast growth (Verbelen *et al*., 2009). During the depletion of oxygen in wort, glycogen accumulates in yeast cells during the exponential growth phase. This exponential growth phase uses up essential lipids, which are diluted between the mother and daughter cells. When the essential lipids are used up, the growth and cell division stop (Aries & Kirsop, 1977). The effect of pitch rate needs to be investigated, because in different pitch rates, the yeast cells still share the same amount of substrates for growth (Suihko *et al*., 1993).

According to Saerens *et al*. (2010), yeast cells share their lipid reserves with their daughter cells in budding stage. This process involves longer chain fatty acids and leaves short-chain fatty acid, which are toxic to the yeast. As a stress response, the yeast combine the short-chain fatty acids with alcohols to synthesize non-toxic esters, which are then released to the fermentation medium (Verbelen *et al*., 2009).

Thurston *et al*. (1982) stated that acetyl CoA is used for both yeast growth and esters formation. A state of equilibrium between the acetyl CoA used for growth and esters formation is present during early stage of fermentation. However, after the lipid reserves for are depleted and the yeast growth ceases, there's an abundance of acetyl CoA for esters production. It is suggested that higher yeast pitching rate, increasing the amount of yeast quantity in the fermenting medium, leads to a longer and more vigorous yeast growth phase. This uses up more acetyl CoA for lipid synthesis, thus limiting the acetyl CoA needed for ester synthesis and decreasing ester synthesis. According to Erten *et al*., (2007), pitching rate had no significant influence on esters except for isoamyl acetate, which increased along with the decrease of yeast pitching rate.

However, Fujii *et al*. (1997) stated that fatty acids and oxygen repress ATF1 gene expression, a gene responsible for acetate esters synthesis. With this theory, it is possible that higher yeast pitching rate leads to lower amount of fatty acid in each cell due to dilution between

mother cells and daughter buds. This will enhance the expression of ATF1 gene and ultimately result in higher concentration of esters.

A slight, but significant increase in isoamyl acetate was found when the yeast pitching rate was increased from 2 g/L to 3 g/L, contradicting the theory of Thurston *et al*. (1982) and Erten *et al*., (2007). However, it should be noted that different combination of fatty acids and higher alcohols produces specific esters (Palmer, 2017), *i.e.* isoamyl alcohol is the precursor of isoamyl acetate, and ethanol is the precursor of ethyl acetate (Nordstorm, 1961). Also, esters production is dependent on yeast strain. Different yeast strains produce not only different amount of ester, it also affects the proportions of each individual ester (Verstrepen *et al*., 2003).

Yeast pitching rate influences the availability of acetyl CoA for esters synthesis and the expression of ATF1 gene. The dynamic between acetyl CoA availability and fatty acids dilution and its impact on ATF1 gene expression plays a role in esters synthesis. For example, it can be seen in Table 2. that the concentration of isoamyl acetate in 10°C fermented beer lowered from 2.992 mg/L in 1 g/L yeast pitching rate to 2.661 mg/L in 2 g/L yeast pitching rate, but increased again to 2.954 mg/L in 3 g/L yeast pitching rate. It is possible that in beer fermented with 2 g/L, there was a short supply of acetyl CoA for esters synthesis. But when the pitch rate was increased to 3 g/L, the short supply of acetyl CoA was balanced with the dilution of fatty acids, thus allowing more ATF1 gene expression and more esters produced.

4.4.The Implementation of Fermentation Temperature Changes in Heineken® Beer

Based on Table 4. and Figure 4 (a and b)., a slight increase in both ethyl acetate and isoamyl acetate can be seen in Heineken® beer from October 2019 to March 2020. The highest concentration of ethyl acetate and isoamyl acetate can be seen in March 2020, where the highest temperature was used. With the exception of September 2019 and January 2020, it can be observed that higher fermentation temperature results in higher esters concentration. This is consistent with Dufour *et al*. (2008), which stated that higher fermentation

temperature will increase the activity of AATase, an enzyme that catalyzes esters formation. Besides increasing enzyme activity, Peddie (1990) suggested that an increase in temperature increases membrane fluidity. This allows more esters to be diffused into fermentation medium. The inconsistency in trend is caused by factors aside from temperature. In industrial scale production of Heineken® beer, there are a lot of elements that determine the final quality of products, such as aeration, filling time, changes of raw material, yeast generation, or internal pressure in fermentation vessel.