1. INTRODUCTION

1.1. Research Background

Beer is the oldest and most popular alcoholic beverage in the world. Across the globe, there are 28 known types of beers classified by the nature of the raw materials, microbial agents used in the fermentation steps, the location of beer production, and the overall production process (Boulton, 2001). To simplify, beers are classified according to their fermentation temperature and yeast species used to ferment them. The most widespread type of beer is lager, which is fermented by bottom fermenting yeast *Saccharomyces pastorianus* in lower temperatures that range from 3.3 to 13.0 °C. The second type is ale beers, using top fermenting *Saccharomyces cerevisiae* in higher temperatures ranging from 16 to 24°C. Lager beers are fermented for long period, usually 4-12 weeks while in contrast, ale beers are fermented in short period of time, ranging from 7-10 days (Humia *et al*., 2019).

One of the most well-known brewing company in the world to brew lager beer is Heineken N.V. Heineken N.V. is a Dutch brewing company which has more than 165 breweries all around the world. In 2018, Heineken® beers are served across 192 countries, with the production of Heineken® beer and other beer brands fully owned by Heineken N.V. reached 3.87 billion litres and 23.38 billion litres respectively (Heineken, 2018). Considering this massive production scale, each and every one of the breweries owned by Heineken N.V. operates under the standards set by Heineken Global Supply Chain to maintain the highest quality possible.

One of the controlled quality parameters is ester compounds concentration, which determine the final flavor of Heineken® beers. The most crucial esters in Heineken® are ethyl acetate and isoamyl acetate, as those two esters produces Heineken's characteristic fruity and estery flavor. As stated in the Heineken Global Basic Recipe, the optimal ratio of isoamyl acetate and ethyl acetate is kept to 1:6 in order to ensure the flavor balance, in which the control range concentration for isoamyl acetate is 3.75 mg/L and 22.5 mg/L for ethyl acetate (Heineken, 2018). These compounds are synthesized during primary and secondary fermentation. During fermentation there are several factors that influence esters synthesis: wort oxygen and lipid content, fermentation temperature, and yeast pitching rate (Verstrepen *et al*., 2003).

Every month, PT Multi Bintang Indonesia as a subsidiary company of the Heineken Company sends a batch their Heineken® beers (known as reference beer/RB) to Heineken® brewery in the Netherlands. These RBs are then analyzed by Global Quality Assurance Laboratory to ensure that the beers made in PT Multi Bintang Indonesia meets the quality standard set by the global supply chain. In order to achieve control range esters concentration, the effect of fermentation variables known to influence esters were investigated.

Research about beer fermentation conditions (fermentation temperature and yeast pitching rate) and their influence to esters concentration are still limited. Furthermore, the effect of fermentation temperature and yeast pitching rate to fermentation speed, which influences ester synthesis are also limited. Therefore, the objectives of this study were to investigate the influence of beer fermentation temperature and yeast pitching rate to final esters concentration. The information gathered from this study is expected to support PT Multi Bintang Indonesia in adjusting their fermentation condition to reach the optimal ratio of isoamyl acetate and ethyl acetate.

1.2. Literature Review

1.2.1. Overview of Beer

Beer is a yeast anaerobic fermentation product of brewer's wort. The principal composition of beer is famously established in the 1516 Bavarian Law of Purity (*reinheitsgebot*), which stated that beer should only be composed of water, barley, yeast, and hops (Willaert, 2007).

During production, beer undergoes three chemical and biochemical reactions (mashing, boiling, fermentation, and maturation) and three solid-liquid separation (wort separation, wort clarification, and beer clarification) (Fillaudeau *et al*., 2006). According to Willaert (2007), the brewing process steps include: malting, milling, mashing, wort separation, wort boiling, wort clarification, wort cooling and aeration, fermentation, maturation, beer filtration, beer stabilization, and beer packaging.

Beers are classified according to their fermentation process as top fermenting and bottom fermenting beer, which refers to yeast behavior during fermentation. Top fermenting yeast congregates at the top of fermentation vessel and produces ale beers while bottom fermentation yeast sinks to the base, producing lager beers (Humia *et al*., 2019). Top fermented beers are usually fermented by *Saccharomyces cerevisiae* at relatively warm temperatures (16-24°C) for 7–10 days, where high temperature generally generates higher levels of flavor components, resulting in fruity characteristics. Bottom fermented beers are fermented at much lower temperature of 3.3-13°C for longer period (4-12 weeks) by *Saccharomyces pastorianus*, resulting in sulfury notes (Humia *et al*., 2019), (Stewart & Russel, 1998).

Although wort compositions are very important in beer production, it's the yeast strain used in fermentation that distinguishes one beer type from another. Yeast plays a vital role in fermentation, as it converts carbohydrate from malt and adjuncts to alcohol and carbon dioxide, with this following reaction:

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

Major products of wort fermentation by yeast are ethanol, carbon dioxide, and glycerol, but it should be noted that they have minimal impact on beer flavor (Stewart & Russel, 1998). Secondary metabolites of the yeast, wort composition, and fermentation conditions primarily determines the flavour of beer. These include higher alcohols, vicinal diketones (VDK), and sulfur (Stewart, 2017).

Heineken® is a pale lager beer with 5% (abv) alcohol content. The main raw materials of Heineken® are pale malted barley with 2-rowed spring, hops, water, and Heineken® A-yeast. Fermentation time and fermentation vessels used in Heineken® beers production distinguish them from other commercial beers. Heineken® beers undergo fermentation in a much longer period than average commercial beers: they ferment for 28 days, in comparison to Bintang beers which undergo 14 days fermentation. For the first five days of fermentation, the wort is fermented in horizontal apollo tank (HORAP) to reduce hydrostatic pressure before transferred to cylindroconical fermenter. The yeast is a patented yeast strain from *Saccharomyces pastorianus* strain used to exclusively brew Heineken® beers which gives them their characteristic estery and fruity flavour. These flavours are obtained by keeping esters level to the control range according to Heineken global supply chain, which is 3.75 mg/L for isoamyl acetate and 22.5 mg/L for ethyl acetate. The optimal ratio of isoamyl acetate and ethyl acetate to ensure flavour balance in Heineken® is 1:6.

Table 1. Specifications of ethyl acetate and isoamyl acetate concentration in Heineken® beer

Parameter	Value	
	Control Range	Tolerance Range
Ethyl acetate (mg/L)	22.5 ± 2.5	22.5 ± 3.5
Isoamyl acetate (mg/L)	3.75 ± 0.5	3.75 ± 0.75

Heineken Global Basic Recipe (HMESC: 02.40.01.001)

1.2.2. Esters

Volatile esters are found only in trace amounts in fermented beverages such as beer, but they hold such a large impact in beer flavour profile. There are two main groups of esters in fermented beverages' flavor. The first group is acetate esters, in which the alcohol group is ethanol or higher alcohols and the acid group is acetate. This group includes ethyl acetate (solvent-like flavor), isoamyl acetate (banana flavor), and phenylethyl acetate (roses & honey flavor). The second group is ethyl esters, in which the acid group is medium chain fatty acid and the alcohol group is ethanol, including ethyl hexanoate (anise & apple flavor) and ethyl octanoate (sour apple flavor) (Pires *et al*., 2014). Mason & Dufour (2000) stated that esters can be formed through following chemical reaction:

$$R'\text{-}OH + R'\text{-}COOH \rightarrow R\text{-}COO\text{-}R' + H_2O$$

This reaction shows that esters formation occurs via reactions between an alcohol or higher alcohols with organic acids. However, the rate of this reaction is too slow to produce the amount of esters present in beer (Peddie, 1990). According to Pronk *et al* (1996) and Stewart (2017), the production of esters mainly occurs during the primary phase of fermentation by this following reaction:

$$\text{Acetyl CoA} + \text{Alcohol} \xleftrightarrow{\text{Alcohol Acetyl Transferase}} \text{Coenzyme A} + \text{Acetate esters}$$

(Pronk *et al*., 1996).

Through this reaction, esters are synthesized in the cytoplasm of yeast (Stewart, 2017) and due to their lipophilic nature, they easily leave the cell. Small chain acetate esters are easier to diffuse through the plasmatic membrane, while medium chain fatty acid esters may have their passage hindered. Due to this circumstance, acetate esters are present in fermentation medium in bigger amounts than their ethyl counterparts. This study will be focused on acetate esters, mainly ethyl acetate and isoamyl acetate as the most prominent flavor compounds in beer industry.

1.2.3. Factors Affecting Esters Concentration

To control the synthesis of esters, their formation mechanisms and what affects them should be understood first. Esters are synthetized intracellular in yeast's cytoplasm, formed during primary fermentation phase by enzymatic chemical condensation of organic acids and alcohols. There are two groups of esters: the lipid soluble acetate esters, which are synthetized from acetic acid with ethanol or a higher alcohol, and the medium-chain fatty acid ethyl esters. This study will mainly discuss the former group, especially isoamyl acetate and ethyl acetate.

Olaniran *et al*., (2017) stated that there are seven factors that could be controlled in order to achieve desired production of esters: using high gravity brewing (15 - 20°P (Stewart, 2010)), altering yeast pitching rate, oxygen, and carbon dioxide, genetic manipulation of yeast, reactor design, and continuous fermentation with immobilized yeast. Kucharczyk & Tuszyński (2018), added that fermentation temperature also affects fermentation and beer volatiles.

PT Multi Bintang Indonesia employs high gravity wort (± 15-16°P), with CO_2 counter pressure at gauge set in 0.30 bar maximum, with aeration rate of 12-34 mg/L. The wort is fermented with patented Heineken® A-yeast, therefore genetic manipulation of yeast is not considered as a variable. Continuous fermentation with immobilized yeast is not a plausible option, since such method gives engineering problems and greatly compromises the flavour of the beer. This research will be focused in controlling the esters level by altering fermentation temperature and yeast pitching rate.

Generally, esters formation increased along with increasing fermentation temperature (Peddie, 1990). This theory is backed up by researches conducted by Engan (1972) and Titica *et al.*, (2000) which found 50 to 75% increase of esters level with temperature increase in the range of 2 – 6 °C. A contradiction of this theory is found in a research conducted by Riverol & Cooney (2007), where ethyl acetate level is independent from temperature.

According to Saerens *et al.* (2010), there are two factors of ester formation: the concentration of co-substrates (acetyl-coenzyme A and alcohols) and the activity of enzymes involved in their hydrolysis and synthesis. Ester synthesis pathway is shown in Figure 1. It can be observed that the substrates of esters are fusel alcohols (also known as higher alcohols) and acetyl CoA. Humia *et al.* (2019) indicates that every variable that modifies the level of the two substrates would influence esters formation. This includes fatty acid, nitrogen and oxygen levels, nutrients availability, fermentation temperature, and fermentation vessel pressure.

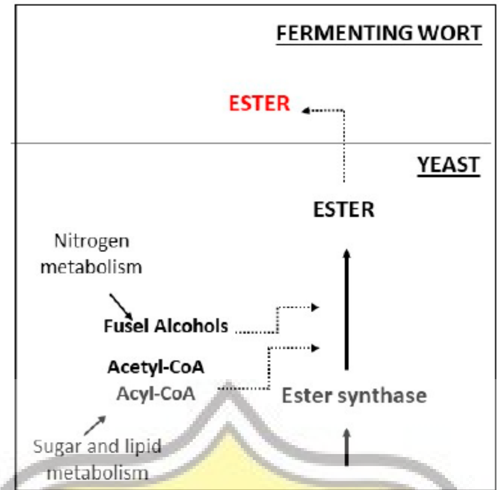

Figure 1. Ester synthesis pathway (Humia *et al.*, 2019)

Higher alcohols are synthetized during fermentation via catabolic pathway (Ehrlich pathway) and anabolic pathway (amino acid metabolism) (Stewart, 2017). In Ehrlich pathway (Figure 2), 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.

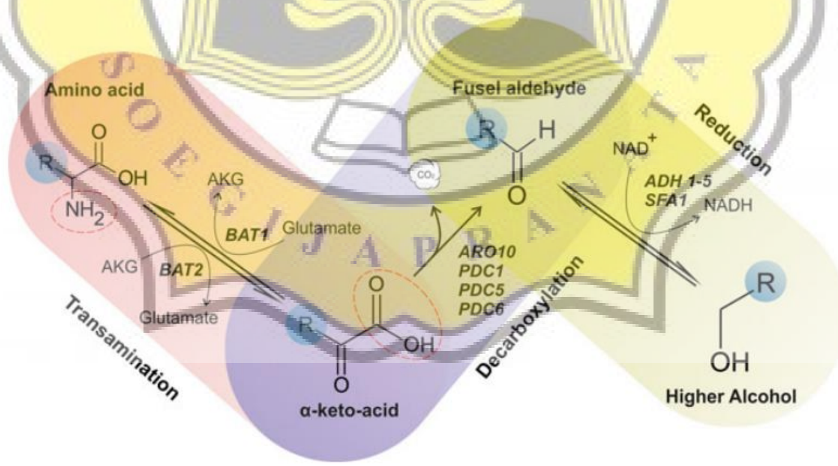

Figure 2. Higher alcohol synthesis via catabolic (Ehrlich) pathway
(Pires *et al.*, 2014)

The importance of anabolic pathway is apparent when the amino acids in wort has been depleted. When the amino acids has been depleted, the anabolic pathway synthetize the higher alcohols 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.

The yeast pitching rate also plays an important role. 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). The growth phase uses up essential lipids, which are diluted between the mother and daughter cells. 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 Thurston *et al*. (1982)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.
Fujii *et al*. (1997) mentioned 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.

Yeast pitching rate affects the production of esters by influencing the amount of acetyl CoA available for esters synthesis and the expression of ATF1 gene. An increase in yeast pitching rate will reduce the amount of acetyl CoA available for esters synthesis, but enhance the expression of ATF1 gene, while a lower pitching rate results in higher amount of acetyl CoA but represses the expression of ATF1 gene.

1.2.4. Gas Chromatography

Gas chromatography is one of the most widely used analytical method for compositional analysis (Al-Bukhaiti *et al*., 2017). In chromatographic methods there are two major phases: the mobile phase and the stationary phase. The sample is dissolved in a mobile phase, including a gas, a liquid, or a supercritical fluid. In gas chromatography, the mobile phase is forced through an immiscible stationary phase, which is fixed in place in a column.

Gas chromatography is a gas-liquid chromatography (GLC). The basic principal of gas chromatography is that greater the affinity of the stationary phase compound, the compound will be more retained by the column and it will take longer before it is eluted and detected. A compound with lower affinity will pass through the column more rapidly and reach the detector at the end of the column faster (Skoog, 2018). Most widely used detector in the food industry is the flame ionization detector (FID) (Al-Bukhaiti *et al*., 2017).

1.3. Objectives

The objectives of this study were to investigate the effect of beer fermentation temperature and yeast pitching rate to final esters concentration and the effect of total higher alcohols concentration to esters concentration.