Development and Characterization of Coffee Beverages by Fermentation of Coffee-Sugar Aqueous Solutions using Different Yeast Varieties

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ABSTRACT

DEVELOPMENT AND CHARACTERIZATION COFFEE BEVERAGE BY COFFEE-SUGAR AQUEOUS SOLUTION USING DIFFERENT YEAST VARIETIES

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Fermented coffee beverage is a relatively unexplored product with limited information in the literature. This study investigated the feasibility of making non-alcoholic beverage by fermenting instant coffee fortified with sucrose. Five different commercial *Saccharomyces* yeast strains were tested for the fermentation of the coffee solutions. Then, the optimal yeast was selected by sensory evaluation to do further study. Further study was focused on making the alcohol content less than 0.5% v/v by fermenting coffee solutions with concentrations from 0.5 to 2.5% w/w. The physiochemical properties and volatile compounds of the products were measured, as well as the sensory profiles evaluating. In result, Safbrew wb-34/70 was selected for this product due to good fermentation performance, the lowest alcohol/ester ratio, and retain typical coffee flavor. Considering the physicochemical and sensory properties, 2% coffee solution with Safbrew wb-34/70 was the most desirable formulation for the preparation of fermented coffee beverage.

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LIST OF ABBREVIATIONS

CSAS	Coffee sugar aqueous solution
HPLC	High-performance liquid chromatography
HS-GC	Headspace gas chromatography
HS-SPME-GC-MS	Headspace solid-phase microextraction with gas chromatography–mass spectrometry
RS	Reducing sugar
FR/RT	Foam retention/retention time
TA	Total Acidity
TS	Total Sugar
TSS	Total soluble solid
VDK	Vicinal diketone
YAN	Yeast assimilable nitrogen

1 Introductions

Coffee is a popular beverage derived from roasted coffee beans and one of the most traded agricultural commodities in the world. Coffee has been implicated for promoting health, and a recent cohort study concluded that coffee drinking could reduce death risk (Van Dam & Feskens, 2002; Gunter et al., 2017), lower the risk of type 2 diabetes (Bhupathiraju et al. 2013), and delay the rise of insulin (Gavrieli et al. 2013).

Coffee is typically brewed by percolating roasted coffee grounds with hot water. The brew is separated from the spent coffee grounds (SCG) by passing the brew through a cellulosic, thermoplastic, or metallic filter, by gravity or hydraulic feed. Grind particle size, grind/water ratio, contact time, and water temperature are the main variables that influence the coffee extraction efficiency (Andueza et al., 2002; Andueza et al., 2003; Andueza, Vila, De Peña, & Cid, 2007; Wang, William, Fu, & Lim, 2016).

Currently, the main coffee products in the market are roasted coffee beans, ground roasted coffee, instant coffee powder, and ready-to-drink coffee beverages, as well as coffee-flavored candies, biscuits, and other food stuff (Boeneke et al. 2010; Passos et al. 2017; Chávez et al. 2017). Volatile and non-volatile compounds have important influences on aroma and flavor perception of coffee beverages (Sunarharum et al. 2014; Kalschne et al. 2018). Among these compounds, furans and pyrazines are considered as the major contributors to coffee aromas (Risticevic et al. 2008).

Furans, one of the most important Maillard reaction products, are formed by thermal degradation of carbohydrates and protein, and oxidation of fatty acids (Bicchi et al., 2001). Volatile furans present malty and sweet roasted aromas. Representative furans in ground coffees are furfuryl alcohol, followed by furfuryl acetate, 2-furfural, 2-methylfuran, 3-methylfuran and 5-methylfurfural (Petisca et al. 2013; Becalski et al. 2016). Hence, coffee can be one of the major sources of furans and hydroxymethylfurfural (HMF) for regular coffee consumers. Since furans are classified as possible human carcinogens (Group 2B) by the International Agency for Research on Cancer (IARC) (FDA, 2004; IARC, 1995; NTP, 2004), researchers have attempted to remove furans from coffees, for example by vacuum treatment, yeast fermentation with adding sucrose, and evaporation during coffee roasting process (Quarta et al. 2012; Akıllıoglu et al. 2014; Anese, 2015).

Pyrazines are also formed during the roasting of coffee beans by the Maillard reaction between amino acids and reducing sugars via α-diketones as intermediates followed by ring condensation (Stadler et al., 2004). The alkyl chain and its position on the pyrazine ring determine the flavor characteristics to the roasted coffee (Bicchi et al., 2001). Researchers have identified a number of characteristic volatile flavor compounds to optimize the roasting of coffee beans, such as 2-methylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, and 2,3,5-trimethylpyrazine (Madihah et al. 2012). Approximately, 70% of the alkylpyrazines present in ground coffee are transferred to beverages during the brewing process (Pickard et al. 2014). The aroma characteristics of pyrazines

from coffee have been well characterized by researchers (Gloess et al. 2018; Caporaso et al. 2014).

The coffee flavor profile is strongly related to the roasting process parameters and brewing methodologies used. As mentioned above, in order to reduce or remove the furan from coffee, some researcher (Akıllıoglu et al. 2014) induce fermentation on brewed and instant coffee by adding yeast and sucrose. Moreover, post-brewing treatment can further alter the sensory attributes of the coffee brew. For example, during the fermentation of the coffee brew, the yeast variety used can contribute to different aromas and sensory characteristics of the resulting beverage due to the formation of volatile acids (e.g., acetic, isovaleric and isobutyric) and fatty acids (Duarte et al. 2010; Callejon et al. 2010; Sun et al. 2011). The compositions of volatile compounds are largely influenced by the nutrients available (Barbosa et al. 2009; Barbosa et al. 2012; Torrea et al. 2011) and redox of the medium (Fariña et al. 2012) during the fermentation process. Thus, yeast could be used for the development of novel fermented coffee beverage products, *Saccharomyces cerevisiae* has also been exploited as an inoculum in on-farm coffee fruit processing to reduce the occurrence of *Aspergillus niger* and *Aspergillus ochraceus*, as well as ochratoxin-A contamination (Velmourougane et al. 2011). *S. cerevisiae* has the ability to decrease furfural and 5-HMF, which can be attributed to the dehydrogenase and reductase activities by the yeast (Park et al. 2011; Jordan et al. 2011).

Many species of *Saccharomyces* yeasts have been used in the production of alcoholic beverages. Acetaldehyde, which contributes to green, grassy or apple-like off-flavors, is produced from ethanol by chemical or

biological oxidation. Diacetyl, characterized by sweet, buttery, creamy and milky sensory attributes, is an important ketone produced by the yeast (Weschenfelder et al. 2015). Acetates of higher alcohols and the esters of fatty acids and ethanol are essential in resulting in desirable and undesirable aromas in fermented products. The utilization of yeasts in the fermentation of roasted or instant coffee-based beverages could be of particular interest. Research and development of novel and healthy fermented coffee products could be essential for expanding coffee product market.

2 Literature Review

2.1 Coffee

2.1.1 Coffee Varieties

The genus *Coffea* belongs to the botanical family of *Rubiaceae* (a family of flowering plants) (Davis et al. 2006) and comprises more than 70 different species. However, there are only three commercially important species that includes *Coffea. arabica* L*., Coffea. canephora* Pierre ex Froehner, and *Coffea. liberica* Bull ex Hiern are of commercial importance (Illy and Viani 2005). The *C. canephora* is usually simply referred as Robusta, since the Robusta is the best-known variety for *C. canephora*. Since the Arabica coffee plants have the poor ability of resisting disease, so the hybrids of *C. arabica* and *C. canephora* have been developed to improve the disease resistance of the Arabica coffee plants. Also, the quality of the Robusta coffee is enhanced due to the hybrids.

2.1.2 Coffee fruit

The coffee fruit is a two-seeded drupe fruit that is commonly called a

berry or cherry. Sometimes, the fruit contains only one round seed called peaberry. The ripe coffee berry consists of a red skin layer called the exocarp or the epicarp, a thick pulp layer, sweet gelatinous-pectic mesocarp, and coffee seeds. Each seed is wrapped in a thin silver skin (rudimentary integument) and protected by a parchment hull (fibrous endocarp) (Wintgens 2004; Illy and Viani 2005). *C. arabica* varieties generally produce an oval convex seed with an S-shaped longitudinal slit (the central cut) on the flat side, while *C.canephora* seeds are more round with a straight central cut. The coffee seeds mostly consist of endosperm with a small embryo at the base of the seed.

2.1.3 Green coffee bean processing

Currently, there are mainly two methods being used for processing coffee cherries to yield the green coffee beans. The wet processing, also known as “washed process”, uses only ripe berries that have been picked manually or are separated mechanically, since unripe cherries or cherries that have partially dried on the tree cannot be handled by the pulping machines. On the other hand, the dry processing, also known as “natural process”, produces dried cherry coffee by drying the whole berries immediately after harvest on patios, racks under the sun, or in mechanical dryers (Clarke and Macrae 1987). This method is simple and less demanding with respect to harvesting.

The wet and dry processed green coffee beans can brew coffee with different sensorial characteristics. The differences in sensory characteristics are because of the coffee beans going through a dry processing, at the same time, they are in contact with the sweet mucilage. Thus, the resulting coffee brew tends to be heavy, sweet, smooth, and complex. On the contrary, wet

processing gives much more consistent and better-quality green coffee beans, which are cleaner and brighter (Clarke and Macrae 1987).

2.1.4 Roasting Process

Roasting of green coffee beans usually requires more than 200°C to provide the better flavors, colors and structural properties compared to green coffee. The influences during roasting in physicochemical properties are complex, which involve interactions between materials and process parameters. Also, in the process of roasting, hot gases or surfaces produce heat, which is transferred to green coffee beans and while reaching higher temperatures, it allows moisture to evaporate by endothermic reaction. As heating continues, concomitant formation of volatiles and CO_2 are caused by exothermic reactions. The increase of the pressure inside the coffee beans cause them to expand and crack. When they reach the desired degree of roast, which are indicated by the temperature produce desirable color, flavor, roast mass and so on, the beans are discharged from the roaster and water quenching and/or air cooling helps them quickly cool down (Clarke and Vitztbum 2001).

High-temperature-short-time (HTST) and low-temperature-long-time (LTLT) are the two main parameters that have affected on the physicochemical and sensory properties of the roasted coffee products, which are important for determining the roasting temperature and roasting time. As well as they control the quantity of heat transferred to the beans (Illy and Viani 1995; Schenker et al. 2000; Schenker et al. 2002; Baggenstoss et al. 2008). High yield or fast roasting coffee is often defined as coffee that takes less than 4 minutes to be roasted (Schenker 2000; Clarke and Vitztbum 2001). High yield roasting

process usually result in coffee beans with larger size, decreased in density, and high porosity. These effect of structures. The fast-roasted beans result in higher yield during brewing, due to water easily penetrating though them and extracting the soluble compounds of coffee (Ortola et al. 1998). However, compared with the traditional roasted coffee, these products have a fair amount of final water content and end up with greater oil sweating phenomenon. Therefore, the beans are more influenced by oxidation reaction and staling during storage (Schenker 2000; Clarke and Vitztbum 2001; Illy and Viani 2005).

2.1.5 Health Benefits of Coffee

Coffee is a popular brewed beverage worldwide. It is one of the most important agricultural commodities in the world with an annual production of more than 150 million 60-kg bags (ICO, 2017). Coffee is a substantial source of antioxidants in daily consumer diets (Brezova et al. 2009). Drinking three or more cups of coffee per day could reduce mortality and cancer risks (Carrieri et al. 2017; Nkondjock, 2009). Moreover, epidemiological studies demonstrated that coffee consumption can decrease the risk of type 2 diabetes (Bhupathiraju et al. 2013), as well as delay the rise of insulin in response to meals and the fall of glucose concentrations (Gavrieli et al. 2013).

Other studies found that adding coffee from hot brew to the culture medium of the virus-infected cells with dose of 20% of normal drinking concentration could inhibited herpes simplex virus type 1 multiplication. Coffee extracts had virucidal activity that could inactivate the infectivity of virus particle and inhibit the progeny infectious virus formation at the late stage of multiplication. (Utsunomiya et al., 2008). Decaffeinated coffee could potentially

delay memory impairment in humans through the inhibition of NF-κB activation and subsequent TNF-α production (Jang et al. 2013). In rat models, caffeinated and decaffeinated instant coffees have been found to reverse high-fat diet-induced metabolic alterations (Caria et al. 2014), induce weight loss, but aggravate the plasma cholesterol profile (Choi et al. 2011), and liver fibrosis (Furtado et al. 2014). Arabinogalactan-protein complex is one of the key polysaccharides present in coffee (Matulová et al. 2011). Certain fractions of arabinogalactan-protein have been shown to possess antitussive and immunomodulating activities (Nosáľová et al. 2011; Capek et al. 2014; Passos et al. 2014; Ferreira et al. 2018). Other health promoting benefits of coffee are summarized by Sharif et al. (2017) and O'Keefe et al. (2018)

2.1.6 Coffee Flavor Substances

The unique aroma and flavor properties of coffee are attributed to a myriad of volatile and non-volatile compounds. Non-volatile compounds include alkaloids (caffeine, trigonelline), chlorogenic acids, carboxylic acids, polysaccharides, lipids, protein, melanoidins and minerals. The major volatile compounds include alcohols, aldehydes, ketones, carboxylic acids, esters, pyrazines, pyrroles, pyridines, sulfur compounds, furans, furanones, and phenols (Sunarharum et al., 2014). Furans and pyrazines are the major volatiles in coffees by quantity, while sulfur-containing compounds and pyrazines are considered the most significant to coffee flavor by quality. These compounds vary substantially in concentration and sensory potency which are dependent on green bean variety, roasting conditions, and brewing method. The complex variation in composition explains the diverse and unique flavor

and aroma characteristics of coffee from different origins, as well as processing/brewing methods used to prepare the beverage (Risticevic et al. 2008; Kalschne et al. 2018; Sunarharum et al. 2014).

Furans are important components in instant and roasted coffee that contribute to malty and roast aroma characteristics. The main furans present in roasted Arabica coffee are 5-hydroxymethylfurfural (HMF), 3-methypropanal, 2-methylfuran, furfural, 2-furfural, furfuryl formate, furfuryl acetate, 5-methyl-2-furancarboxyaldehyde, 4-ethylguaiacol, 3-methylthiophene, 2-furanmethanol acetate, 2-ethyl-3,6-dimethylpyrazine, 5-methylfurfural, and 1-(2-furanyl)-2-butanone (Ribeiro et al., 2009; Petisca et al. 2013). In a study looking at furan levels of commercially available coffees from the Spanish market, Altaki et al. (2011), evaluated furan concentrations in regular, decaffeinated, and instant coffee and commercial packed capsules. The results indicated that brewed regular coffees obtained by drip coffee makers (paper filtered) tended to have lower furan levels than those from espresso coffee machines. Also, levels of furan in decaffeinated and regular coffees were similar. Overall, instant coffee brews had the lowest level of furan while single-serve coffee capsules were highest among all samples.

Although furan is an important contributor to roasted coffee aroma, it is classified as a possible human carcinogen (Group 2B) by the International Agency for Research on Cancer (IARC) (FDA, 2004; IARC, 1995; NTP, 2004) due to its genotoxic and mutagenic effects. To mitigate this risk, Quarta and Anese (2012) investigated a method to remove furfurals from roasted coffee ground by vacuum treatment. Particularly, the anhydrous commercial coffee

powder sample was hydrated to a water activity of 0.7, then applied under vaccum treatment at 2.7 kPa and 60 °C for 10 min. The results showed that 20% HMF and 100% furfural were successfully removed. Although a reduction of furfurals could be achieved with this approach, a significant decrease in the total headspace volatiles was reported, resulting in a lowering of odor intensity of the samples. Baker's yeast (*Saccharomyces cerevisiae*) could mitigate HMF in instant coffee by fermentation (Akıllıoglu et al. 2014), and other potential mitigation strategies have been summarized by Anese (2015).

Pyrazines are another family of aroma compound formed during roasting by the Maillard reaction between amino acids and reducing sugars, with α-diketones as intermediates of transamination, followed by ring condensation. Depending on the alkyl chain and its position in the pyrazine molecules, they contribute to different flavor characteristics in roasted coffees. Approximately, 70% of the alkylpyrazines present in ground coffee are transferred to the brew (Pickard et al. 2014). Slow roasting speeds favor the pyridine formation (Petisca et al. 2013). In another study, 2-methylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, and 2,3,5-trimethylpyrazine were the landmark volatile flavor compounds in roasted Robusta coffee beans (Madihah et al. 2012). Moreover, Caporaso et al. (2014) investigated the chemical characteristics among Neapolitan, espresso, American, and moka coffee brews. Specifically, hexanal, β-damascenone and pyrazines were detected in Neapolitan brew, which was considered as the unique flavor aspect among other brews. Therefore, pyrazine played an important role on the contribution to coffee aromas

2.1.7 *Saccharomyces* Yeasts

Saccharomyces cerevisiae and *Saccharomyces bayanus* have long been extensively applied in alcoholic beverages, such as beer (Gallone et al. 2018), wine (Varela et al. 2016), sparkling wine (Borrull et al. 2016), fruit wine (Berenguer et al. 2016), and various kinds of spirits (Satora et al. 2010; Amorim et al. 2016; Bovo et al. 2015). In coffee, *Saccharomyces* yeasts are indigenous residents on coffee bean fruit which have been used in wet processing of coffee (Pereira et al. 2014), and its ability of producing aromatic compounds in coffee pulp simulation medium and pectinolytic activities were evaluated. The results indicated that *Saccharomyces* yeasts had the best pectinase-producing performance compared to the most frequent isolated yeast strains-*Pichia fermentans*, *Pichia kluyveri*, and significantly improve the formation of volatile compounds during the fermentation process. In addition, during the coffee semi-dry processing (Evangelista et al. 2014a), *Saccharomyces cerevisiae* yeast strains were also applied as starter culture during coffee fermentation to detect and monitor the unique volatile compounds and organic acids. Furthermore, *Saccharomyces cerevisiae* yeast was also applied as starter culture on the dry fermentation of coffee beans (Evangelista et al. 2014b). The study indicated that coffee inoculated with *Saccharomyces cerevisiae* yeasts had higher flavor sensations with increased levels of alcohol, furan, and aldehydes than the coffee without the fermentation. As starter cultures in dry processed coffee, *S. cerevisiae* yeasts could produce typical flavors of caramel, herbs, and fruit. There was another study used hydrothermal treatment on spent coffee ground to extract aroma compounds, fermented the extracts with sucrose and *S. cerevisiae* yeasts adding, and distilled fermented broth to make

spirits (Sampaio et al., 2013). The final product presented the desirable sensory characteristics (including clarity and brilliance, coffee, roasted, alcohol, elegance, frankly, bitter, astringent and pungent, and finesse) that was contributed by volatile compounds (including alcohols, esters, aldehydes, and acids) generated during fermentation.

2.1.8 Flavor Substances Produced during Fermentation

During fermentation, higher alcohols are produced when keto acids corresponding to the carbon skeleton from the different amino acids undergo decarboxylation and reduction. Undesirable higher alcohols, such as n-propanol and isobutanol are normally below the sensory threshold in the fermented products. However, isoamylol, which is the precursor for some esters, has a negative sensory impact on the fermented products (Gamero et al., 2011).

Acetaldehyde, which is a major kind of low boiling point volatiles generated by fermentation, is mainly reduced to ethanol by yeast. However, a small residual quantity of acetaldehyde might present in the medium, giving rise to green, grassy or apple-like off-flavor. Acetaldehyde can also be produced from ethanol by chemical or biological oxidation. High levels of acetaldehyde have been found in oxidized wine and chartered by fruity and rotting apple odor (Pinto et al., 2018). Diacetyl, recognized by sweet, buttery, creamy, and milky flavor characteristics (Weschenfelder et al. 2015), is the most important ketone formed outside the yeast cell in soluble coffee solutions. Its precursor, acetolactate is excreted by yeasts into the surrounding medium, which is spontaneously converted into diacetyl. The reaction is enhanced by low pH and increasing temperature.

Different yeasts can contribute to different aromas and sensory characteristics. For example, *S. cerevisiae* yeast has the ability to decrease furfural and 5-hydroxymethylfurfural (Park et al. 2011; Jordan et al. 2011). The addition of nitrogen can influence the formation of aroma compounds by different strains of *S. cerevisiae* in wines (Barbosa et al. 2009), especially the major volatile compounds (Barbosa et al. 2012). For example, nitrogen supplements, in the form of amino acids and ammonium nitrogen, to Chardonnay grape juices can substantially modulate volatiles composition and perceived aroma, by increasing both acetate and medium chain fatty acid esters and decrease higher alcohols (Torrea et al. 2011).

2.2 Analysis of Flavor Substances

2.2.1 Volatile Compounds Analysis

Headspace gas chromatography (HS-GC) is commonly applied to detect low boiling point volatiles in foods. HS-GC is an official technique endorsed by the European Brewery Convention (EBC) to analyze dimethyl sulphide and other lower boiling point volatile compounds in beer (Analytica-EBC 9.39, 2000). Tian (2010) applied HS-GC with a flame ionization detector (FID) to detect acetaldehyde in beer, using n-propanol as internal standard (HS-GC/FID method).

In addition, head space-solid phase microextraction (HS-SPME) and GC/FID could be used on leaves, pulps, and peels of FICUS carica to detect low molecular weight volatiles including acetaldehyde, ethyl acetate, methanol,

ethanol, hexanal, limonene, (*E*)-2-hexenal and octanal (Oliveira et al. 2010). Furthermore, solid phase extraction with gas chromatography-mass spectrometry (GC-MS) was also applied to cinnamon bark volatile oils to extract the main volatile compounds- *C. zeylanicum* and identify the composition of volatile compounds- Ecinnamaldehyde, which indicated that Ecinnamaldehyde had the highest area percentage of 79.60% in the cinnamon bark volatile oils (Golmohammad et al. 2012).

HS also can be combined with GC-MS method to detect the volatile compounds during the production of fruit vinegars (Ubeda et al. 2011). Headspace solid-phase microextraction (HS-SPME) combined with gas chromatography mass spectrometry (GC-MS), is widely utilized to detect aroma and flavor compounds in coffee (Bressanello et al. 2017). Using this technique, Yang et al. (2016) detected volatile marker compounds of roast defects for coffee, including indole, 4-ethyl-2-methoxyphenol, phenol, maltol, and 2,5-dimethylfuran, which are indicative of “light”, “scorched”, “dark”, “baked”, and “underdeveloped” defects, respectively. Bicchi et al. (2011) applied a HS-SPME-GC-MS method to quantify furan and 2-methyl-furan in roasted coffee suspended in water with high repeatability and sensitivity. Similarly, headspace solid-phase microextraction with gas chromatography mass spectrometry (HS-SPME-GC-MS), has been applied in other studies to determine volatile compounds in wine (Maturano et al. 2017; Sagandykova et al. 2017; Xiao et al. 2015), beer (Rodriguez-Bencomo et al. 2012; Pizarro et al. 2010), fruits (Lasekan et al. 2013; Nardini et al. 2013), and other products (Tian et al. 2013).

2.2.2 Caffeine and Organic Acids Analysis

Caffeine is one of the key components in coffee and tea beverages. It has some physiological impacts on the human body such as stimulation to the central nervous system, elevation of blood pressure, metabolic rate increasing, and diuretic effect (Higdon & Frei 2006). In green coffee, caffeine content is highly affected by coffee species. Specifically, arabica coffee tends to have lower caffeine levels than Robusta. Furthermore, during the coffee roasting process, caffeine is relatively heat-stable. However, because of the sublimation loss at increased roast temperatures, there were higher caffeine levels detected in light roast coffee than in dark roast (Hečimović et al. 2011). The grinding and brewing methods such as solids-to-water ratio and grinding particle size could have effects on caffeine content in brewed coffee. The values of caffeine levels varied from 50 to 143 mg per 177 mL (6 oz) in coffee brews (McCusker et al. 2011).

Belguidoum et al. (2014) performed a quantitative determination of phenolic compounds and caffeine in different brands of coffee including roasted, green, and instant in the Algerian market. Eight phenolic acids, three flavonoids, and caffeine with total polyphenol and caffeine level were analyzed and measured based on different varieties of packaging, roasting degree, grain size, instantaneity, and decaffeination. The final results came out with total polyphenol level varying from 12.37 ± 0.55 to 200.08± 6.47 mg/L, as well as caffeine content ranging from 3 8.00 ± 1.89 to 136.00 ± 6.45 mg/L. Jeon et al. (2019) applied a high-performance liquid chromatography with a diode array detector (HPLC-DAD) method to determine the chlorogenic acids and caffeine

in instant coffee, roasted, and ground coffee, ready-to-drink coffee, and Americano coffee sold in supermarkets and coffee shops in Korea. The results indicated that the highest level of 194.1 ± 67.7 mg/serving of caffeine was detected in Americano coffee sold in coffee shops.

Roasted coffee contains organic acids such as acetic, ascorbic, citric, malic, and succinic acids. According to Alcázar et al. (2003), acetic, ascorbic, citric, malic, and succinic acids contents were analyzed by HPLC in green and roasted coffee from the varieties of Arabica and Robusta. Typically, succinic acids were not detected in all samples. Arabica green coffee had the levels of acetic, ascorbic, citric, and malic acids of 0.58, 3.37, 8.52, and 4.14 mg/g, respectively, while Robusta green had 0.40, 3.08, 6.08, and 2.95 mg/g, respectively. For the roasted coffee, ascorbic acid was not detected on both varieties. Thus, arabica roasted coffee contained acetic, citric, and malic acids levels of 1.74, 5.22, and 1.74 mg/g, respectively, while Robusta roasted coffee contained 1.78, 4.60, and 0.50 mg/g, respectively. Overall, Arabica coffee tended to have higher organic acids contents than Robusta.

During industrial fermentation of liquors and alcoholic beverages with *S. cerevisiae*, *S. paradoxus*, and *S. bayanus* yeasts, acetic, lactic, and succinic acids are intermediate metabolites produced by these yeasts. However, malic and citric acids could vary based on different strains of yeasts usage. Some yeasts strains are able to produce malic acid content. Specially, *S. bayanus* yeast was applied on the apple juice fermentation during ice cider production to reduce malic acid contents, as well as *S. cerevisiae* NF66 yeasts applied on fermentation of red wine making (Bedriñana et al. 2017; Francesca et al. 2014).

During the sake making process, *S. cerevisiae* sake yeast strain No. 28 was applied on sake fermentation could produce malic acid (Nakayama et al. 2012). Similarly, during pomegranate wine making, *S. cerevisiae* yeasts strains of Viniferm (including Viniferm Revelación, Viniferm SV and Viniferm PDM) performed similar fermentation characteristics in organic acids changing, which produced citric acid pomegranate juice fermentation (Berenguer et al 2016). On the contrary, using *S. cerevisiae* yeasts on are able to assimilate citric acid during the fermentation of Garcinia must (Rai et al. 2014).

3 Hypothesis and Objectives

Based on the literature reviewed, the information on fermentation processes, as well as aroma and flavor sensory profiles of fermented coffee is limited. It is hypothesized that instant coffee beverages, when fortified with sucrose, can be fermented into low alcoholic beverages with a unique foam head, physicochemical, and sensory properties. Therefore, using yeasts to ferment beverages may present new product development opportunities.

Specific objectives of this research include:

1). Develop coffee fermentation process.

2). Compare different commercial *Saccharomyces* yeast strains.

3). Analyze key parameters during fermentation.

4). Evaluate aroma and flavor sensory profiles of fermented coffee.

To achieve these objectives, five strains of *Saccharomyces cerevisiae* yeasts (Safbrew wb-06, Safbrew wb-34/70, SafAle US-05, Lalvin EC-1118 (EC-500), and SIHA Active Yeast 3 were evaluated for fermenting instant coffee-sugar aqueous solution (CSAS). Fermentation processes were compared by monitoring free amino nitrogen, total soluble solid, reducing and total sugar, alcohol content, total acidity, pH value, and vicinal diketones.

The volatile compounds, including acetaldehyde, ethyl acetate, isobutyl alcohol, n-propanol, isoamylol, isoamyl acetate, and ethyl hexanoate and ethyl octanoate were analyzed. Moreover, the foam stability and sensory

characteristics, CO_2, and caffeine contents were characterized in the final CSAS products.

4 Fermentation and Characterization of Instant Coffee using Commercial *Saccharomyces* Yeast strains

4.1 Introduction

The unique aroma and flavor sensory profiles, along with its stimulating effect of caffeine, are among the main sought-after characteristics by coffee consumers. Yeasts have been used in the fermentation of coffee cherries to improve coffee flavors (Pereira et al. 2014; Evangelista et al. 2014a; Evangelista et al. 2014b; Martinez et al. 2017; Bressani et al. 2018) and the development of innovative uses of coffee wastes (Sampaio et al, 2013; Mussatto et al. 2012; Rocha et al. 2014; Tehrani et al. 2015). However, the application of yeast for the fermentation of coffee beverage is a relatively new concept.

Recently, “nitro” coffee has started to gain traction in consumer acceptance. The coffee is prepared by pressurizing the cold brew with N_2 (typically 30-50 psi) and stored in a keg maintained at refrigerated temperature. Instead of pressurizing the brew with CO_2 which can impart acidity, N_2 is used to avoid this sensory issue, as well as to produce smaller bubbles and creamier foam texture. “Nitro” coffee is a relatively new product popularized in North America over the past decade. It is prepared by pressurizing cold brew coffees with nitrogen (30 to 50 psi) and stored in keg or bottle at refrigerated temperature. When the containers are opened, the oversaturation of the dissolved nitrogen forms small bubbles that raise to the top to generate a stable foam head, resulting in beverages with unique sensory characteristics. Based on this concept, it is conceivable that by introducing carbon dioxide into coffee

beverage through fermentation, low alcoholic beverages with unique foam head, physicochemical, and sensory properties can be derived. Compare with nitro coffee, it is anticipated that fermented coffee beverages would have more complex sensory profiles due to the introduction of metabolites from yeast fermentation.

As reviewed in Chapter 2, furans are important components in instant and roasted coffee that contribute to malty and roast aroma characteristics. However, it is classified as a possible human carcinogen (Group 2B) by the International Agency for Research on Cancer (IARC) (FDA, 2004; IARC, 1995; NTP, 2004) due to its genotoxic and mutagenic effects. Therefore, a fermentation process could be applied to brew coffee to reduce the furan level. Among the various brewing methods currently available, instant coffee brew has the lowest level of furan when compared to regular, decaffeinated, and commercial packed capsule coffees (Altaki et al., 2013). Therefore, instant coffee powder would be a preferred choice for the development of fermented coffee beverages with low furan level. Furthermore, since instant brew lacks the sucrose required to support the fermentation process, the coffee brew needs to be fortified with sugar.

The main goal of this study is to develop the fermentation technique of instant coffee-sugar aqueous solutions (CSAS) using commercial *Saccharomyces* yeast strains. Five commercial active dry yeast strains were tested for the fermentation of instant coffee solution fortified with sugar. The parameters during fermentation process were monitored, including pH, nitrogen, total soluble solid, reducing and total sugar, total acidity, alcohol and vicinal

diketone content. Low boiling point volatile compounds were analyzed using the HS-GC method, including acetaldehyde, ethyl acetate, isobutyl alcohol, n-propanol, isoamylol, isoamyl acetate, ethyl hexanoate, and ethyl octanoate. For the final fermented CSAS products, CO_2 level, caffeine content and foam retention time, as well as sensory characteristics were evaluated. Using a HS-SPME-GC-MS technique, volatile species, including furans, pyrazines, esters, and acids between samples were compared.

4.2 Materials and Methods

4.2.1 Materials and chemicals

Instant coffee (Nescafé, Nestle, Canada), white sugar, and purified water were purchased from a local grocery store. Caffeine, citric acid, pyruvic acid, malic acid, succinic acid, lactic acid, and acetic acid, were chromatographic purity purchased from Sigma-Aldrich (St. Louis, MO, USA). The volatile compound standards (i.e., acetaldehyde, ethyl acetate, isobutanol, n-propanol, isoamylol, isoamyl acetate, ethyl hexanoate, ethyl octanoate, n-butanol, 3-octanol) were obtained from Sigma and Aldrich (St Louis, MO, USA). All other chemicals and solvents were analytical reagent grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

4.2.2 Yeasts

Five strains of dry *S. cerevisiae* yeasts were evaluated, including Safbrew wb-06, Safbrew wb-34/70 and SafAle US-05 (Fermentis, Marcq-en-

Baroeul, France), Lalvin EC-1118 (EC-500) (Lallemand Inc., Montreal, Canada), and SIHA Active Yeast 3 strain WET 136 (E. Begerow GmbH & Co., Langenlonsheim, Germany). The dosages used for the yeasts are summarized in Table 4.1.

Table 4.1 - Yeast characteristics and their dosage for fermentation.

Yeast	Cell level (g/L)*	Dosage (%, w/w)*
Safbrew wb-06	0.50-0.80	0.077
Safbrew wb-34/70	0.80-1.20	0.077
SafAle US-05	0.50-0.80	0.077
Lalvin EC-1118	0.25-0.40	0.033
SIHA Active Yeast 3	0.15-0.25	0.033

*Information provided by the suppliers.

4.2.3 Preparation of CSAS for Fermentation

Natural spring water (450 g) was boiled in a stainless-steel pot, followed by the addition of instant coffee powder (20 g), sugar (80 g) and then mixed to dissolve thoroughly. Deionized water (450 g) was cooled down in a refrigerator to 4 ^{o}C, and then 2/3 of the cooled water was added into the pot, mixed, and then transferred to a fermentation vessel. The remaining 1/3 portions of cooled water was used to rinse the residual solution in the pot into the fermentation vessel. The solution was allowed to cool down to 18 ^{o}C for fermentation.

4.2.4 Inoculation and Fermentation

The activated dry yeasts were sprinkled in 10 times of its weight of sterile water at 27 ± 3 ^{o}C, gently stirred for 20 min. The resultant cream was pitched

into the fermentation vessels. The main fermentation was carried out at 18 ^{o}C for 168 h (i.e. one week), following which, the brews were bottled and further fermented at 18 ^{o}C for an additional 24 h. The bottles were then cooled to 4 ^{o}C in a refrigerator for one week to produce the final products.

4.2.5 Analysis Methods

4.2.5.1 Yeast Assimilable Nitrogen (YAN)

YAN was determined according to Analytica EBC (European Brewery Convention) Method 8.10.1. Ninhydrin and glycine were used as color reagent and standard, respectively. Measurement was carried out at 570 nm in a 10 mm cuvette using a spectrophotometer (2600 UV/VIS; UNICO Co., Shanghai, China). The results were expressed as mg glycine/L.

4.2.5.2 Total Soluble Solid (TSS), Reducing/Total Sugars

TSS was detected by a refractometer (PAL-1, ATAGO Co., Ltd., Tokyo, Japan). For reducing sugar determination, samples were diluted by purified water to 2-4 g/L glucose equivalent. For total sugar determination, 1 mL of sample, 5 mL of hydrochloric acid solution (1:1 hydrochloric acid:water), and 20 mL purified water were added into a 100 mL volumetric flask and mixed thoroughly. The mixtures were heated to 68 ^{o}C in a water bath for 15 min and then then cooled to room temperature. The mixtures were neutralized by 200 g/L NaOH solution to pH of 8.2, and then diluted with purified water to the 100 mL scale of the flask. The reducing and total sugar contents were determined according to AOAC Official Method 923.09 “Invert Sugar in Sugars and Syrups Lane-Eynon General Volumetric Method”, expressed as g/L glucose.

4.2.5.3 Alcohol Content

The alcohol content was determined using an Alcolyzer Beer Analyzing System (Anton Paar GmbH, Austria). Samples were degassed, equilibrated to 20^{o}C in water bath, filtered into a vial by a 0.45 μm filter, and then loaded onto the analyzer for analysis. The alcohol content was expressed as % by volume.

4.2.5.4 Total Acidity and pH Value

The total acidity was detected according to AOAC Official Method 920.92 Acidity (Total) of Roasted Coffee. An aliquot of 50 mL of sample was degassed in a 40 ^{o}C water bath for 30 min and titrated with 0.1 M NaOH solution to pH 8.2 with a potentiometric titrator (METTLER TOLEDO, Shanghai, China). The pH value of the degassed sample was recorded just before titrating. The total acidity was expressed as mL 1M NaOH required neutralizing total acidity of 100 mL test sample.

4.2.5.5 Vicinal Diketones, CO_2, and Foam Retention Time

Vicinal diketones content was determined according to Analytica-EBC protocol (9.24.1 Vicinal Diketones in Beer: Spectrophotometric Method) using a spectrophotometer (2600 UV/VIS Spectrophotometer, UNICO, Shanghai, China), and results were expressed in mg/L. CO_2 content was determined according to Analytica-EBC (9.28.3 Carbon Dioxide in Beer: Pressure Method), and results were expressed as % by mass. Foam retention time (FRT) was assessed following an Analytica-EBC method (9.42.1 Foam Stability of Beer) using a foam stability tester (FST-100PTH, Zhaoqing, China). The FRT test cup used was colorless and transparent, with an internal diameter of 60 mm, height 120 mm, and wall thickness 2 mm.

4.2.5.6 HS-GC Analysis

The low boiling point volatile compounds detected were acetaldehyde, ethyl acetate, isobutyl alcohol, n-propanol, isoamylol, isoamyl acetate, and ethyl hexanoate and ethyl octanoate. These compounds were detected according to an Analytica-EBC protocol (9.39 Dimethyl Sulphide and Other Lower Boiling Point Volatile Compounds in Beer by Gas Chromatography) with some modification. An aliquot of 5 mL of sample, spiked with 1.6 mg/L of n-butanol internal standard at 4 ^{o}C was injected into a 20 mL vial (Derian Instrument Co., Shanghai, China) using a syringe. The vial was then sealed immediately with crimp caps (Derian Instrument Co., Shanghai, China). Each analysis was undertaken in triplicate. To minimize the loss of volatile compounds, the samples were kept at 4 ^{o}C until they were to be analyzed. The gas chromatograph (Agilent 6890N capillary gas chromatograph, Agilent Co., USA), connected to a static headspace auto-sampler (Agilent 7694E headspace auto-sampler, Agilent Co., USA), was equipped with a capillary column (Chrompack 7773, 50 m×0.32 mm i.d., external diameter 0.45 mm, liquid phase CP WAX 52 CB, thickness 1.11 μm) and a flame ionization detector. The parameters of the auto-sampler were listed as follows: sample temperature 60 ^{o}C; needle temperature 110 ^{o}C; transfer temperature 110 ^{o}C; injection temperature 110 ^{o}C; thermostat time 20 min; pressurization time 30 s, and injection time 0.08 min. Initial oven temperature of the gas chromatograph was 75 ^{o}C for for 6 min, followed by ramping at 25 ^{o}C/min to 110 ^{o}C and held for 3 min. FID temperature was 250 ^{o}C. Nitrogen was used as carrier gas maintained at 3.5 mL/min flow rate, using the split ratio of 5:1.

4.2.5.7 HS-SPME-GC-MS Analysis

Volatile compounds were determined according to the method of Molina-Calle et al. (2017) with some modification. An aliquot of 25 mL sample was poured into a 60 mL glass vial containing 5 g of sodium chloride. The vial was sealed and incubated at 40 ^{o}C for 10 min using a hotplate stirrer (VWR Co., USA). Volatiles were extracted with a solid phase microextraction (SPME) fiber coated with divinylbenzene / carboxen / polydimethylsiloxane (DVB/CAR/PDMS 50/30 μm) (Supelco, Bellefonte, PA, USA). The SPME fiber was exposed to the sample headspace for 40 min at 40 ^{o}C to allow for volatile absorption, and then transferred to the injection port (GC-MS; TQ8030, Shimadzu Co., Japan) for 5 min to ensure total desorption. The GC was equipped with a VF-Wax MS column (60 m × 0.25 mm × 0.5 μm, Agilent J & W, USA). The injector temperature was fixed at 230 ^{o}C and injection was carried out in 1/30 split mode. The gas flow was set at 1 mL/min. Column temperature was held initially at 30 ^{o}C for 4 min, and then ramped at 5 ^{o}C /min to 100 ^{o}C, held for 5 min. This was followed by a second ramp at 3 ^{o}C /min to 220 ^{o}C and held for 10 min. The MS parameters were listed as follows: ion source temperature 230 ^{o}C in full scan mode (m/z 29.00–360.00); solvent delay 3 min; total analysis time 73 min; data acquisition time 13 to 73 min. The compound identifications were achieved by comparing their retention indices (RI) and mass spectra fragments with those obtained from a database (NIST11 & NIST11s Library, Shimadzu Co., Japan). When standards were not available, volatile compounds were tentatively identified using GC–MS spectra only. The volatile compounds were identified by similarity of no less than 70%. The quantities of the volatile compounds were reported in relative contents (%),

which was calculated by taking the peak area of the identified volatile compound divided by the sum of all peak areas.

4.2.5.8 HPLC Analysis for Caffeine

The determination of caffeine was done according to National Food Safety Standards of P.R. China (GB 5009.139 Determination of Caffeine in Beverages), with some modification. Briefly, for the instant coffee powder, 1.000 g sample was transferred into a 250 mL conical flask, 200 mL purified water was added, and mixed thoroughly. The flask was placed into a boiling water bath for 30 min with intermittent shaking. The flask was then cooled under running water, followed by the addition of 5 g of magnesium oxide and mixed by a vortex mixer (QL-901, Haimen, China) for one min. The flask was placed into the boiling water bath and heated again for 20 min, cooled to 20 ^{o}C, transferred the solution into a 250 mL volumetric flask, and then diluted with water to the scale. The supernatant was filtered by 0.45 and 0.22 micro filter in sequence and injected into the column for caffeine analysis. For the fermenting and fermented CSAS, the sample was degassed for 10 min by a sonicator (KQ-250DE, Kunshan, China). An aliquot of 5 mL of the degassed sample was transferred into a test tube, and 0.5 g magnesium oxide were added，mixed completely by a Vortex (QL-901, Haimen, China), and then the supernatant was filtered by 0.45 and 0.22 μm filter in sequence and injected into the column for caffeine analysis.

Caffeine content in samples was determined by an external standard method using a high-performance liquid chromatograph (LC-20AT; Shimadzu Co, Japan) equipped with a C18 column (4.6mm×250 mm, 5 μm, Waters Co.,

USA) and a diode-array detector (DAD) (SPD-M20A, SHIMADZU Co, Japan). Water: methanol (24:76, v/v) was used as a mobile phase at a flow rate of 1.0 mL/min. The column temperature was 25 ^{o}C. Before use, the mobile phase was degassed by an ultrasonic apparatus (KQ-250DE, Kunshan, China) and filtered (0.45 μm nylon filter). An aliquot of 10 μL sample was injected into the column by an autosampler (SIL-20A, SHIMADZU Co, Japan) and the analytes were detected at 273 nm.

4.2.6 Sensory Evaluation

Descriptive sensory evaluations were performed according to Analytica-EBC method (13.13 Sensory Analysis: Routine Descriptive Test Guideline) with some modification. The evaluation was conducted by Shandong Taishan Beer Co. Ltd. (Taian, China), using their trained sensory panel, following the Chinese Institute of Brewing sensory analysis procedures. The tasting panel was comprised of five female and six male sensory assessors, with ages ranging from 20 to 50 years old and who consumed coffee on a regular basis (once or twice per week). The panel judged the sample quality by appearance, foam, aroma, taste, and coffee flavor typicality.

4.2.7 Statistical Analysis

Analyses were performed in duplicate. Data were statistically analyzed using SPSS Statistics 22 software and results were expressed as mean ± 1.0 standard deviation. Significant differences between the treatments were examined by least significant difference (LSD) method, with $p < 0.05$ being considered as statistically significant.

4.3 Results and Discussion

4.3.1 Changes in Physicochemical Properties

4.3.1.1 Total Soluble Solid (TSS)

The TSS (Figure 4.1) decreased with increasing fermentation time, mainly due to the depletion of sugar by yeasts. After 168 h of fermentation, Safbrew wb-34/70, Lalvin EC-1118 and SIHA Active Yeast 3 had the lowest TSS (~5.5 Brix), while SafAle US-05 had significantly higher TSS value (~7 Brix).

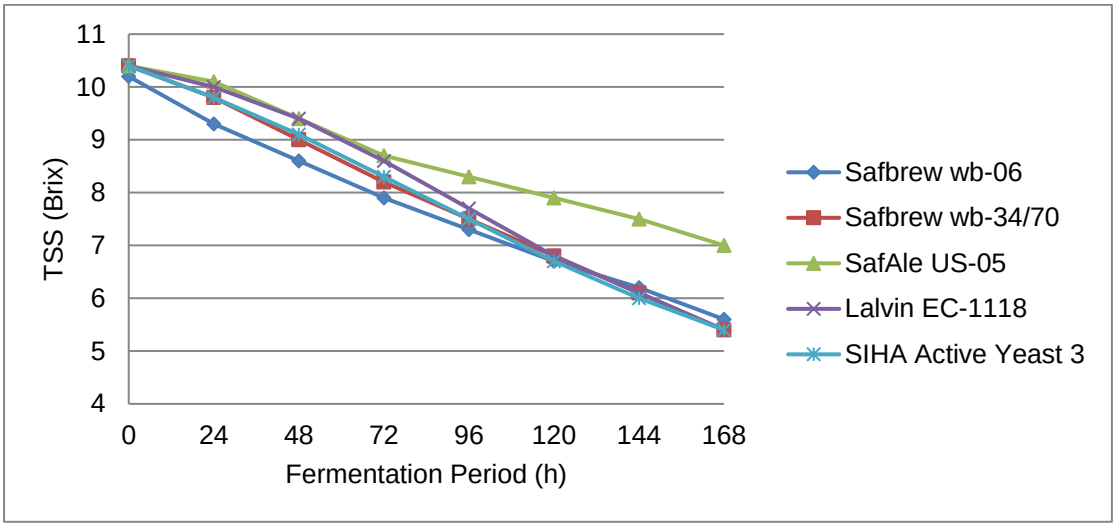


Figure 4.1 - Changes in total soluble solid (TSS) during preliminary fermentation.

4.3.1.2 pH Value and Total Acidity (TA)

The pH values for all samples showed decreasing trends as fermentation progressed due to the formation of organic acids (Figure 4.2). At 168 h, Lalvin EC-1118 had the highest pH value of 4.15, while SafAle US-05 showed the lowest (pH 3.99). The pH values of brewed samples prepared from Safbrew wb-06, Safbrew wb-34/70, and SIHA Active Yeast 3 were between this range. The total acidity showed a similar trend during the early phase of fermentation

(Figure 4.3). Overall, samples brewed from Lalvin EC-1118 had the lowest total acidity (2.5 mL 1M NaOH/100 mL) during the whole main fermentation, which was followed by SafAle US-05, SIHA Active Yeast 3, Safbrew wb-06, and Safbrew wb-34/70 (2.6, 2.7, 2.8, and 2.8 mL 1 M NaOH/100 mL, respectively). Comparing Figures 4.2 and 4.3, the minimal changes of pH values towards the end of fermentation period can be attributable to the formation of other metabolites by the yeasts that exhibited pH buffering capacity in CSAS.

4.3.1.3 Reducing Sugar (RS) and Total Sugars (TS)

In general, during the fermentation process, the yeasts converted the fermentable sugar into biomass, alcohols (mainly ethanol) and other by-products. In the present study, however, the sugar profiles displayed complex trends dependent of the yeast strains (Fig. 4.4).

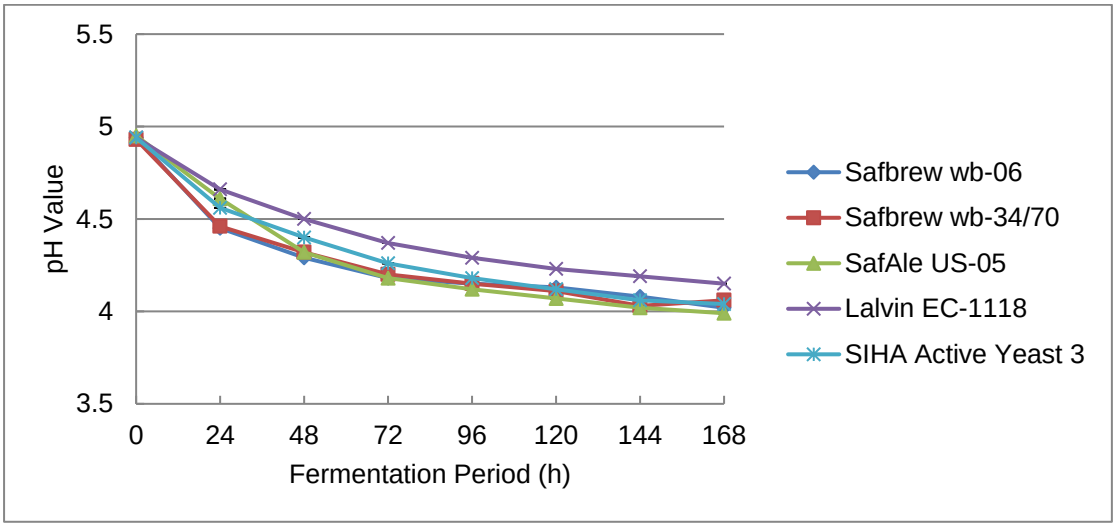


Figure 4.2 - Changes in pH value during preliminary fermentation.

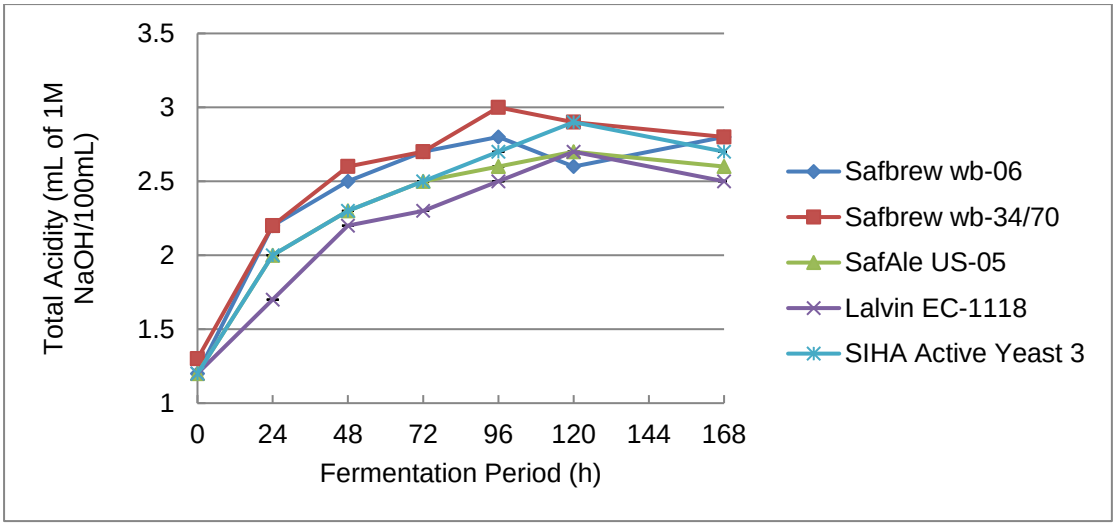


Figure 4.3 - Changes in total acidity during preliminary fermentation.

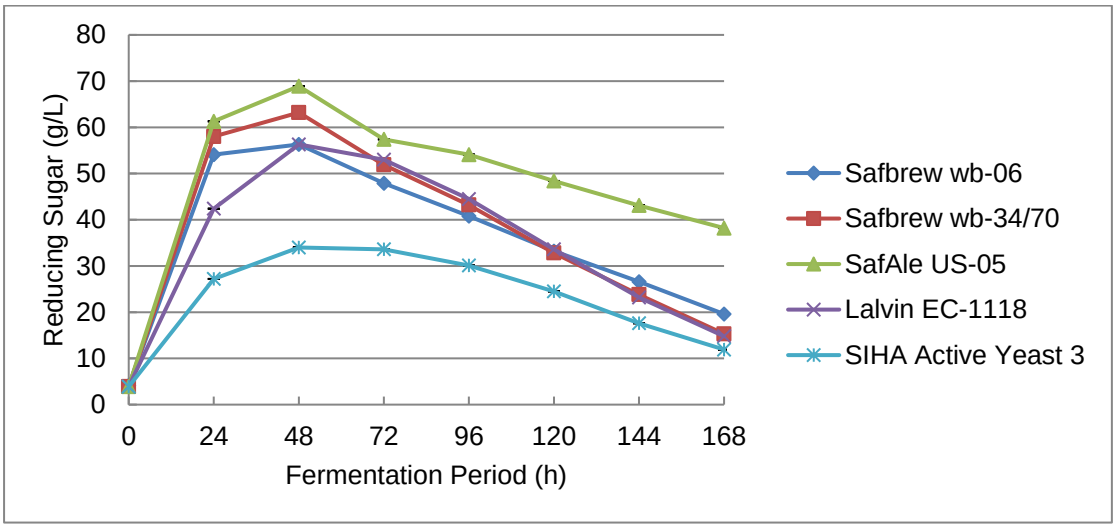


Figure 4.4 - Changes of reducing sugar during preliminary fermentation.

Before yeast inoculation (0 h), the reducing sugar (RS) contents ranged from 3.9 to 4.1 g/L (Figure 4.4). After inoculation, the yeasts secreted invertase which hydrolyzed sucrose into glucose and fructose, but with different invertase activities (Evangelista et al., 2014). The reducing sugar contents reached the highest level at around 48 h, up to 45.3 g/L for samples derived from Lalvin EC-

1118, followed by those fermented by SIHA Active Yeast 3 (26.3 g/L in). At 168 h, brews from SIHA Active Yeast 3 had the lowest RS content (11.9 g/L), while SafAle US-05 the highest (38.2 g/L). The more reducing sugar left in the samples, the sweeter flavor would present in the beverages. Therefore, SafAle US-05 tended to have highest level of sweetness in sensory aspect.

TS in all brew samples decreased linearly during fermentation with the higher TS was found in samples prepared using SafAle US-05 (Figure 4.5). The lower the TS at the end of the fermentation process, the higher the fermentation capacity the yeast possessed. The greatest decrease in TS was found in samples fermented by Safbrew wb-06 and wb-34/70 during the first 24 h (Figure 4.4). This observation implies that Safbrew wb-06 and wb-34/70 had excellent capacities to start alcohol fermentation.

On the contrary, samples prepared from Lalvin EC-1118 and SIHA Active Yeast 3 possessed higher reducing and total sugar contents on the first 24 h, suggesting that these two yeasts had lower rate of starting fermentation. However, both yeasts gave the lowest residual sugar at the end of the 168-h fermentation period. On the other hand, SafAle US-05 possessed lower invertase activity and moderate alcohol fermentation rate on the first 24 h (Figures 4.4 & 4.5). It could be a good candidate for the production of low alcoholic coffee beverages due to easier manipulation of alcohol content as compared to other yeast strains tested.

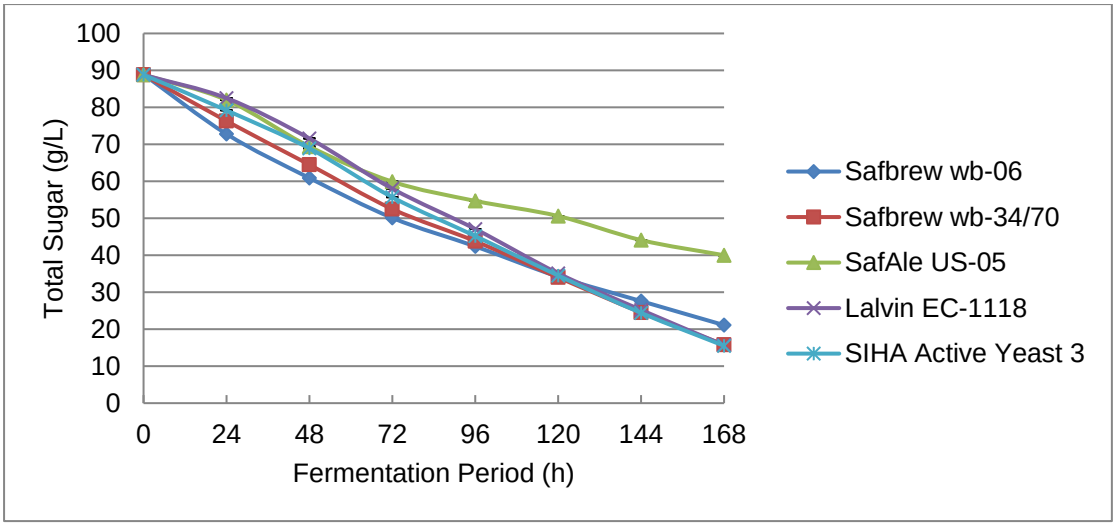


Figure 4.5 - Changes in total sugar during preliminary fermentation.

4.3.1.4 Yeast Assimilable Nitrogen (YAN)

The CSAS, before fermentation, contained approximately 35 mg/L of free YAN (Figure 4.6). At the initial stage of fermentation, the yeasts could uptake YAN for their multiplication, resulting in a decrease in free YAN. However, during the later stage of the fermentation, the yeasts could produce amino acids which were released into the fermenting solution, causing an increase in YAN value. As shown in Figure 4.6, most of YAN (34.4 mg/L) was utilized by yeast SIHA Active Yeast 3 within the first 24 h, but some quantities of YAN were released into the CSAS from 48 h onwards. At 168 h the highest level of YAN of 9.5 mg/L was determined in samples from Safbrew wb-34/70, followed by those from SIHA Active Yeast 3 (8.7 mg/L). Free YAN values for brews from Safbrew wb-06, SafAle US-05 and Lalvin EC-1118 were not significantly different ($p>0.05$). Based on the results from Figure 4.6, it can be concluded that 35.8 mg/L of YAN is appropriate to support yeast multiplication and alcohol fermentation.

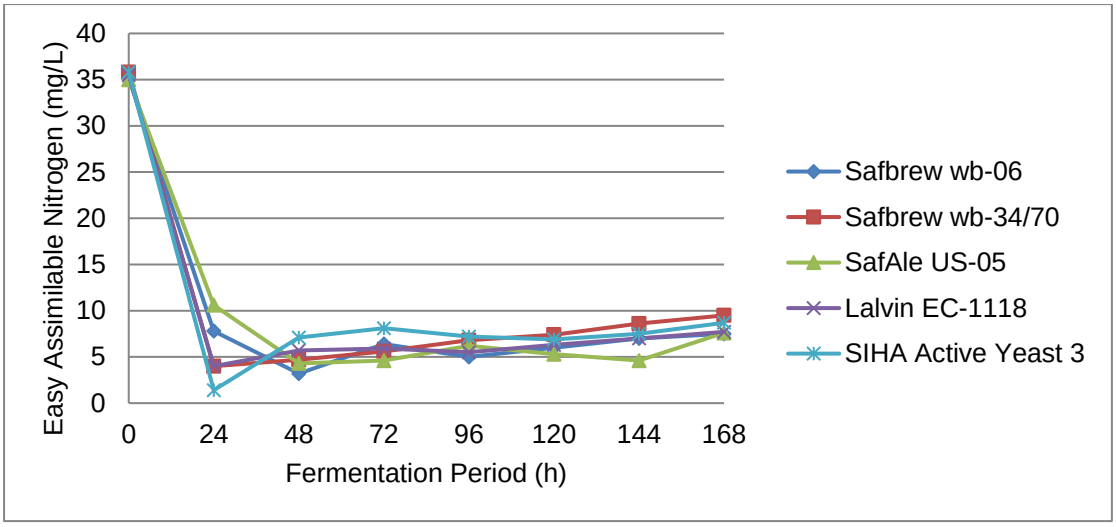


Figure 4.6 - Changes in free YAN during preliminary fermentation.

4.3.1.5 Alcohol Content

During fermentation, yeasts converted fermentable sugar such as glucose, fructose, and sucrose into alcohol. The rates of alcohol production among samples varied, especially during the early phase of fermentation. At 24 h, alcohol contents were 0.58, 0.56, 0.38, 0.24, and 0.20 % for samples derived from Safbrew wb-06, Safbrew wb-34/70, SIHA Active Yeast 3, SafAle US-05, and Lalvin EC-1118, respectively. After 24 h, the differences of alcohol content among the samples became smaller, with SafAle US-05 exhibited the lowest alcohol producing rate. Accordingly, for the production of low or non-alcoholic fermented coffee beverages, SafAle US-05, Lalvin EC-1118, and SIHA Active Yeast 3 would be advantageous.

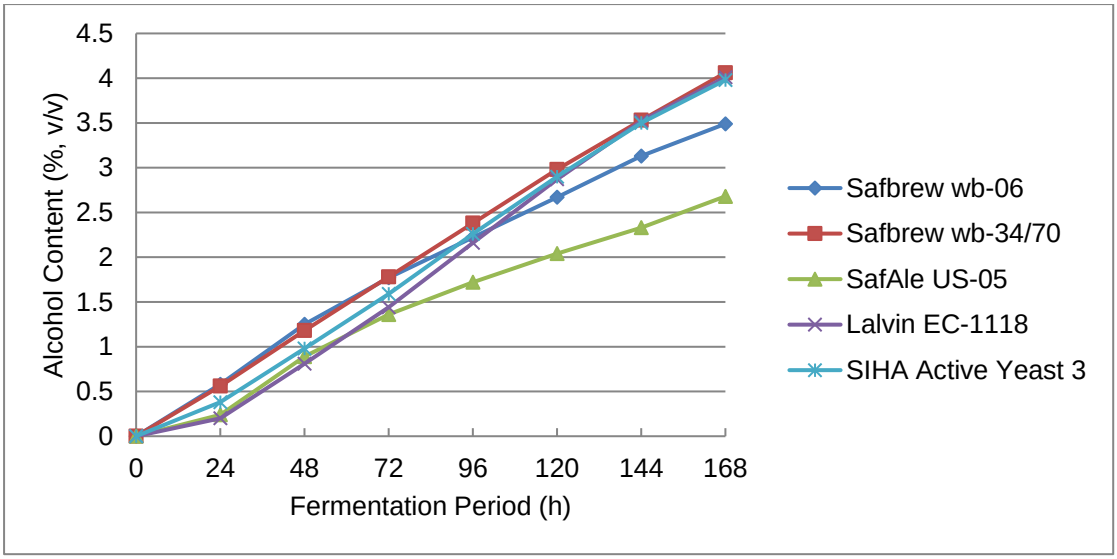


Figure 4.7 - Changes in alcohol content during preliminary fermentation.

4.3.1.6 Vicinal Diketone (VDK) Content

VDK is a family of aroma diketones with two adjacent carbonyl groups in the molecular structure. VDK compounds commonly present in fermented alcohol beverages include diacetyl (2,3-butanedione) and 2,3-pentanedione. Diacetyl and 2,3-pentanedione are also naturally present in roasted coffee characterized by their sweet, buttery, creamy, and milky flavor (Weschenfelder et al. 2015). The sensory threshold of diacetyl was about ten time lower than that of 2,3-pentanedione, and therefore, the former has a relatively higher sensory importance. In the fermented products, both diacetyl and 2,3-pentanedione were detected by the spectrophotometry method via reaction with o-phenylenediamine to produce quinoxalines. The VDK compounds could be produced by yeast during the early stage of fermentation, which entered into the solution from the yeast cells.

As shown in Figure 4.8, the VDK content in CSAS before fermentation was 0.57 mg/L. After inoculating with yeast, VDK contents samples increased

at different rates, with a maximal level occurred at 48 h for SafAle US-05 (4.21 mg/L), Safbrew wb-34/70 (1.27 mg/L), and Lalvin EC-1118 (1.11 mg/L). Maximal VDK was observed at 24 h for SIHA Active Yeast 3 (1.29 mg/L) and Safbrew wb-06 (0.95 mg/L). Since VDK could be reduced by the yeasts, the VDK levels in fermenting CSASs of Safbrew wb-34/70, SIHA Active Yeast 3, and Safbrew wb-06 were lower than that of their corresponding CSASs respectively at 168 h. Safbrew wb-06 possessed the potential to ferment good-quality fermented drink with lower VDK level and following by SIHA Active Yeast 3, Lalvin EC-1118, and Safbrew wb-34/70.

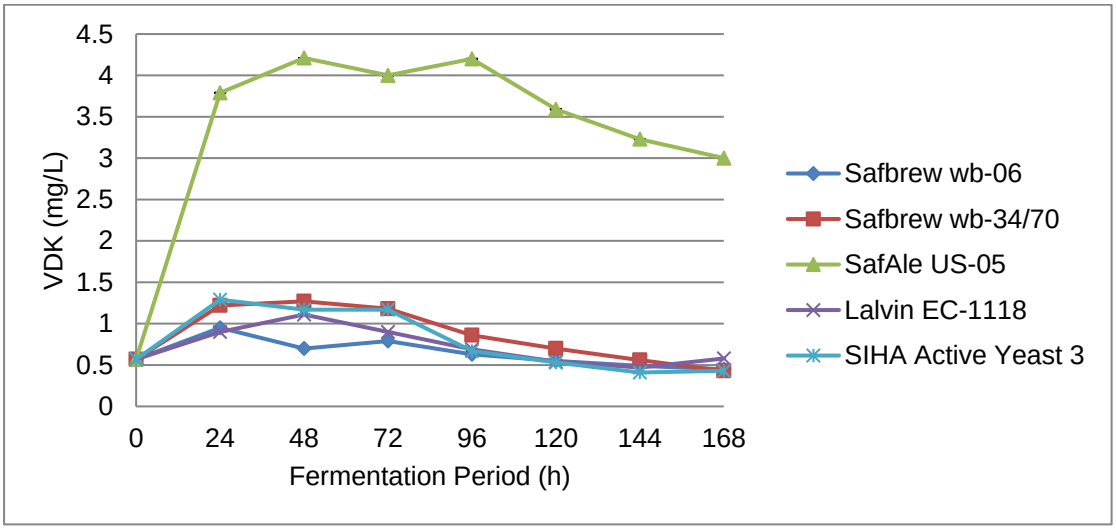


Figure 4.8 - Changes in VDK during preliminary fermentation.

4.3.1.7 Caffeine

During the 8-day fermentation period, the changes in caffeine content were monitored (Figure 4.9). The concentration of caffeine in CSAS samples ranged from 600 to 900 mg/L. On day 7, decreases in caffeine concentrations on day 7 were observed among samples. The reason for this observation is unknown. However, considering that there was a recovery of caffeine

concentration on day 8 and there are no known mechanisms that would generate new caffeine during the storage, the anomaly was likely due to an experimental error. Overall, during the entire fermentation process, there was no significant changes in caffeine contents in samples for the five strains of yeast tested.

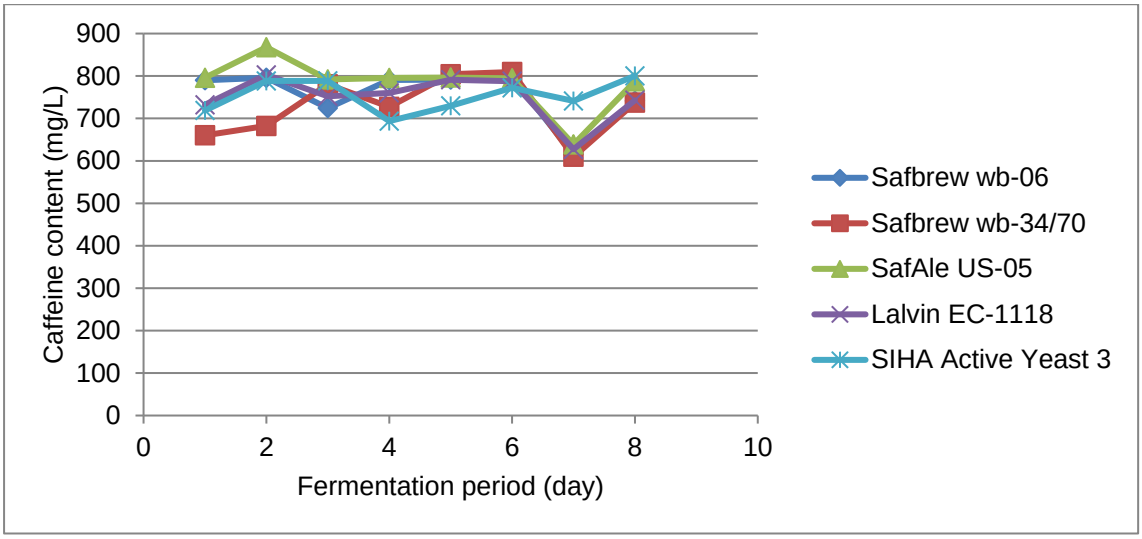


Figure 4.9 - Changes in caffeine content during fermentation.

4.3.2 Physicochemical Properties of Finished Product

Physicochemical properties of the final products after fermentation are summarized in Table 4.2. All samples had comparable pH values ranging from 3.95 (SafAle US-05) to 4.13 (Lalvin EC-1118). TA values spanned from 2.7 (Lalvin EC-1118) to 3.1 mL/100 mL (Safbrew wb-06, Safbrew wb-34/70, and SafAle US-05). The contents of reducing and total sugars were determined by the fermentation rate of the yeasts. The highest levels of reducing and total sugars were detected in samples prepared from SafAle US-05, followed by that of Safbrew wb-06, Lalvin EC-1118, Safbrew wb-34/70, and SIHA Active Yeast 3 ($p<0.05$). For total alcohol, the highest level was found in SIHA Active Yeast

3, followed by that of Safbrew wb-34/70, Lalvin EC-1118, Safbrew wb-06, and SafAle US-05, at 4.64, 4.54, 4.44, 3.85, and 3.03 (%, v/v), respectively. As one of the most important flavor components, VDK was detected in all samples. However, all yeasts demonstrated strong VDK-reducing capacity, except SafAle US-05, which exhibited similar level of VDK as in CSAS before fermentation (Table 4.2). Compare with data in Figure 9, the level of caffeine did not change significantly after fermentation (Table 4.2).

During the later stage of fermentation, considerable quantities of CO_2 were generated in the bottle. The CO_2 contents among samples were similar (0.3-0.4%) (Table 4.2). All the samples exhibited distinctive foaming characteristic with different foam stability upon decompression. The longest foam retention time was observed for SIHA Active Yeast 3 (324 s), while the shortest (252 s) in Lalvin EC-1118 (Table 4.2). Sensory characters were also different among the samples prepared from different strains of yeast. The final fermented CSAS by Safbrew wb-06 showed typically complex flavors of yeast and coffee, while those from Safbrew wb-34/70 gave typical coffee favor. The samples from Lalvin EC-1118 possessed green/astringent flavor, while those by SIHA Active Yeast 3 had neutral and astringent flavor. Among all fermented samples, SafAle US-05 presented a balanced flavor profile.

Table 4.2 - Physicochemical indices of final fermented CSAS products.

Parameters	Safbrew wb-06	Safbrew wb-34/70	SafAle US-05	Lalvin EC-1118	SIHA Active Yeast 3
pH	4.02±0.00^{c}	4.05±0.00^{b}	3.95±0.00^{d}	4.13±0.00^{a}	4.02±0.00^{c}
TA (mL/100 mL)	3.1±0.0^{a}	3.1±0.0^{a}	3.1±0.0^{a}	2.7±0.0^{c}	3.0±0.0^{b}

RS (g/L)	14.5±0.0^{b}	10.4±0.1^{d}	34.4±0.0^{a}	10.7±0.0^{c}	8.4±0.0^{e}
TS (g/L)	15.1±0.1^{b}	10.9±0.1^{d}	35.7±0.0^{a}	11.4±0.0^{c}	10.3±0.0^{e}
YAN (mg/L)	4.4±0.1^{c}	6.0±0.1^{b}	4.2±0.1^{c}	5.9±0.1^{b}	6.5±0.1^{a}
VDK (mg/L)	0.10±0.0^{c}	0.11±0.0^{b}	0.58±0.0^{a}	0.10±0.0^{c}	0.10±0.0^{c}
CA (mg/L)	795.84±0.28^{d}	800.59±0.17^{b}	797.6±0.09^{c}	788.48±0.04^{e}	802.92±0.15^{a}
AL (%)	3.85	4.54	3.03	4.44	4.64
FR (second)	293	284	256	252	324
CO_2 (%, v/v)	0.34	0.38	0.38	0.33	0.37
Sensory characteristic	Yeast, coffee, complex	Typical coffee, pure	Balance	Green, Astringent	Neutral, astringent

Note: CSAS, coffee sugar aqueous solution. TA, total acidity (mL/100 mL); RS, reducing sugar (g/L); TS, total sugar (g/L); YAN, easy assimilable nitrogen (mg/L); VDK, vicinal diketones (mg/L); CA, caffeine (mg/L); AL, alcohol (%, v/v); FR, foam retention (second); CO_2, carbon dioxide (%, v/v). Same lowercase letter indicates no significant difference ($p>0.05$).

Table 4.3 - Low boiling point volatiles in final fermented CSASs.

Volatile compounds (threshold, mg/L*)	CSAS	Safbrew wb-06	Safbrew wb-34/70	SafAle US-05	Lalvin EC-1118	SIHA Active Yeast 3
Acetaldehyde (10 mg/L)	0.47±0.02^{e}	9.11±0.35^{d}	11.39±0.04^{c}	10.86±0.07^{c}	34.36±0.00^{a}	21.81±0.47^{b}
Ethyl formate (150 mg/L)	0	0.13±0.00^{b}	0.20±0.01^{a}	0.20±0.00^{a}	0.14±0.00^{b}	0.20±0.00^{a}
Ethyl acetate (33 mg/L)	0	8.87±0.12^{b}	10.30±0.121^{a}	3.10±0.04^{e}	7.28±0.08^{c}	6.21±0.08^{d}
Isobutyl acetate (1.6 mg/L)	0	0.04±0.00^{a}	0.04±0.001^{a}	0.03±0.00^{a}	0.04±0.00^{a}	0.034±0.00^{a}
n-Propyl alcohol (800 mg/L)	0	12.08±0.01^{a}	7.08±0.80 ^{bc}	8.35±0.34^{b}	6.23±0.12^{c}	6.64±0.30^{c}
Isobutanol (200 mg/L)	0	25.29±0.20^{c}	17.70±0.24^{d}	34.37±0.19^{a}	25.28±0.02^{c}	28.97±0.32^{b}
Isoamyl acetate (1.6 mg/L)	0	0.09±0.01^{c}	0.20±0.01^{a}	0.02±0.00^{d}	0.10±0.00^{c}	0.15±0.00^{b}
Isoamylol (65 mg/L)	0.47±0.19^{e}	114.99±1.72^{a}	90.68±0.82^{c}	58.12±1.64^{d}	110.04±1.16^{ab}	107.28±1.76^{b}
Ethyl hexanoate (0.23 mg/L)	0	0.08±0.00^{a}	0.04±0.00^{bc}	0.03±0.00^{c}	0.08±0.00^{a}	0.05±0.00^{b}
Ethyl octanoate (0.9 mg/L)	0.50±0.52^{a}	0.23±0.19^{a}	0.02±0.00^{a}	0.15±0.08^{a}	0.30±0.40^{a}	0.36±0.12^{a}
Total esters	0.51±0.10^{f}	9.434±0.21^{b}	10.82±0.10^{a}	3.54±0.03^{e}	7.94±0.21^{c}	7.02±0.03^{d}

Total alcohols	0.44±0.20^{e}	152.35±1.36^{a}	115.46±0.16^{c}	100.84±1.54^{d}	141.55±0.89^{b}	142.88±1.68^{b}
Alcohol/ester	0.50	16.14	10.67	28.45	17.83	20.36

Note: CSAS, coffee sugar aqueous solution; same lowercase letter indicates no significant difference (p>0.05); *threshold values cited from Briggs et al. (2004).

4.3.3 Analysis of Volatiles by HS-SPME-GC-MS

As shown in Table 4.3, acetaldehyde and isoamylol contents in the fermented samples were higher than their sensory thresholds (10 and 65 mg/L, respectively) according to the values provided by Briggs et al. (2004). Acetaldehyde is a precursor to ethanol production during yeast fermentation, and a lower level acetaldehyde is perceived as a fruity flavor, while at a higher level it gives a distinctive green apple aroma. Acetaldehyde levels for Safbrew wb-06, Safbrew wb-34/70, and SafAle US-05 were comparable, ranging from 9.11 to 11.39 mg/L. Brews from Lalvin EC-1118 and SIHA Active Yeast 3 had significantly higher ($p<0.05$) acetaldehyde contents, which were 34.46 and 21.81 mg/L, respectively.

Isoamyl alcohol (i.e. 3-methyl-1-butanol) has pleasant vinous, banana, and sweet flavor with perception threshold 65 mg/L. However, at higher levels, isoamyl alcohol will present off-flavor in the beverage. The lowest level of isoamyl alcohol was found in SafAle US-05, followed by Safbrew wb-34/70, SIHA Active Yeast 3, Lalvin EC-1118, and Safbrew wb-06, which were 58.12, 90.68, 107.28 and 110.04 ($p>0.05$), and 114.99 mg/L, respectively. SIHA Active Yeast 3, Lalvin EC-1118, and Safbrew wb-06 had comparable isoamyl alcohol contents (107.28, 110.04 and 114.99 mg/L, respectively), while Safbrew wb-

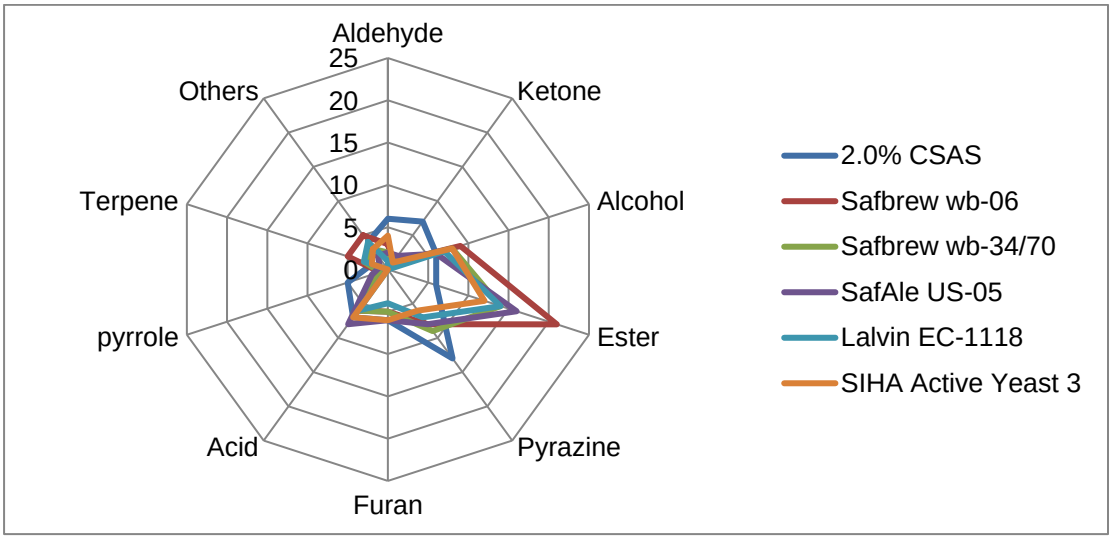
34/70 and Safbrew wb-34/70 had significantly lower ($p < 0.05$) isoamyl alcohol levels (90.68 and 58.12 mg/L, respectively).

The presence of other volatiles could also influence the flavor of the fermented products. Researchers have looked at the total alcohols, total esters, and the ratio of alcohol to ester to evaluate the flavor quality of the final products. In total, 62 volatile components were detected in CSAS before fermentation. By contrast, 64, 52, 53, 46, and 49 volatiles were detected in final brew products from, Safbrew wb-06, Safbrew wb-34/70, SafAle US-05, Lalvin EC-1118, and SIHA Active Yeast 3, respectively. The relatively large quantities of volatiles detected may explain the more complex flavor characteristics of Safbrew wb-06 as compared to other brews. Safbrew wb-34/70 possessed the lowest alcohol/ester ratio of 10.67, followed by Safbrew wb-06 and Lalvin EC-1118, while SafAle US-05 had the highest alcohol/ester ratio of 28.45 (Table 4.3). The higher alcohol/ester ratio was, the more desirable sensory characteristics would show up in the beverage. Therefore, Safbrew US-05 had the most desirable sensory features among five samples.

The categories of compounds detected are summarized in Figure 4.10, showing that the aldehydes, ketones, pyrazines, and pyrroles content decreased after fermentation. In particular, ketones and pyrroles decreased considerably. On the other hand, alcohols increased in Safbrew wb-06 and wb-34/70, Lalvin EC-1118, and SIHA Active Yeast 3, but minimal changes were observed in SafAle US-05. Two terpenes were found in CSAS, five in Safbrew wb-06, three in Safbrew wb-34/70 and Lalvin EC-1118, while only one in SafAle US-05. Yeast produced ester as by-products and 21, 15, 16, 14, 12 esters was

found in Safbrew wb-06, Safbrew wb-34/70, SafAle US-05, Lalvin EC-1118, and SIHA Active Yeast 3, but only six esters were detected in CSAS.

Six furans were found in CSAS, SafAle US-05 and SIHA Active Yeast 3, five in Safbrew wb-06 and Safbrew wb-34/70, and four in Lalvin EC-1118. Eight organic acids were detected in SafAle US-05, seven in CSAS and SIHA Active Yeast 3, while six in Safbrew wb-06, Safbrew wb-34/70 and Lalvin EC-1118.



Note: “Others” include ethers, pyridine, alkenes, hydrocarbons, lactone, and phenols.

Figure 4.10 - Number of compounds detected in key categories of volatiles in CSAS before fermentation and final brew products.

4.3.4 Specific Compounds

4.3.4.1 Aldehydes

Before fermentation, several aldehydes were detected in CSAS samples, including acetaldehyde, 2-methylpropanal, hexanal, trans-2-methyl-2-butenal, decanal, and benzaldehyde. After fermentation, 2-methylpropanal, hexanal, decanal, benzaldehyde, and trans-2-methyl-2-butenal in CSAS were

disappeared or depleted considerably. Notably, 10-undecenal and undecanal were detected only in SIHA Active Yeast 3 (Table 4.4).

Table 4.4 - Aldehydes in the final products by HS-SPME-GC-MS analysis.

RT	Volatiles	CAS No.	Relative content (%)					
			CSAS	1	2	3	4	5
5.245	Acetaldehyde	75 - 07 - 0	0.18	0.03	0.8	0.99	0.07	1.13
8.093	2-Methylpropanal	78 - 84 - 2	2.12	-	-	-	-	-
17.853	Hexanal	66 - 25 - 1	0.18	-	-	-	-	-
18.394	trans-2-Methyl-2-butenal	497 - 03 - 0	0.27	-	-	-	-	-
32.449	10-Undecenal	112 - 45 - 8	-	-	-	-	-	0.12
37.422	Decanal	112 - 31 - 2	0.28	0.08	0.06	-	-	0.08
38.425	Benzaldehyde	100 - 52 - 7	3.42	0.18	-	0.09	-	-
46.59	Undecanal	112 - 44 - 7	-	-	-	-	-	0.04

RT, retention time; CSAS, coffee sugar aqueous solution; “-”, not detected; 1, Safbrew wb-06; 2, Safbrew wb-34/70; 3, SafAle US-05; 4, Lalvin EC-1118; and 5, SIHA Active Yeast 3.

4.3.4.2 Ketones

As showed in Table 4.5, ketones that were initially present in CASAs depleted considerably after fermentation, including 2-methyl-3-pentanone, 2,3-butanedione, 3-hexanone, 2,3-pentanedione, cyclohexanone, 3-methyl-1,2-cyclopentanedione, and 6,10-dimethyl-5,9-undecadien-2-one. However, a new ketone, cyclopentanone, was formed in Safbrew wb-06, Safbrew wb-34/70 and SafAle US-05. Other ketones, 2-methyl-3-heptanone and 2-methyl-3-thiolanone, were detected in fermented samples from SIHA Active Yeast 3 and SafAle US-05, respectively.

Table 4.5 - Ketones in the final fermented products by HS-SPME-GC-MS analysis.

RT	Volatiles	CAS No.	Relative content (%)					
			CSAS	1	2	3	4	5
13.909	2-Methyl-3-pentanone	565 - 69 - 5	0.48	-	-	-	-	-
13.985	2,3-Butanedione	431 - 03 - 8	1.4	-	-	-	-	-
16.691	3-Hexanone	589 - 38 - 8	0.29	-	-	-	-	-
16.959	2,3-Pentanedione	600 - 14 - 6	1.47	-	-	-	-	-
22.266	Cyclopentanone	120 - 92 - 3	-	0.03	0.04	0.04	-	-
27.976	Cyclohexanone	108 - 94 - 1	0.56	-	-	-	-	-
35.311	2-Methyl-3-heptanone	13019 - 20 - 0	-	-	-	-	-	0.12
39.477	2-Methyl-3-thiolanone	13679 - 85 - 1	-	-	-	0.12	-	-
51.689	3-Methyl-1,2-cyclopentanedione	765 - 70 - 8	0.6	-	-	-	-	-
52.2	6,10-Dimethyl-5,9-undecadien-2-one*	689 - 67 - 8	1.07	-	-	-	-	-

RT, retention time; CSAS, coffee sugar aqueous solution; “-”, not detected; 1, Safbrew wb-06; 2, Safbrew wb-34/70; 3, SafAle US-05; 4, Lalvin EC-1118; and 5, SIHA Active Yeast 3.

4.3.4.3 Alcohols

Table 4.6 summarizes various alcohols detected (excluding ethanol) in CSAS before fermentation, with 3-methyl-1-butanol being the major fraction (4.27%), followed by phenylethyl alcohol of (1.84%). Many of these alcohols disappeared after fermentation, such as isobutanol, 2-heptanol and 1-hexanol. As one of the major by-products of alcoholic fermentation, 3-methyl-1-butanol appeared in all the fermented products, with relative contents (the percentage of alocohol content in total volatile compounds contetn) in Safbrew wb-06, Safbrew wb-34/70, SafAle US-05, Lalvin EC-1118, and SIHA Active Yeast 3 at 30.5, 37.9, 27.5, 36.2, and 43.9%, respectively. Similarly, the relative contents of

phenylethyl alcohol, which is one of the by-products of alcoholic fermentation, increased in Safbrew wb-06, Safbrew wb-34/70, SafAle US-05, Lalvin EC-1118, and SIHA Active Yeast 3, up to 27.9, 12.6, 10.6, 8.2, and 11.4%, respectively. Other traces of alcohols detected were, 2-methyl-1-propanol, 1-octanol, 1-propanol, 1-butanol, isohexanol, 3-ethoxy-1-propanol, 1-heptanol, 1-decanol, and 1-dodecanol.

Table 4.6 - Alcohols in the final fermented products by HS-SPME-GC-MS analysis.

RT	Volatiles	CAS No.	Relative content (%)					
			CSAS	1	2	3	4	5
15.73	1-Propanol	71 - 23 - 8	-	0.19	0.24	0.22	-	0.12
17.726	3-Methyl-1-propanol	78 - 83 - 1	-	1.64	1.72	3.94	1.99	2.9
17.933	Isobutanol	78 - 83 - 1	0.16	-	-	-	-	-
19.776	1-Butanol	71 - 36 - 3	-	0.01	0.03	-	-	-
22.556	isoamylol	123 - 51 - 3	4.27	30.46	37.86	27.47	36.24	43.94
27.998	Isohexanol	626 - 89 - 1	-	0.02	-	-	-	-
28.188	2-Heptanol	543 - 49 - 7	0.35	-	-	-	-	-
30.009	1-Hexanol	111 - 27 - 3	0.22	-	-	-	-	-
31.425	3-Ethoxy-1-propanol	111 - 35 - 3	-	-	-	-	0.08	-
31.92	3-Octanol	589 - 98 - 0	0.56	-	0.11	-	-	-
35.025	1-Heptanol	111 - 70 - 6	-	0.07	0.05	0.84	-	0.04
39.775	1-Octanol	111 - 87 - 5	-	0.04	0.04	0.05	0.03	0.11
48.315	1-Decanol	112 - 30 - 1	-	0.04	-	-	0.06	0.1
54.708	Phenylethyl Alcohol	1960/12/8	1.84	27.93	12.63	10.59	8.15	11.4
55.894	1-Dodecanol	112 - 53 - 8	-	-	--	-	0.08	0.1

RT, retention time; CSAS, coffee sugar aqueous solution; “-”, not detected; 1, Safbrew wb-06; 2, Safbrew wb-34/70; 3, SafAle US-05; 4, Lalvin EC-1118; and 5, SIHA Active Yeast 3.

4.3.4.4 Terpenes

Eucalyptol and citronellol were detected in unfermented CSAS, which disappeared or decreased significantly in the final fermented products. However, one new terpene, trans-nerolidol, was formed in all fermented samples with relative content stranging from 0.14 (SafAle US-05) to 0.85% (Lalvin EC-1118). Other detected new terpenes were linalool, citronellyl butyrate, and citronellal, which were detected only in Safbrew wb-06. On the other hand, 2,3-dihydro-6-trans-farnesol was detected in Safbrew wb-06 and Lalvin EC-1118. Among the yeast strains, brews prepared from Safbrew wb-06 exhibited the largest total relative contents of terpene (Table 4.7).

Table 4.7 - Terpenes in the final fermented products by HS-SPME-GC-MS analysis.

RT	Volatiles	CAS No.	Relative content (%)					
			CSAS	1	2	3	4	5
23.232	Eucalyptol	470 - 82 - 6	0.19	-	0.08	-	-	-
39.264	Linalool	78 - 70 - 6	-	0.06	-	-	-	-
48.514	Citronellyl butyrate	141 - 16 - 2	-	0.02	-	-	-	-
48.516	Citronellol	106 - 22 - 9	0.5	-	0.09	-	0.12	0.06
58.425	Trans-Nerolidol	40716 - 66 - 3	-	0.5	0.23	0.14	0.85	0.28
66.06	2,3-Dihydro-6-trans-farnesol	20576 - 54 - 9	-	0.16	-	-	0.24	-
69.681	Citronellel	106 - 23 - 0	0	0.25	-	-	-	-

RT, retention time; CSAS, coffee sugar aqueous solution; “-”, not detected; 1, Safbrew wb-06; 2, Safbrew wb-34/70; 3, SafAle US-05; 4, Lalvin EC-1118; and 5, SIHA Active Yeast 3.

4.3.4.5 Esters

There were six esters detected in unfermented CSAS, among which ethyl decanoate and ethyl trans-4-decenoate were the two major esters. The alcoholic fermentation process resulted in considerable increases in the ester contents for all the fermented samples (Table 4.8). For example, isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl trans-4-decenoate, and 2-phenethyl acetate, which were initially present in CSAS, increased considerable after fermentation in most of the yeast strains. New esters formed include ethyl dodecanoate, isoamyl decanoate, ethyl undecenoate, and ethyl hydrocinnamate. Ethyl octanoate and ethyl decanoate were most abundant esters present in the fermented samples.

Table 4.8 - Esters in the final fermented products by HS-SPME-GC-MS analysis.

RT	Volatiles	CAS No.	Relative content (%)					
			CSAS	1	2	3	4	5
8.674	Ethyl formate	109 - 94 - 4	-	-	0.19	-	-	-
9.467	Ethyl Acetate	141 - 78 - 6	-	2.63	-	1.21	-	-
13.174	Ethyl propanoate	105 - 37 - 3	-	-	0.26	-	0.17	-
15.11	Ethyl hexanoate	123 - 66 - 0	-	0.12	-	-	-	-
15.567	Ethyl butanoate	105 - 54 - 4	-	0.04	-	-	-	-
19.008	Isoamyl acetate	123 - 92 - 2	0.15	0.42	0.98	0.14	0.49	0.87
21.845	Isoamyl propionate	105 - 68 - 0	-	0.03	-	-	-	-
24.041	Ethyl hexanoate	123 - 66 - 0	0.14	0.73	0.74	0.84	1.29	1.02
25.325	Ethyl heptanoate	106 - 30 - 9	-	0.06	-	0.05	-	-
34.122	Ethyl octanoate	106 - 32 - 1	1.17	6.28	10.65	15.35	15.08	11.45
38.875	Ethyl nonanoate	123 - 29 - 5	-	-	0.07	-	0.05	-

43.331	Ethyl decanoate	110 - 38 - 3	2.09	6.24	10.66	9.36	14.42	7.86
44.168	Octyl phenylacetate	122 - 45 - 2	-	0.24	-	-	-	-
44.24	Isoamyl octanoate	2035 - 99 - 6	-	-	0.24	0.13	0.29	0.25
45.027	Ethyl benzoate	93 - 89 - 0	-	0.2	-	-	-	-
45.585	Ethyl trans-4-decenoate	76649 - 16 - 6	1.35	2.93	5.91	2.59	7.14	5.81
51.092	2-Phenethyl acetate	103 - 45 - 7	0.68	3	2.88	0.64	0.85	1.89
51.399	Ethyl dodecanoate	106 - 33 - 2	-	4.9	0.87	3.08	0.7	0.53
52.103	Isoamyl decanoate	2306 - 91 - 4	-	0.07	0.06	0.08	0.27	0.12
53.474	Ethyl undecenoate	692 - 86 - 4	-	0.11	0.17	0.09	0.16	0.23
53.636	Ethyl hydrocinnamate	2021 - 28 - 5	-	0.69	0.2	0.57	0.07	0.2
58.692	Ethyl tetradecanoate	124 - 06 - 1	-	0.71	-	0.37	-	-
60.066	Ethyl Oleate	111 - 62 - 6	-	0.25	-	-	-	-
65.462	Ethyl hexadecanoate	628 - 97 - 7	-	0.7	-	0.7	0.18	-
65.493	Ethyl decanoate	110 - 38 - 3	-	-	-	-	-	0.13
66.576	Ethyl 9-hexadecenoate	54546 - 22 - 4	-	0.14	-	0.18	-	-

RT, retention time; CSAS, coffee sugar aqueous solution; “-”, not detected; 1, Safbrew wb-06; 2, Safbrew wb-34/70; 3, SafAle US-05; 4, Lalvin EC-1118; and 5, SIHA Active Yeast 3.

4.3.4.6 Furans

Furans detected in unfermented CSAS include 2-furfuryl methyl ether, furfural, acetylfuran, furfuryl acetate, 2-formyl-5-methylfuran, and 2-furanmethanol with 2-furanmethanol and furfural. After fermentation, all the original furans present in coffee were disappeared (2-furfuryl methyl ether and 2-formyl-5-methylfuran) or decreased substantially. Two new furans were detected after fermentation, namely 5-methyl-2-furfurylmercaptan and ethyl 2-furanpropionate (Table 4.9).

Table 4.9 - Furans in the final fermented products by HS-SPME-GC-MS analysis.

RT	Volatiles	CAS No.	Relative content (%)					
			CSAS	1	2	3	4	5
24.656	2-Furfuryl methyl ether	13679 - 46 - 4	0.74	-	-	-	-	-
28.275	5-Methyl-2-furfurylmercaptan	59303 - 05 - 8	-	0.08	0.08	0.14	0.09	0.11
35.232	Furfural	98-01-01	6.51	0.08	-	0.1	-	0.03
38.171	Acetylfuran	1192 - 62 - 7	1.25	0.03	0.03	0.08	-	0.06
39.175	Furfuryl acetate	623 - 17 - 6	3.75	0.06	0.09	0.24	0.21	0.2
41.304	2-Formyl-5-methylfuran	620 - 02 - 0	4.78	-	-	-	-	-
44.635	2-Furanmethanol	98 - 00 - 0	9.36	0.3	0.35	0.47	0.21	0.31
45.382	Ethyl 2-furanpropionate	10031 - 90 - 0	-	-	0.21	0.17	0.06	0.27

RT, retention time; CSAS, coffee sugar aqueous solution; “-”, not detected; 1, Safbrew wb-06; 2, Safbrew wb-34/70; 3, SafAle US-05; 4, Lalvin EC-1118; and 5, SIHA Active Yeast 3.

4.3.4.7 Pyrazines

In total, 13 pyrazines were detected in unfermented CSAS (Table 4.10). Overall, fermentation led to significant decreases in the pyrazines, while several pyrazines were totally depleted after fermentation, such specifically, 2,3-dimethylpyrazine, trimethylpyrazine, 2-propylpyrazine, 2,6-diethylpyrazine, and 2-methyl-3,5-diethylpyrazine.

Table 4.10 - Pyrazines in the final fermented products by HS-SPME-GC-MS analysis.

RT	Volatiles	CAS No.	Relative content (%)

			CSAS	1	2	3	4	5
26.152	2-Methylpyrazine	109 - 08 - 0	5.08	0.11	0.21	0.26	0.16	0.16
28.959	2,5-Dimethylpyrazine	123 - 32 - 0	3.25	0.09	0.15	0.17	0.09	0.11
29.242	2,6-Dimethylpyrazine	108 - 50 - 9	3.24	0.07	0.13	0.17	0.06	0.11
29.632	2-Ethylpyrazine	13925 - 00 - 3	3.54	0.05	0.14	0.13	0.07	0.07
30.299	2,3-Dimethylpyrazine	5910 - 89 - 4	0.67	-	-	-	-	-
32.081	2-Ethyl-6-methylpyrazine	13925 - 03 - 6	3.20	0.09	0.14	0.18	0.13	0.14
32.416	2-Ethyl-5-methylpyrazine	13360 - 64 - 0	2.56	0.07	0.15	0.18	0.06	-
33.02	Trimethylpyrazine	14667 - 55 - 1	0.84	-	-	-	-	-
33.09	2-Ethyl-3-methylpyrazine	15707 - 23 - 0	1.07	0.04	0.04	0.05	-	-
33.878	2-Propylpyrazine	18138 - 03 - 9	0.23	-	-	-	-	-
34.52	2,6-Diethylpyrazine	13067 - 27 - 1	1.80	-	0.03	-	-	-
34.901	3-Ethyl-2,5-dimethylpyrazine	13360 - 65 - 1	2.55	0.04	0.07	0.11	0.05	0.05
37.271	2-Methyl-3,5-diethylpyrazine	18138 - 05 - 1	0.59	-	-	-	-	-

RT, retention time; CSAS, coffee sugar aqueous solution; “-”, not detected; 1, Safbrew wb-06; 2, Safbrew wb-34/70; 3, SafAle US-05; 4, Lalvin EC-1118; and 5, SIHA Active Yeast 3.

4.3.4.8 Organic Acids

Table 4.11 summarizes the volatile organic acids detected in CSAS before and after fermentation. In CSAS, the volatile acids detected were 3-methylbutanoic, 2-methylpropanoic, acetic, propanoic, octanoic, hexanoic, and n-decanoic acids. Among these acids, 2-methylpropanoic and acetic acids decreased considerably after fermentation, while propanoic acid was not detected in the final fermented products.

Table 4.11 - Acids in the final fermented products by HS-SPME-GC-MS analysis.

RT	Volatiles	CAS No.	Relative content (%)

			CSAS	1	2	3	4	5
36.664	Acetic acid	64 - 19 - 7	3.13	0.96	0.76	0.38	0.54	0.83
40.334	Propanoic acid	1979/9/4	0.29	-	-	-	-	-
41.442	2-Methylpropanoic acid	79 - 31 - 2	0.49	0.05	0.07	0.25	0.03	0.1
45.666	3-Methylbutanoic acid	503 - 74 - 2	5.55	-	-	0.31	-	-
45.676	4-Methylpentanoic acid	646 - 07 - 1	-	0.23	-	-	-	-
49.125	Diethylacetic acid	1988/9/5	-	-	-	-	-	0.09
52.472	Hexanoic acid	142 - 62 - 1	0.43	0.62	0.57	1.21	0.53	0.54
56.265	Heptanoic acid	111 - 14 - 8	-	-	-	0.09	-	-
59.881	Octanoic acid	124 - 07 - 2	1.73	2.45	3.53	9.14	3.9	3.32
67.044	n-Decanoic acid	334 - 48 - 5	0.92	1.82	3.61	5.12	3.12	1.45
69.678	9-Decenoic acid	14436 - 32 - 9	-	-	0.77	0.5	0.87	0.35

RT, retention time; CSAS, coffee sugar aqueous solution; “-”, not detected; 1, Safbrew wb-06; 2, Safbrew wb-34/70; 3, SafAle US-05; 4, Lalvin EC-1118; and 5, SIHA Active Yeast 3.

4.3.4.9 Pyrroles

As shown in Table 4.12, five pyrroles were detected in the unfermented CSAS, namely, 1-ethyl-1H-pyrrole-2-carbaldehyde, 1-methyl-2-formylpyrrole, 2-acetyl-1-methylpyrrole, 1-furfurylpyrrole, 2-acetylpyrrole, and pyrrole-2-carboxaldehyde. Overall, all these pyrroles decreased or disappeared after fermentation. One exception being 1-furfurylpyrrole, which was absent initially in CSAS, but was detected in brews prepared from Safbrew wb-34/70, but not in brews from all other yeast strains.

Table 4.12 - Pyrroles in the final fermented products by HS-SPME-GC-MS analysis.

RT	Volatiles	CAS No.	Relative content (%)					
			CSAS	1	2	3	4	5
43.039	1-Ethyl-1H-pyrrole-2-carbaldehyde	2167 - 14 - 8	0.36	-	-	-	-	-
43.6	1-Methyl-2-formylpyrrole	1192 - 58 - 1	0.89	-	-	0.08		-
44.861	2-Acetyl-1-methylpyrrole	932 - 16 - 1	0.42	-	-	-	-	-
51.649	1-Furfurylpyrrole	1438 - 94 - 4		-	0.1	-	-	-
56.994	2-Acetylpyrrole	1072 - 83 - 9	0.91	0.1	-	0.15	-	-
59.072	Pyrrole-2-carboxaldehyde	1003 - 29 - 8	1.59	-	-	-	-	-

RT, retention time; CSAS, coffee sugar aqueous solution; “-”, not detected; 1, Safbrew wb-06; 2, Safbrew wb-34/70; 3, SafAle US-05; 4, Lalvin EC-1118; and 5, SIHA Active Yeast 3.

4.3.4.10 Other Organic Compounds.

Other volatile in the samples are summarized in Table 4.13. Compounds initially present in CSAS, such as ethyl n-propyl ether, pyridine, butyrolactone, guaiacol, and phenol were all disappeared after fermentation. Pyridine is characterized by rotten fish, burned matter flavor and smoky. Guaiacol is described as phenolic, chemical, spicy, smoky, and sweet (Kalschne et al. 2018). Styrene, characterized by balsamic and gasoline odors, was detected as a new compound in all the fermented samples. Styrene is found in low concentration as a naturally-occurring component in foods, such as coffee (Yener et al. 2016), meat (Zhou et al. 2015; Acevedo et al. 2012), malts (Langos et al. 2017), and wheat germ oil (Zou et al. 2018). Styrene is probably derived from cinnamic acid decarboxylation by yeast or a thermal decarboxylation (Schwarz et al. 2011).

Table 4.13 - Others in the final fermented products by HS-SPME-GC-MS analysis.

RT	Volatiles	CAS No.	Relative content (%)					
			CSAS	1	2	3	4	5
16.066	Ethyl n-propyl ether	628 - 32 - 0	0	-	-	-	0.2	-
42.579	Carbitol	111 - 90 - 0	-	0.21	0.05	0.19	-	-
22.041	Pyridine	110 - 86 - 1	0.6	-	-	-	-	-
25.793	Styrene	100 - 42 - 5	-	0.11	0.81	0.26	0.06	0.15
49.219	5-(1-Methylpentylidene)-1,3-cyclopentadiene	61039 - 45 - 0	-	0.09	-	-	-	-
58.707	3,4-Dimethoxystyrene	6380 - 23 - 0	-	-	-	-	0.38	0.55
40.929	2,6,11-Trimethyldodecane	31295 - 56 - 4	-	0.02	0.05	-	-	-
41.222	Hexadecane	544 - 76 - 3	-	-	-	-	-	0.1
45.295	2-Methyltridecane	1560 - 96 - 9	-	0.03	-	-	-	-
54.171	(3,3-Dimethylpentyl)cyclohexane	61142 - 22 - 1	-	-	-	-	0.11	-
44.248	Butyrolactone	96 - 48 - 0	0.5	-	-	-	-	-
52.915	Guaiacol	1990/5/1	0.42	-	-	-	-	-
58.142	Phenol	108 - 95 - 2	0.77	-	-	-	-	-

RT, retention time; CSAS, coffee sugar aqueous solution; “-”, not detected; 1, Safbrew wb-06; 2, Safbrew wb-34/70; 3, SafAle US-05; 4, Lalvin EC-1118; and 5, SIHA Active Yeast 3.

4.4 Conclusion

This study showed that 35.8 mg/L of YAN in 2% instant coffee CSAS is appropriate for the yeast multiplication and alcohol fermentation. No significant changes in caffeine content were observed after the fermentation process in all yeast strains tested. Yeast Safbrew wb-06 and wb-34/70 had excellent capacities to initiate the alcohol fermentation process. Lalvin EC-1118 and

SIHA Active Yeast 3 exhibited strong invertase activity. After 24 h of fermentation, the alcohol contents were 0.58, 0.56, 0.38, 0.24, and 0.20 % (v/v) for brews derived from Safbrew wb-06 and wb-34/70, SIHA Active Yeast 3, SafAle US-05, and Lalvin EC-1118, respectively. Based on the results from this preliminary study, to make low-alcohol coffee fermented drink, yeasts Safbrew wb-06 and wb-34/70 would be ideal candidates but a decrease in inoculum size is required. If a higher level of VDK is preferred in the fermented drink, SafAle US-05 would be a more suitable starter, while other strains tend to produce fermented drinks with lower VDK levels. Each strain of the yeasts tested produced brews with substantially different sensory characteristics. From the informal sensory evaluation by the expert panel of Shandong Taishan Beer Co. Ltd., Safbrew wb-06 was being characterized as having yeast and coffee flavor, Safbrew wb-34/70 pure and typical coffee flavor, SafAle US-05 balanced flavor, Lalvin EC-1118 green and astringent flavor, and finally SIHA Active Yeast 3 neutral and astringent flavor. Safbrew wb-34/70 possessed the lowest alcohol/ester ratio of 10.67 while SafAle US-05 had the highest alcohol/ester ratio of 28.45.

Through HS-SPME-GC-MS analyses, 62 volatile components were detected in CSAS. In the fermented samples, 64, 52, 53, 46, and 49 components were detected in Safbrew wb-06, Safbrew wb-34/70, SafAle US-05, Lalvin EC-1118, and SIHA Active Yeast 3, respectively. The complex flavor of Safbrew wb-06 may be attributed to the large varieties of volatiles present in the brews. The fermentation process resulted in overall decreases in aldehydes, ketones, pyrazines, and pyrroles, especially ketones and pyrroles. Pyrazine

and furan were dominant volatiles in CSAS, while alcohols and esters are the key components in the flavor of fermented products. Safbrew wb-34/70 had good fermentation performance, which retained the typical coffee favor. Therefore, this strain of yeast was selected for further research.

5 Fermentation Process by Selected *Saccharomyces* Yeast Strain

5.1 Introduction

In Canada, the Canadian *Food and Drug Regulations* mandate that beverages with an ethanol (alcohol) content of greater that 1.1% (v/v) to declare the alcohol content on the package label. Also, consuming alcohol mixed with caffeine has some potential health impacts related to altered subjective states such as decreased perceived intoxication, increased stimulation, offset fatigue from drinking, and facilitated drinking and increased the desire to keep drinking (Marczinski et al. 2014; Mcketin et al. 2015). Considering the potential health impact when the two compounds are consumed in combination, this study is focused on the development of a non-alcoholic beverage that would fit into the non-alcoholic beverage product category. Moreover, since consumers tend to have a negative impression of beverages containing both alcohol and caffeine, non-alcohol coffee beverages likely are more receptive by general consumers.

The main objective of this study is to develop a fermented coffee beverage with a final ethanol content of lower than 0.5% (v/v). Based on the preliminary results from coffee-sugar aqueous solution (CSAS) fermentation trials, Safbrew wb-34/70 yeast with typical coffee flavor was selected for further experimentation. Thus, this specific strain of yeast was selected as an inoculum for further experiments. To this end, the inoculation size of the yeast was reduced in order to decrease the fermentation rate and make fermentation easy to monitor in short period. Two techniques were used to characterize the volatile species of the fermented products, namely headspace gas chromatography (HS-GC) and headspace solid-phase microextraction GC-mass (HS-SPME-

GC-MS). The key non-volatile components analyzed were caffeine and organic acid, both characterized by high performance liquid chromatography (HPLC).

5.2 Materials and Methods

5.2.1 CSAS Preparation

Safbrew wb-34/70 (*S. cerevisiae*) was procured from Fermentis, Marcq-en-Baroeul, France. The dosage for CSAS is shown in Table 5.1. CSAS samples with 0.5, 1.0, 1.5, 2.0, and 2.5% w/w instant coffee powder for fermentation were prepared according to Table 5.1. Natural water (2 kg) was boiled in a stainless-steel pot, and then added with the weighed instant coffee and sugar, followed by thorough mixing. A 2/3 portion of sterile deionized water, previously cooled to 4^{o}C, was added into the pot and then the cooled coffee solution was transferred from the pot to the fermentation vessel. The remaining sterile purified water was applied to rinse the residual solution in the pot and then transferred to the fermentation vessel. Finally, the CSAS was cooled down to 18^{o}C to beginning the fermentation. Total soluble solids (TSS), pH, total acid (TA), reducing sugar (RS), total sugar (TS), vicinal diketone (VDK), yeast assimilable nitrogen (EAN), volatile compounds, organic acid, and caffeine of the samples were determined before inoculation.

Easy Assimilable Nitrogen (EAN) was determined according to Analytica EBC (European Brewery Convention) Method 8.10.1 Free Amino Nitrogen in Wort by Spectrophotometry – Manual method. Ninhydrin was used as a color reagent and glycine as a standard. Measurement was carried out at 570 nm in a 10 mm cell using a 2600 UV/VIS spectrophotometer (UNICO Co., Shanghai, China). Results were expressed as mg glycine/L.

Table 5.1 - Formula of coffee aqueous solution for fermentation.

Composition	Concentration of coffee in CSAS (%, w/w)				
	0.5	1.0	1.5	2.0	2.5
	(F1)	**(F2)**	**(F3)**	**(F4)**	**(F5)**
Instant coffee (g)	50	100	150	200	250
White sugar (g)	500	500	500	500	500
Deionized water (g)	4725	4700	4675	4650	4625
Natural spring water (g)	4725	4700	4675	4650	4625
Total (g)	10000	10000	10000	10000	10000

5.2.2 Inoculation, Fermentation, and Inactivation

Five grams of Safbrew wb-34/70 yeast was dissolved in purified water at 27 ± 3 ^{o}C, gently stirred for 20 min and pitched the resultant cream into the 10 kg of 0.5, 1.0, 1.5, 2.0, and 2.5% (w/w) CSAS fermentation vessels, respectively, and mixed thoroughly. The inoculated CSAS samples were allowed to stand for 30 min, bottled in 500 mL glass bottles, and allowed to ferment at 18 ^{o}C. The TSS of the fermenting solutions was monitored every four hours until the level decreased down to 0.5 ^{o}Brix. The bottles were placed into a 70 ^{o}C hot water bath heated on a hotplate to inactive the yeasts, using a thermocouple probe (VWR International, USA) to monitor the temperature. The bottles were maintained at 65^{o}C for 15 min, cooled down to room temperature by running water, and then transferred to a 4^{o}C refrigerator where the samples were stored for one week to obtain the final products, herein known as non-alcoholic carbonated coffee drinks. Further analyses conducted included EAN,

pH, TA, RS, TS, TTS, VDK, alcohol, CO_2, foam stability, volatile, organic acid, caffeine, and sensory evaluation.

5.2.3 Analysis

Refer to Section 4.2.5.

5.3 Results and Discussion

5.3.1 The Change of Total Soluble Solid

As shown in Figure 5.1, F5 had highest reduction in TSS to 0.5 ºBrix, during 24-hour fermentation, followed by F4/F3 (28 h), F2 (36 h), and F1 (65 h). The longer fermentation time was required for the samples with lower coffee concentrations, probably due to lack of nutrient present in the fermenting broth, such as YAN (Table 5.2).

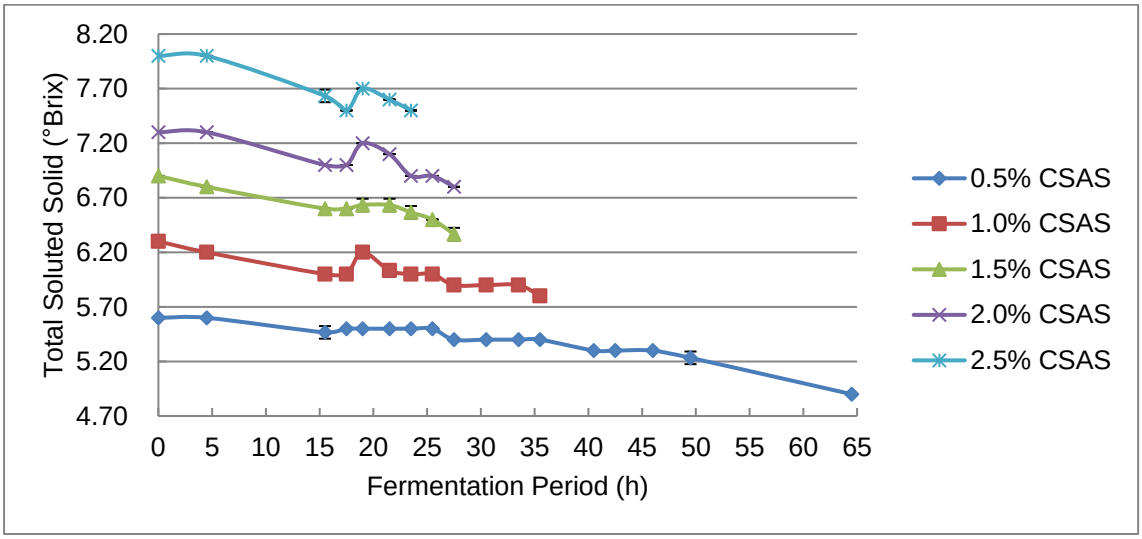


Figure 5.1 - Change of TSS in CSASs during alcoholic fermentation. CSAS: coffee sugar aqueous solution.

5.3.2 Physicochemical Characteristics

As shown in Table 5.2, after fermentation and pasteurization, the TSS in F1, F2, F3, F4, and F5 decreased by 0.7, 0.5, 0.6, 0.5, and 0.5ºBrix, respectively. The final products of these samples had an alcohol content of 0.61, 0.41, 0.36, 0.36, and 0.40% (v/v), CO_2 concentration of 0.52, 0.38, 0.36, 0.42% (w/w), and foam retention time of 438, 410, 378, 416, and 403 s, respectively. The lowest foam retention time in F3 might be due to the low CO_2 concentration in the sample.

The pH decreased from 4.90 to 4.81, while the TA value increased from 0.3 to 1.5 as the coffee content increased from 0.5 to 2.5% (w/w). At the end of fermentation, F1 exhibited the highest TA of 2.7 mL/100mL, probably due to the lowest EAN content (8.8 mg/L). Although an increase in TA was found in the final F4 and F5 fermented products, minimal changes in pH value were observed because of the increase buffer capacity as coffee concentration increased. On the other hand, VDK values increased with coffee concentration in both CSAS and final samples. Because of the unfinished alcoholic fermentation, the diacetyls produced by yeast were retained in the final products.

Table 5.2 - Physical-chemical indices of the fermented CSASs.

Indices		**Sample***				
		F1	F2	F3	F4	F5
TSS (Brix)	Pre-inoculation	5.6±0.0	6.3±0.0	6.9±0.0	7.3±0.0	8.0±0.0

	Final product	4.9±0.0	5.8±0.0	6.3±0.0	6.8±0.0	7.5±0.0
	Decrease	0.7	0.5	0.6	0.5	0.5
pH value	Pre-inoculation	4.90±0.00	4.87±0.00	4.84±0.00	4.82±0.00	4.81±0.00
	Final product	4.13±0.00	4.40±0.00	4.51±0.00	4.49±0.00	4.52±0.00
	Decrease	0.77	0.47	0.33	0.33	0.29
TA (mL/100mL)	Pre-inoculation	0.3±0.0	0.6±0.0	0.9±0.0	1.2±0.0	1.5±0.0
	Final product	2.7±0.0	1.2±0.0	1.5±0.0	1.9±0.0	2.4±0.0
	Increase	2.4	0.6	0.6	0.7	0.9
YAN (mg/L)	Pre-inoculation	8.8±0.5	18.9±1.0	28.6±1.6	37.5±1.3	41.8±0.2
	Final product	0.9±0.0	1.9±0.1	2.9±0.1	3.8±0.1	4.2±0.0
	Decrease	7.9	17.0	25.7	33.7	37.6
VDK (mg/L)	Pre-inoculation	0.11±0.00	0.19±0.00	0.21±0.0	0.27±0.0	0.43±0.0
	Final product	0.68±0.00	0.63±0.00	0.97±0.00	1.90±0.00	2.60±0.00
	Increase	0.57	0.44	0.76	1.63	2.17
Alcohol (%, v/v)	Final product	0.61	0.41	0.36	0.36	0.40
Foam retention (s)	Final product	438	410	378	416	403
CO_2 (%, m/m)	Final product	0.52	0.38	0.36	0.43	0.42

*Sample F1, F2, F3, F4, and F5 contained 0.5, 1.0, 1.5, 2.0, and 2.5% coffee CSAS, respectively. TSS, pH value, TA, EAN, and VDK were detected in triplicate; alcohol, foam retention and CO_2 were detected in duplicate. CSAS, coffee sugar aqueous solution; TSS, total soluble solid; TA, total acidity; EAN, easy assimilable nitrogen; VDK, diacetyl.

5.3.3 Organoleptic Evaluation

Images of the foam produced in the final products are shown in Figure 5.2, while the results of sensory evaluation are summarized in Table 5.3. All the fermented products possessed unique foam characteristics (Tables 4.2 and 4.3, Figure 5.2), with typical coffee aroma and flavor. The foam of the fermented products displayed darker coloration with the increase in coffee content. Among all the fermented products, F4 resulted in the most attractive appearance, flavor, and mouthfeel.

Table 5.3 - Organoleptic evaluation of the fermented CSASs.

Sample	Evaluation
F1	Clean white and fine persistent foam; sweet and roasted aroma; light coffee flavor; minimal mouthfeel.
F2	Fine persistent foam with light brown hue; baking and caramel aroma; typical coffee flavor; good mouthfeel.
F3	Fine persistent foam with brown hue; baking, caramel and coffee bitter aroma; typical coffee flavor; better mouthfeel.
F4	Fine persistent foam with dark brown hue; distinct cream, caramel and coffee aroma; clear coffee flavor; full mouthfeel.
F5	Fine and persistent dark brown foam; distinct coffee and baking aroma; bitter aftertaste of coffee; rich mouthfeel.

Note: CSAS, coffee sugar aqueous solution. Sample F1, F2, F3, F4, and F5 contained 0.5, 1.0, 1.5, 2.0, and 2.5% coffee CSAS, respectively.

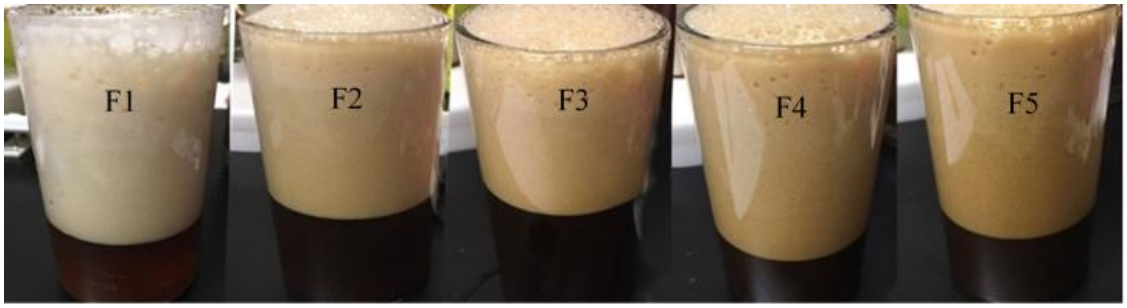


Figure 5.2 - The foam imagines of the final CSASs by yeast Safbrew wb-34/70. Sample F1, F2, F3, F4, and F5 contained 0.5, 1.0, 1.5, 2.0, and 2.5% coffee CSAS, respectively.

5.3.4 Changes in Organic Acid Profiles

As shown in Table 5.4, among the acids detected, citric acid had the highest concentration in the coffee solutions, followed by acetic, lactic, malic, succinic, and pyruvic acids. After fermentation, citric acid in F2, F3, F4, and F5 decreased, but an increased was observed in F1. On the other hand, succinic and acetic acids in F1, F2, F3, and F4 increased but a decrease was observed in F5. On the other hand, pyruvic and lactic acids both increased in all samples. Malic increased in F1 and F2 after fermentation, but decreases were observed for F3, F4, and F5, suggesting that Safbrew wb-34/70 was capable of utilizing the malic acid during the fermentation process when the coffee concentration was low. Citric acid content was also the highest among the organic acids detected in both the fermented CSASs and the final pasteurized products, followed by acetic, lactic, malic, succinic, and pyruvic acids. The relationship between the total organic acid content and coffee concentration of samples was compared and summarized in Figure 5.3. The linear regression equations of total organic acids and coffee concentration in CSAS before fermentation (y_{Pf}), after fermentation (y_{Af}), and after pasteurization (final products; y_{Fp}) are y_{Pf} =

0.0009x - 0.4552, y_{Af} = 0.001x - 0.7358, and y_{Fp} = 0.001x - 0.7449, respectively. The coefficient of determination (R^2) values were all greater than 0.99, indicating that the total organic acids and coffee concentration possessed strong positive linear relationships.

Table 5.4 – Organic acid contents during fermentation and pasteurization.

Component/Threshold	Sample	Pf	Af	Fp	Fp-Pf
Citric acid/49.96**	F1	252.19±0.42^{Ce}	271.20±1.51^{Be}	286.46±0.21^{Ae}	34.27±0.21
	F2	493.23±0.29^{Bd}	498.40±1.89^{Ad}	470.17±0.28^{Cd}	-23.06±0.57
	F3	735.15±1.69^{Ac}	724.22±2.23^{Bc}	696.52±0.86^{Cc}	-38.63±0.83
	F4	941.27±6.05^{Ab}	915.12±1.20^{Bb}	916.39±1.22^{Bb}	-24.88±4.83
	F5	1247.94±0.37^{Aa}	1152.56±0.62^{Ca}	1164.46±0.84^{Ba}	-83.49±0.47
Pyruvic acid/300*	F1	0.79±0.02^{Be}	38.16±0.55^{Ac}	1.12±0.10^{Be}	0.34±0.08
	F2	1.51±0.02^{Cd}	61.52±0.13^{Aa}	3.03±0.01^{Bd}	1.52±0.03
	F3	3.57±0.09^{Cc}	40.50±0.02^{Ab}	6.44±0.03^{Bc}	2.87±0.12
	F4	4.57±0.32^{Cb}	17.70±0.41^{Ad}	9.90±0.04^{Bb}	5.32±0.28
	F5	8.11±0.05^{Ba}	2.91±0.49^{Ce}	11.40±0.02^{A}a	3.30±0.02
Malic acid/49.48**	F1	240.29±0.10^{Be}	258.80±4.05^{Ad}	253.08±1.16^{Ae}	12.79±1.06
	F2	248.05±0.07^{Cd}	291.48±0.98^{Ac}	281.53±1.16^{Bd}	33.48±0.53

	F3	338.27±0.52^{Ac}	258.04±2.85^{Bd}	340.75±1.37^{Ac}	2.48±0.85
	F4	408.55±3.10^{Ab}	344.75±2.02^{Cb}	398.02±1.16^{Bb}	-10.53±1.93
	F5	474.20±1.42^{Aa}	467.80±2.96^{Aa}	466.68±1.42^{Aa}	-7.52±0.00
Succinic acid/106.28**	F1	57.97±0.71^{Cd}	116.20±1.00^{Ae}	113.19±0.10^{Bc}	55.22±0.61
	F2	56.66±1.34^{Cd}	194.75±0.31^{Aa}	102.21±0.81^{Bd}	45.54±0.54
	F3	94.35±0.47^{Cc}	180.66±1.22^{Ab}	103.28±0.79^{Bd}	8.93±1.26
	F4	135.69±0.87^{Bb}	129.08±1.09^{Cd}	140.50±0.17^{Ab}	4.81±0.69
	F5	189.94±2.27^{Aa}	148.83±0.43^{B}c	146.47±1.59^{Ba}	-43.47±0.68
Lactic acid/400*	F1	161.57±1.44^{Ce}	196.57±1.86^{Be}	242.18±0.41^{Ae}	80.61±1.03
	F2	327.56±0.56^{C}d	415.36±0.02^{Ad}	362.67±0.97^{Bd}	35.11±1.52
	F3	490.97±1.25^{Cc}	554.07±1.78^{Ac}	506.68±1.09^{Bc}	15.71±0.16
	F4	645.42±0.40^{Cb}	673.34±0.38^{Ab}	663.20±0.24^{Bb}	17.77±0.65
	F5	822.25±0.63^{Aa}	824.12±0.63^{Aa}	824.38±1.54^{Aa}	2.13±0.92
Acetic acid/175*	F1	173.13±1.58^{Ce}	188.35±1.43^{Ae}	182.13±0.34^{Be}	8.99±1.24
	F2	371.07±0.94^{B}d	274.01±4.98^{Cd}	416.35±0.84^{Ad}	45.28±0.10
	F3	551.34±3.00^{Ac}	360.17±0.47^{Bc}	555.33±2.21^{Ac}	3.99±0.79
	F4	714.84±0.78^{Ab}	715.28±4.04^{Ab}	722.72±4.23^{Ab}	7.89±3.45

F5	923.02±2.42^{Aa}	901.42±1.74^{Ba}	910.61±3.60^{Ba}	-12.41±1.18

Note: Sample F1, F2, F3, F4, and F5 contained 0.5, 1.0, 1.5, 2.0, and 2.5% coffee CSAS, respectively. Pf, Pre-fermentation; Af, after-fermentation; Fp, final product. Same upper-case letters in the same row indicate of each group no significant difference (P > 0.05); same lower-case letters in the same column indicate of each group no significant difference (P > 0.05). *, threhold values were excerpted from Briggs et al. (Briggs et al. 2004). **, threshold values were excerpted from Hufnagel et al. (Hufnagel et al. 2008).

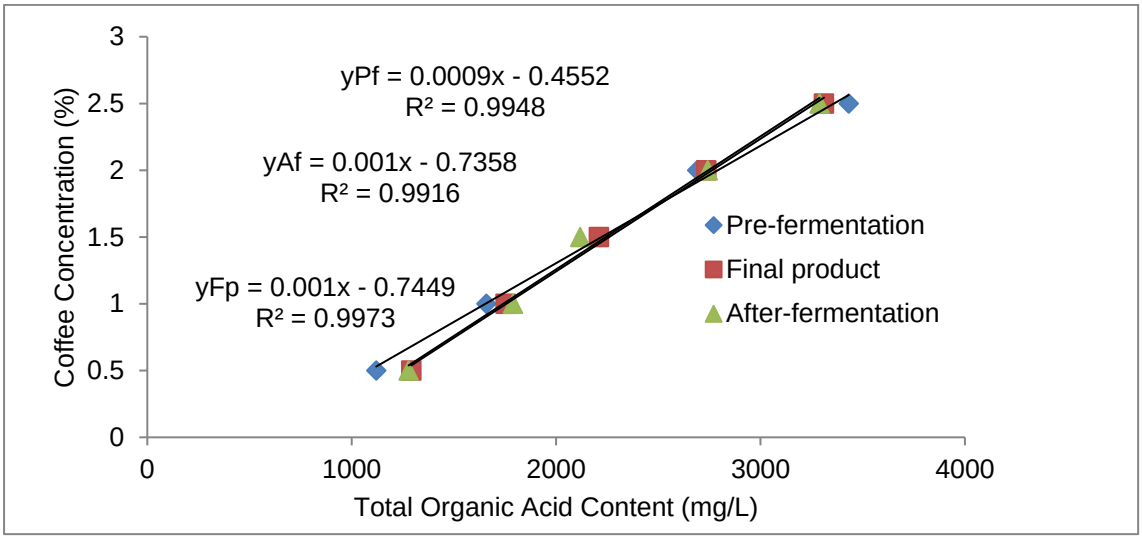


Figure 5.3 - Relationship between total organic acid content and coffee concentration. Note: The total organic acid included citric, pyruvic, malic, succinic, lactic, and acetic acids. y_{Pf}, relationship between total organic acid content and coffee concentration in CSAS; y_{Af}, relationship between total organic acid content and coffee concentration in fermented CSAS; y_{Fp}, relationship between total organic acid content and coffee concentration in final product.

5.3.5 Caffeine in the Fermented CSASs

The changes in caffeine content during fermentation are summarized in Table 5.5 and Figure 5.4. Comparing between pre-, post-fermentation and the final product, it can be seen that the differences in caffeine content in the samples, within the same coffee concentration, were small although significant

($P < 0.05$). As expected, strong positive linear correlations were observed for pre-fermentation ($y_{Pf} = 411.4x - 24.055$; $R^2 = 0.9989$), after-fermentation ($y_{Af} = 389.07x - 4.105$; $R^2 = 0.9995$), and final ($y_{Fp} = 383.86x - 6.8594$; $R^2 = 0.9997$) samples. These results suggested that the caffeine content remained fairly stable throughout the fermentation and pasteurization processes.

Table 5.5 - The caffeine contents of the fermentation liquids of different coffee concentration.

Sample	Pre-fermentation	Post-fermentation	Final product
F1	192.42 ±0.04^{a}	192.28 ±0.01^{b}	190.25 ±0.02^{c}
F2	382.96 ±0.14^{a}	378.49 ±0.11^{b}	374.58 ±0.01^{c}
F3	576.83 ±0.54^{b}	563.80 ±0.23^{c}	580.33 ±0.05^{a}
F4	801.19 ±0.71^{a}	784.42 ±0.44^{b}	757.67 ±0.18^{c}
F5	1011.80 ±0.13^{a}	961.99 ±0.03^{b}	958.37 ±0.04^{c}

Sample F1, F2, F3, F4, and F5 contained 0.5, 1.0, 1.5, 2.0, and 2.5% coffee CSAS, respectively. same lower-case letters in the same row indicate no significant difference ($P > 0.05$).

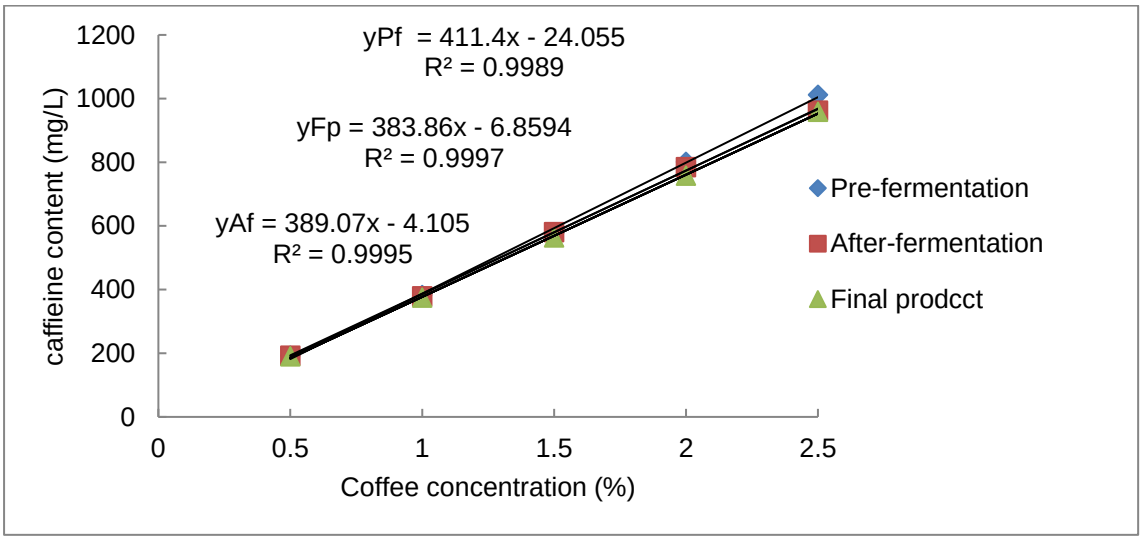


Figure 5.4 - The change of caffeine contents of fermented coffee drinks of different concentrations at the pre-, post -fermentation and post-storage period. y_{Pf}: pre-fermentation; y_{Pf}: post-fermentation; y_{Ps}: post-storage.

5.3.6 Low Boiling Point Volatiles by HS-GC Analysis

Low boiling point volatiles produced during alcoholic fermentation were detected using HS-GC method. In total, 13 different volatiles were detected (Table 5.6). In the CSAS of F1, F2, F3, F4, and F5, only acetaldehyde, ethyl acetate, n-propyl alcohol, isobutanol, and isoamylol were found. Acetaldehyde has pungent, ripe apple odor; isoamylol has fusel, alcohol, sweet, and fruity odor (Sunarharum et al., 2014). These two compounds would contribute flavor characteristics to the final products.

Table 5.6 - Changes of Volatiles in CSASs after fermentation and pasteurization detected by a HS-GC analysis.

Component Threshold (mg/L)*		F1	F2	F3	F4	F5
Acetaldehyde /10	Pf	11.08±0.04^{Bb}	9.94±0.03^{Cb}	9.46±0.1473^{Db}	12.32±0.0017^{Ab}	12.25±0.02^{Ab}
	Fp	17.06±0.62^{Ba}	15.26±0.76^{Ba}	22.13±0.00^{Ba}	26.46±5.10^{Aa}	20.15±2.50^{Aa}
Ethyl formate /150	Pf	0	0	0	0	0
	Fp	0.03±0.00^{A}	0.06±0.02^{A}	0.03±0.00^{A}	0.03±0.01^{A}	0.04±0.00^{A}
Ethyl acetate /33	Pf	0.69±0.02^{Ab}	0.30±0.01^{Bb}	0.16±0.02^{Eb}	0.24±0.00^{Cb}	0.20±0.02^{Db}
	Fp	9.90±0.11^{Aa}	9.45±0.06^{Ba}	2.88±0.00^{Ea}	8.81±0.13^{Ca}	6.96±0.10^{Da}
Isobutyl acetate /1.6	Pf	0	0	0	0	0
	Fp	0.02±0.00^{A}	0.02±0.00^{A}	0.02±0.00^{A}	0.02±0.00^{A}	0.02±0.00^{A}
n-Propyl alcohol /800	Pf	1.11±0.00^{Db}	1.36±0.04^{Cb}	1.41±0.01^{Cb}	1.74±0.00^{Bb}	1.85±0.01^{Ab}
	Fp	8.26±0.02^{Aa}	4.16±0.02^{Da}	5.38±0.00^{Ba}	4.11±0.00^{Ea}	4.69±0.01^{Ca}
Isobutanol /200	Pf	3.59±0.03^{Ab}	2.51±0.03^{Cb}	2.05±0.01^{Eb}	2.70±0.00^{Bb}	2.17±0.01^{Db}
	Fp	20.34±0.02^{Da}	13.42±0.02^{Ea}	27.55±0.00^{Aa}	22.29±0.54^{Ca}	26.21±0.01^{Ba}

Isoamyl acetate /1.6	Pf	0	0	0	0	0
	Fp	0.11 ± 0.00^{C}	0.19 ± 0.00^{A}	0.02 ± 0.00^{D}	0.12 ± 0.00^{C}	0.16 ± 0.00^{B}
Isoamylol /65	Pf	16.12 ± 0.29^{Ab}	9.48 ± 0.11^{Bb}	6.66 ± 0.03^{Db}	7.61 ± 0.01^{Cb}	5.96 ± 0.04^{Eb}
	Fp	92.39 ± 0.51^{Ba}	72.17 ± 0.14^{Ca}	47.23 ± 0.00^{Da}	97.51 ± 1.97^{Aa}	94.71 ± 0.90^{Aa}
Ethyl hexanoate /0.23	Pf	0	0	0	0	0
	Fp	0.06 ± 0.00^{B}	0.03 ± 0.00^{D}	0.03 ± 0.00^{D}	0.08 ± 0.00^{A}	0.05 ± 0.00^{C}
Ethyl octanoate /0.9	Pf	0	0	0	0	0
	Fp	0.07 ± 0.00^{B}	0.07 ± 0.00^{B}	0.06 ± 0.00^{B}	0.15 ± 0.02^{A}	0.10 ± 0.00^{B}
Total esters	Pf	0.70	0.32	0.18	0.25	0.21
	Fp	10.21	9.83	3.04	9.23	7.34
Total alcohols	Pf	20.82	13.35	10.12	12.06	9.98
	Fp	120.98	89.75	80.12	123.91	125.61
Alcohols/ esters	Pf	29.66	41.55	56.57	47.75	47.81
	Fp	11.85	9.13	26.34	13.43	17.12

Sample F1, F2, F3, F4, and F5 contained 0.5, 1.0, 1.5, 2.0, and 2.5% coffee CSAS, respectively. Pf, Pre-fermentation; Fp, final product. Same upper-case letters in the same row indicate of each group no significant difference ($P > 0.05$); same lower-case letters in the same column indicate that each component between pre-fermentation and final product was no significant difference ($P > 0.05$). *Threshold values were obtained from Briggs et al. (2004).

5.3.7 HS-SPME-GC-MS Analysis

5.3.7.1 Aldehydes

The aldehyde profile of the fermented CSAS is summarized in Table 5.7. Among the aldehydes detected, 2-methylpropanal, hexanal, and trans-2-methyl-2-butenal were present in CSAS. On the other hand, acetaldehyde, decanal, and benzaldehyde were detected in both CSAS and the final fermented products. The relative content of acetaldehyde increased while those for decanal and benzaldehyde decreased with increasing coffee concentration.

Octanal and nonanal were new aldehydes produced after the fermentation process. Acetaldehyde was an intermediate product produced before the formation of ethanol, which was reduced to the ethanol by alcohol dehydrogenase. At the end of CSAS fermentation, residual acetaldehyde might have persisted in the samples. Acetaldehyde has pungent and ripe apple odor. It could play a role in the flavour complexity of the fermented CSASs (Peinado et al. 2004).

Table 5.7 - Aldehyde in the fermented CSAS by Safbrew wb-34/70.

RT	Volatiles	CAS No.	Relative content (%)					
			Control	F1	F2	F3	F4	F5
5.546	Acetaldehyde	75 - 07 - 0	0.18	2.39	2.42	2.28	2.71	2.7
8.093	2-Methylpropanal	78 - 84 - 2	2.12	-	-	-	-	-
17.853	Hexanal	66 - 25 - 1	0.18	-	-	-	-	-
18.394	Trans-2-Methyl-2-butenal	497 - 03 - 0	0.27	-	-	-	-	-
27.185	Octanal	124 - 13 - 0	-	0.07	0.08	-	-	-
32.434	Nonanal	124 - 19 - 6	-	0.34	0.34	-	-	-
37.474	Decanal	112 - 31 - 2	0.28	0.1	0.1	0.15	0.18	0.14
39.27	Benzaldehyde	100 - 52 - 7	3.42	2.81	2.83	0.94	0.95	1.07
44.509	Benzeneacetaldehyde	122 - 78 - 1	-	-	-	0.27	-	-

RT: retention time; Control: unfermented 2% CSAS (coffee sugar aqueous solution); F1, F2, F3, F4, and F5 denote 0.5, 1.0 1.5, 2.0 and 2.5% coffee concentrations, respectively.

5.3.7.2 Ketones

Seven ketones were detected in the CSAS, including 2-methyl-3-pentanone, 3-hexanone and dihydropseudoionone, which disappeared in the final fermented products (Table 5.8). Flavor compounds, such as 2,3-butanedione and 2,3-pentanedione present in CSAS persisted in all the fermented products. Acetoin with an odor threshold of 800 µg/L (Peinado et al. 2004), an intermediate metabolite of 2,3-butanedione, was also found in all the fermented products. It possesses fatty, wet, and buttery sensory characteristics (Chang et al. 2015). Complex ketone profiles were observed among samples. For example, 6-methyl-5-hepten-2-one was detected in F2, F3 and F4, but not F1 and F5. On the other hand, 2-cyclopenten-1-one tended to dominate in samples with higher coffee concentrations (F3, F4 and F5). For 3-methylcyclopentane-1,2-dione, it was detected in unfermented CSAS and F3, but not in other samples (Table 5.8).

Table 5.8 - Ketones in the fermented CSAs by Safbrew wb-34/70.

RT	Volatiles	CAS No.	Relative content (%)					
			Control	F1	F2	F3	F4	F5
13.909	2-Methyl-3-pentanone	565 - 69 - 5	0.48	-	-	-	-	-
13.814	2,3-Butanedione	431 - 03 - 8	1.4	0.25	0.25	0.52	0.96	1.17
16.691	3-Hexanone	589 - 38 - 8	0.29	-	-	-	-	-
16.9	2,3-Pentanedione	600 - 14 - 6	1.47	0.12	0.13	0.25	0.54	0.69
27.445	Acetoin	513 - 86 - 0	-	0.13	0.07	0.19	0.21	0.2
27.976	Cyclohexanone	108 - 94 - 1	0.56	-	-	-	-	-

29.649	6-Methyl-5-hepten-2-one	110 - 93 - 0	-	0.28	0.27	-	0.83	-
31.206	2-Cyclopenten-1-one	930 - 30 - 3	-	-	-	0.11	0.12	0.16
51.705	3-Methylcyclopentane-1,2-dione	765 - 70 - 8	0.6	-	-	0.34	-	-
52.2	Dihydropseudoionone	689 - 67 - 8	1.07	-	-	-	-	-

RT: retention time; Control: unfermented 2% CSAS (coffee sugar aqueous solution); F1, F2, F3, F4, and F5 denote 0.5, 1.0 1.5, 2.0 and 2.5% coffee concentrations, respectively.

5.3.7.3 Alcohols

In terms of alcohols (Table 5.9), isobutanol, isoamylol, 3-octanol, and phenylethyl alcohol initially present in CSAS were detected in all of the fermented products, with isobutanol and isoamylol increased considerably. 1-Decanol and 1-octoal were detected in all the fermented samples. Isoamylol is characterized by fuel, alcoholic, sweet, and fruity odor (Baiano et al. 2017). Phenylethyl alcohol, the second most abundant volatile in the fermented CSASs, has a threshold of 125 mg/L (Briggs et al. 2004). These two volatile alcohols are expected to play an important role in the flavor of the fermented CSAS.

Table 5.9 - Alcohols in the fermented CSAS by Safbrew wb-34/70.

RT	**Volatiles**	**CAS No.**	**Relative content (%)**					
			Control	F1	F2	F3	F4	F5
16.14	1-Propanol	71 - 23 - 8	-	-	-	0.39	-	0.6
17.88	Isobutanol	78 - 83 - 1	0.16	1.95	1.97	1.73	2.32	1.97
22.643	Isoamylol	123 - 51 - 3	4.27	46.03	46.42	32.92	37.64	32.42

28.205	2-Heptanol	543 - 49 - 7	0.35	-	-	-	0.06	0.13
28.732	1-Hexanol	111 - 27 - 3	0.22	0.06	0.07	-	-	-
31.914	3-Octanol	589 - 98 - 0	0.56	0.32	0.33	0.39	0.57	0.77
32.927	1-Heptanol	111 - 70 - 6	-	-	-	0.12	-	-
39.781	1-Octanol	111 - 87 - 5	-	0.13	0.13	-	0.15	0.27
48.331	1-Decanol	112 - 30 - 1	-	0.14	0.15	0.2	0.22	0.19
54.733	Phenylethyl alcohol	60-12-8	1.84	4.86	4.91	3.16	2.36	1.85
55.921	1-Dodecanol	112 - 53 - 8	0	-	-	0.38	-	-

RT: retention time; Control: unfermented 2% CSAS (coffee sugar aqueous solution); F1, F2, F3, F4, and F5 denote 0.5, 1.0 1.5, 2.0 and 2.5% coffee concentrations, respectively.

5.3.7.4 Esters

Four esters, i.e., isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate were detected in all fermented samples (Table 5.10). These volatiles are key contributors to the unique odour profiles for the beverage. For example, isoamyl acetate was characterized by banana-like odour; ethyl hexanoate has apple-, banana-, and wine-like odor; ethyl octanoate is characterized by banana, floral, pear, pineapple, and wine odour; ethyl decanoate has grape, oily, and wine-like odor (Baiano et al. 2016). The thresholds for these compounds are 1.6, 0.23, 0.9, and 1.5 mg/L, respectively (Briggs et al. 2004). The ethyl decanoate and ethyl octanoate contributed more flavor to the unfermented CSAS, while ethyl octanoate, ethyl hexanoate, and ethyl decanoate gave the fermented CSASs more intensive flavor. Ethyl trans-4-decenoate and 2-phenethyl acetate in unfermented CSAS were also found in

F1, F2, F3, F4. Newly produced ethyl dodecanoate was detected in all the fermented samples.

Table 5.10 - Esters in the fermented CSAS by Safbrew wb-34/70.

RT	Volatiles	CAS No.	Relative content (%)					
			Control	F1	F2	F3	F4	F5
16.105	Propyl formate	110 - 74 - 7	-	-	-	-	0.73	-
19.161	Isoamyl acetate	123 - 92 - 2	0.15	0.37	0.38	0.45	1.52	1.04
24.134	Ethyl hexanoate	123 - 66 - 0	0.14	0.5	0.51	0.37	1.31	1.33
34.157	Ethyl octanoate	106 - 32 - 1	1.17	2.79	2.82	2.23	2.79	2.54
43.337	Ethyl decanoate	110 - 38 - 3	2.09	2.99	3.02	3.03	3.47	2.8
45.594	Ethyl trans-4-decenoate	76649 - 16 - 6	1.35	1.01	1.02	0.89	0.6	-
51.097	2-Phenethyl acetate	103 - 45 - 7	0.68	0.3	0.3	0.31	0.3	-
51.441	Ethyl dodecanoate	106 - 33 - 2	-	0.5	0.51	0.88	0.97	0.86

RT: retention time; Control: unfermented 2% CSAS (coffee sugar aqueous solution); F1, F2, F3, F4, and F5 denote 0.5, 1.0 1.5, 2.0 and 2.5% coffee concentrations, respectively.

5.3.7.5 Furans

Furans are the most abundant group of volatiles present in coffee which contribute to important sensorial or aroma profiles in the brewed beverages. Specifically, volatile furans present malty and sweet roasted aromas in coffee. The most representative furans in ground coffees are furfuryl alcohol, followed by furfuryl acetate, 2-furfural, 2-methylfuran and 5-methylfurfural (Petisca et al. 2013). Table 5-11 shows that 2-furanmethanol in the CSAS was detected in all the fermented products without 2-furfuryl methyl ether. These observations

suggest that the yeast was capable of removing some furans through extended fermentation. Previous report also declared that baker's yeast (*S. cerevisiae*) could mitigate HMF in instant coffee by fermentation (Akıllıoglu et al. 2014). However, other new furans could be produced, such as 5-methyl-2-furfurylmercaptan and ethyl 2-furanpropionate, when the fermentation period was extended.

Table 5.11 - Furans in the fermented CSAs by Safbrew wb-34/70.

RT	Volatiles	CAS No.	Relative content (%)					
			Control	F1	F2	F3	F4	F5
24.656	2-Furfuryl methyl ether	13679 - 46 - 4	0.74	-	-	-	-	-
36.224	Furfural	1998/1/1	6.51	0.69	0.7	1.51	1.28	1.87
38.19	2-Acetylfuran	1192 - 62 - 7	1.25	-	-	0.13	0.15	0.31
39.205	Furfuryl acetate	623 - 17 - 6	3.75	-	-	-	-	0.37
41.32	5-Methylfurfural	620 - 02 - 0	4.78	0.06	0.06	0.25	0.31	0.53
44.652	2-Furanmethanol	98 - 00 - 0	9.36	0.53	0.54	2.3	2.29	2.96

RT: retention time; Control: unfermented 2% CSAS (coffee sugar aqueous solution); F1, F2, F3, F4, and F5 denote 0.5, 1.0 1.5, 2.0 and 2.5% coffee concentrations, respectively.

5.3.7.6 Pyrazines

Several pyrazines were detected in unfermented CSAS and fermented samples, including 2-methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2-ethyl-6-methylpyrazine, and 3-ethyl-2,5-dimethylpyrazine (Table 5.12). On the other hand, 2-propylpyrazine and 2-methyl-3,5-diethylpyrazine were detected in unfermented CSAS but not the fermented

products. Trimethylpyrazine was detected in unfermented CSAS and F3, F4 and F5. However, 2-methyl-3,5-diethylpyrazine was newly appeared in F3, F4 and F5. Pyrazine is the second most abundant flavor compound follow by furans in the roasted coffee (Hurtado-Benavides et al. 2016). The main pyrazines are 2-methylpyrazine, 2,3- dimethylpyrazine, 2,5-dimethylpyrazine, and trimethylpyrazine (Madihah et al. 2012). As shown in Table 5.12, 2-ethyl-5-methylpyrazine, 2-ethyl-3-methylpyrazine, and 2-ethyl-3,5-dimethylpyrazine, appeared only in samples with coffee contents of less than 1.5%, which may be important in contributing to coffee characteristics to the fermented products.

Table 5.12 – Pyrazines in the fermented CSAS by Safbrew wb-34/70.

RT	Volatiles	CAS No.	Relative content (%)					
			Control	F1	F2	F3	F4	F5
26.228	2-Methylpyrazine	109 – 08 – 0	5.08	0.19	0.19	0.75	0.79	1.16
29.029	2,5-Dimethylpyrazine	123 – 32 – 0	3.25	0.09	0.09	0.55	0.58	0.89
29.299	2,6-Dimethylpyrazine	108 – 50 – 9	3.24	0.13	0.16	0.54	0.51	0.81
29.718	2-Ethylpyrazine	13925 – 00 – 3	3.54	-	-	0.4	-	1.17
30.314	2,3-Dimethylpyrazine	5910 – 89 – 4	0.67	-	-	-	0.11	-
32.161	2-Ethyl-6-methylpyrazine	13925 – 03 – 6	3.2	0.2	0.2	0.51	0.55	0.9
32.486	2-Ethyl-5-methylpyrazine	13360 – 64 – 0	2.56	-	-	0.67	0.64	1.02
33.039	Trimethylpyrazine	14667 – 55 – 1	0.84	-	-	0.1	0.12	0.3
33.15	2-Ethyl-3-methylpyrazine	15707 – 23 – 0	1.07	-	-	0.16	0.18	0.29
33.878	2-Propylpyrazine	18138 – 03 – 9	0.23	-	-	-	-	-
34.535	2,6-Diethylpyrazine	13067 – 27 – 1	0.5	-	-	-	-	0.14
34.932	3-Ethyl-2,5-dimethylpyrazine	13360 – 65 – 1	2.55	0.07	0.06	0.25	0.28	0.66

35.77	2,6-Diethylpyrazine	13067 – 27 – 1	1.3	-	-	-	-	-
35.795	2-Ethyl-3,5-dimethylpyrazine	13925 – 07 – 0	-	-	-	0.16	0.18	0.28
37.271	2-Methyl-3,5-diethylpyrazine	18138 – 05 – 1	0.59	-	-	-	-	-

RT: retention time; Control: unfermented 2% CSAS (coffee sugar aqueous solution); F1, F2, F3, F4, and F5 denote 0.5, 1.0 1.5, 2.0 and 2.5% coffee concentrations, respectively.

5.3.7.7 Acids

Acetic, hexanoic, octanoic, and n-decanoic acids were detected in unfermented CSAS and all fermented samples (Table 5.13). Propanoic acid was detected only in unfermented CSAS and F5, while 2-methylpropanoic acid was found in all the samples except F3. Organic acids are important contributors to product flavors: acetic acid has vinegar odor; hexanoic acid has cheese, fatty, and sour odor; octanoic acid has fatty acid, dry, and dairy flavor; n-decanoic acid has fatty acid, dry, and woody odor; nonanoic acid is cheese and waxy character; 2-methylpropanoic acid has fruity, waxy, sweaty, fkavors; 3-methylbutanoic acid (isovaleric acid) has rancid, cheese and rotten fruit sensory characters (Baiano et al. 2017; Styger et al. 2011).

Table 5.13 – Acids in the fermented CSAs by Safbrew wb-34/70.

RT	**Volatiles**	**CAS No.**	**Relative content (%)**					
			Control	F1	F2	F3	F4	F5
36.657	Acetic acid	64 – 19 – 7	3.13	1.57	1.59	1.69	1.64	1.85
40.33	Propanoic acid	29102	0.29	-	-	-	-	0.19
41.434	2-Methylpropanoic acid	79 – 31 – 2	0.49	0.23	0.23	-	0.26	0.26

45.679	3-Methylbutanoic acid	503 – 74 – 2	5.55	0.81	0.82	0.76	0.66	1.02
50.814	Senecioic acid	541 – 47 – 9	-	-	-	-	-	0.21
52.472	Hexanoic acid	142 – 62 – 1	0.43	0.88	0.89	1.03	0.79	0.99
59.886	Octanoic acid	124 – 07 – 2	1.73	12.33	11.73	13.13	8.68	8.36
63.331	Nonanoic acid	112 – 05 – 0	-	0.21	-	0.48	-	0.39
67.044	n-Decanoic acid	334 – 48 – 5	0.92	12.78	12.91	20.21	16.75	16.26

RT: retention time; Control: unfermented 2% CSAS (coffee sugar aqueous solution); F1, F2, F3, F4, and F5 denote 0.5, 1.0 1.5, 2.0 and 2.5% coffee concentrations, respectively.

5.3.7.8 Pyrrole

As showed in Table 5.14, no pyrrole was found in F1 and F2 samples, which might be due to the lower coffee concentrations involved in the fermentation broths. On the other hand, 1-methyl-2-formylpyrrole was found in unfermented CSAS, F4 and F5, while 2-acetylpyrrole was detected in unfermented CSAS, F3 and F5. Other pyrroles, such as 2-formylpyrrole was detected in unfermented CSAS, F3, F4 and F5, while 1-furfurylpyrrole could only be detected in F5.

Table 5.14 - Pyrrole in the fermented CSAs by Safbrew wb-34/70.

RT	**Volatiles**	**CAS No.**	**Relative content (%)**					
			Control	F1	F2	F3	F4	F5
43.039	1-Ethyl-1H-pyrrole-2-carbaldehyde	2167 - 14 - 8	0.36	-	-	-	-	-
43.63	1-Methyl-2-formylpyrrole	1192 - 58 - 1	0.89	-	-	-	0.21	0.46
44.861	2-Acetyl-1-methylpyrrole	932 - 16 - 1	0.42	-	-	-	-	-
51.652	1-Furfurylpyrrole	1438 - 94 - 4	-	-	-	-	-	0.44
57.02	2-Acetylpyrrole	1072 - 83 - 9	0.91	-	-	0.33	-	0.46

59.104	2-Formylpyrrole	1003 - 29 - 8	1.59	-	-	0.54	0.43	0.78

RT: retention time; Control: unfermented 2% CSAS (coffee sugar aqueous solution); F1, F2, F3, F4, and F5 denote 0.5, 1.0 1.5, 2.0 and 2.5% coffee concentrations, respectively.

5.3.7.9 Terpene and others

Two terpenes, namely eucalyptol and citronellol, were detected in unfermented CSAS. Eucalyptol was also detected in F4 and F5, while citronellol was detected in F1, F2, and F5 (Table 5.15). Curcumene and trans-geranylacetone were newly produced in the fermented samples, with the former being detected in F1 to F4, while the latter in F1 to F3. Eucalyptol possesses camphor, minty, and sweet flavor (Chang et al. 2015). Citronellol has a rose odor and trans-Nerolidol characters by rose, apple, green, citrus, and woody (Peinado et al. 2004). Styrene and 2,6,11-trimethyldodecane were not detected in all fermented samples (Table 5.15). Carbitol was found in all the fermented products. Butyrolactone, characterized by sweet, cake, caramel, and fruity odour (Peinado et al. 2004), existed only in unfermented CSAS and F5.

Table 5.15 - Terpene and other components in the fermented CSAS by Safbrew wb-34/70.

Group	RT	Volatiles	CAS No.	Relative content (%)					
				Control	F1	F2	F3	F4	F5
Terpenes	23.25	Eucalyptol*	470 - 82 - 6	0.19	-	-	-	0.77	1.21
	48.52	Citronellol*	106-22-9	0.5	0.11	0.11	-	-	0.22
	49.286	Curcumene	644 - 30 - 4	0	0.49	0.49	0.27	0.16	-

	52.203	trans-Geranylacetone	3796 - 70 - 1	0	0.1	0.1	0.65	-	-
Phenols	52.915	Guaiacol	1990/5/1	0.42	-	-	-	-	-
	58.142	Phenol	108 - 95 - 2	0.77	-	-	-	-	-
Pyridines	22.041	Pyridine	110 - 86 - 1	0.6	-	-	-	-	-
ethers	42.865	Carbitol*	111 - 90 - 0	0	0.09	0.1	0.13	0.17	0.14
	44.248	Butyrolactone	96 - 48 - 0	0.5	-	-	-	-	0.2

RT: retention time; Control: unfermented 2% CSAS (coffee sugar aqueous solution); F1, F2, F3, F4, and F5 denote 0.5, 1.0 1.5, 2.0 and 2.5% coffee concentrations, respectively.

There were 42, 41, 49, 48, and 53 volatiles detected in the final fermented F1, F2, F3, F4 and F5, respectively, while 62 volatiles were detected in 2% unfermented CSAS. Yeast fermentation produces aldehydes, ketones, esters, and acids, especially alcohols as main by-products, and fermented coffee also contained aldehydes, ketones, alcohols, esters, and acids. In general, after fermentation, aldehydes and ketones contents were decreased, while alcohol increased.

Based on Figure 5.5, pyrazine, furan, pyrrole, and terpene were the main volatiles of the roasted coffee. Pyrazine and furan played key roles in the coffee flavor, which were 28.26% (pyrazine) and 26.39% (furan) in 2% fermented CSAS, other important volatile substances included acid (12.54%), alcohol (7.4%), aldehydes (6.45%), ketone (5.87%), ester (5.58%), pyrrole (4.17%), and small amount of pyridine and phenol. In the fermented samples, alcohol was the most abundant volatiles, followed by acid, ester, aldehyde, and furan. Pyrazine and furan were decreased significantly ($p<0.05$) in the fermented sample.

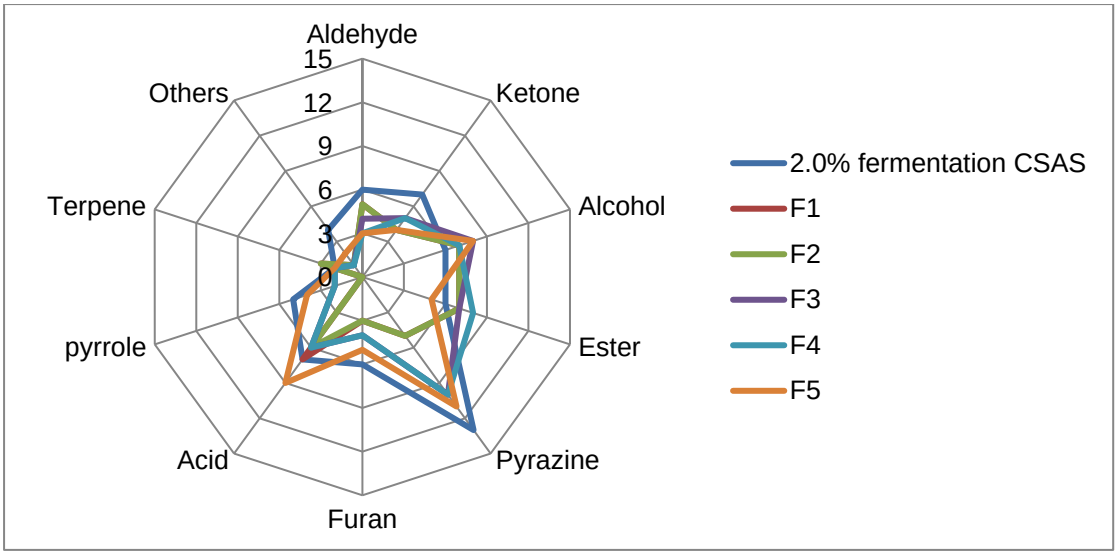


Figure 5.5 - Volatiles detected by HS-SPME-GC-MS analysis in the second fermentation. CSAS, coffee sugar aqueous solution. The 0.5, 1.0 1.5, 2.0 and 2.5% final fermented CSAS expressed as F1, F2, F3, F4, and F5.

5.4 Conclusion

This study showed that the nutrients in CSAS samples with 1.5 to 2.5 % instant coffee (F3, F4 and F5) can support the growth and fermentation of Safbrew wb-34/70 yeast. Considering the fermentation rate, acceptable alcohol content (<0.5%, v/v), as well as the physicochemical and organoleptic properties, F4 is the most desirable formulation for the preparation of the fermented coffee beverage. Among the organic acids detected in CSAS, citric acid was found to be of the highest level, followed by acetic, lactic, malic, succinic, and pyruvic acids. Minimal changes in the individual organic acid contents were observed after fermentation and pasteurization, due to weak physiological metabolic activity of the yeast.

HS-GC analysis revealed that low boiling point volatiles, including acetaldehyde, ethyl acetate, n-propyl alcohol, isobutanol, and isoamylol were

detected in all CSAS samples. In the final fermented products, all volatile levels were increased, including acetaldehyde, ethyl formate, ethyl acetate, isobutyl acetate, n-propanol, isobutanol, isoamyl acetate, isoamyl alcohol, ethyl hexanoate, and ethyl octanoate. Pyrazine, ketone, alcohol, acid, furan, and ester were the major volatiles detected in CSAS. In the fermented samples, the main volatiles detected were pyrazine, alcohol, acid, ester ketone, aldehyde, and furan.

6 Thesis Conclusion and Future Work

6.1 Thesis Conclusion

In this thesis, Chapters 1 and 2 reviewed the background information of flavor substances of coffee and the fermentation applied in coffee industry. Pyrazines and furans, which are the two major products from the Maillard reaction in roasted coffees, were reviewed. Also, the use of *Saccharomyces* yeast for fermentation was reviewed in these two chapters. To date, information related to the fermentation application on the coffee beverage is limited, implying that there are opportunities for the development of innovative fermented coffee beverages. In Chapter 3, the research hypothesis and objectives are presented to develop a fermented coffee beverage, along with the product parameters needed to be investigated.

Chapter 4 presented the results of a preliminary study to look at the feasibility of fermenting instant coffee with added sugar. Five yeasts were applied into fermentation process and evaluated based on the performance during the fermentation process. The 2% CSAS had 35.8 mg/L of yeast assemble nitrogen which was able to provide proper conditions for yeast multiplication and alcohol fermentation. Yeasts Safbrew wb-06 and wb-34/70 was selected for further research based on the test results due to their excellent capacity to initiate alcohol fermentation in the first 24 h; while the other three yeasts began to show the strong activities after 24 h. In order to make low alcohol drinks, wb-06 and wb-34/70 could be applied with reducing inoculum size. Moreover, HS-SPME-GC-MS analysis detected 62 volatile components in CSAS, and 64, 52, 53, 46, and 49 components were detected in wb-06, wb-

34/70, SafAle US-05, Lalvin EC-1118, and SIHA Active Yeast 3, respectively. Apparently, wb-06 had the most varieties of volatiles that led to the most complex flavor in the final product. However, wb-34/70 had the lowest alcohol/ester ratio of 10.67 that contributed to the better flavor with low alcohol content. Also, wb-34/70 retained the original flavor of coffee while wb-06 had more yeast and alcohol taste profile. Hence, Safbrew wb-34/70 was chose to applied on the next step of research.

In Chapter 5, results indicated that the Safbrew wb-34/70 yeast were able to grow and ferment with the sufficient sugar from the CSAS samples with 1.5 to 2.5 % w/w instant coffee concentrations. CSAS with a concentration of 2% was the best formulation for producing fermented coffee drinks. In order to control alcohol content (< 0.5%, v/v), the fermentation period required a short time to limit yeast's activities, resulting in sugar residual in the product that contributed to sweet taste. Based on the organoleptic evaluation, 2% CSAS had provided the desirable sweetness. Furthermore, in physiochemical aspects, since the metabolic activity of yeast was restricted by the short fermentation period, the organic acids contents only had the slight variations after fermentation and pasteurization. Thus, the acidity of the product had minimal impact on the organoleptic properties of the product. From HS-GC analysis, a number of volatiles in the CSAS were detected. In CSAS, volatiles detected were acetaldehyde, ethyl acetate, n-propyl alcohol, isobutanol, and isoamylol. After fermentation, ethyl formate, isobutyl acetate, isoamyl acetate, ethyl hexanoate, and ethyl octanoate were detected in the final products.

In conclusion, this study showed that it is feasible to manufacture fermented coffee beverage with low alcohol content from CSAS with desirable sensory properties. Two fermentation experiments based on different yeast and concentrations indicated that there was no significant change in caffeine content. The sensory characteristics and alcohol content could be controlled and adjusted by choosing appropriate yeast species, decreasing inoculum size, and shortening the fermentation period.

6.2 Future Work

This study reported the feasibility of making fermented coffee beverage by using instant coffee. With addition of sucrose, there were several varieties of coffee that could be applied on the further investigation:

- Applying decaffeinated coffee beans or coffee powder to reduce the caffeine content in the beverage.
- Applying different varieties of raw coffee beans such as Arabica or Robusta with various levels of roasting and grinding size (e.g. medium and dark, coarse and fine) on the fermentation process to investigated the differences.

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