School of Science and Health

Optimisation of processing conditions and quality evaluation of honey mead

by

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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Abstract

Honey based beverages are the oldest known fermented drinks. Fermentation of these beverages is a time consuming process with the average fermentation time for honey mead is 264 hours (11 days). The aim of this study was to development a method to reduce the fermentation time of honey mead. In this research, the effects of type of inoculum, inoculum level and the addition nutritional supplements were investigated.

Honey was dissolved in sterile water and pasteurised at 65°C for 10 minutes. The samples were inoculated with 1 and 2g yeast of strains EC1118 and 71B. The effect of no nutrition or with DAP (diammonium phosphate) or lupin flour as nutritional (nitrogen) supplement was investigated. Analysis of the amount of ethanol present in the honey mead was performed by using a GC-FID according to the methods used by (Debebe, Redi-Abshiro, & Chandravanshi, 2017). The highest concentration of ethanol 12.79 % (w/w) was obtained after fermenting with 71B yeast strain for 96 hr at 24°C with DAP supplementation. The lowest concentration of ethanol 3.89 % was obtained after fermenting the sample with 71B yeast strain for 24 hr at 24°C without any nutritional supplement. Compared to no nutritional supplement, addition of DAP significantly increased the amount of ethanol, whereas addition of lupin flour decreased the amount of ethanol produced. The overall amount of ethanol produced was not dependent on the quantity of inoculation. However the rate of ethanol production in the first 48 h was normally faster with 2g inoculation for both yeast strains. For the fermentation performed with no nutritional supplement or DAP yielded 8-10% ethanol within 2-3 days. Analysis of the aroma compounds was performed using a DVB/CAR/PDMS SPME fibre and measured by GC-MS. Acetic acid was the predominant aroma compound found in the honey mead samples other than ethanol.

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1. Introduction

Honey is a natural sweet and biologically active substance. The chemical composition of the honey depends upon the floral origin, geographic location and climatic conditions of the harvest. The development of the honey based products is an innovative idea for the farmers as it increases the employment and improves economic value. Honey mead is a fermented alcoholic beverage with ethanol concentration 8-12% (w/w). Although the existence of honey mead is known since ages, the production and development of this beverage is difficult and not preferred by the industries. The problems such as lengthy fermentation periods, depletion of the nutrient's, increased acidity and off flavour of the final product are well known conditions and are often troublesome. The quality of the honey mead depends upon the proportions of honey and water. The water and honey in proportions of 3:1 and 2:1 yielded high ethanol content. The temperature at which fermentation was performed also plays vitals as increase or decrease in the temperature can slow down the rate of fermentation and give off flavours to the final honey mead. Saccharomyces yeast strains are preferred microbes by the wineries. Various strains of saccharomyces are commercially available, but the rate of fermentation and amount of ethanol produced are strain specific and depends upon the added nutritional supplement. The mass of the yeast inoculations is a vital factor, as the ethanol production is dependent on the mass of inoculation. The aroma compounds present in the honey mead are alcohols, esters, fatty acids, carbonyl compounds and volatile phenols. The flavour of the honey mead depends on the aroma compounds present in the mead. Commercial mead industries preferred to develop a mead with reduced fermentation time and improve the amount of ethanol with desirable flavour and aroma. This study was designed to develop honey mead product by fermenting the honey and water inoculated with yeast strains EC1118 and 71B without and with nutritional supplements.

Objectives

- To reduce the fermentation time of the honey mead development
- To investigate the amount of ethanol produced with yeast strains used
- To analyse the amount of ethanol produced without and with nutritional supplement

2. Literature Review

2.1. Honey

Honey is natural sweet product produced by the honeybee *Apis mellifera.* It is produced from the secretions of the plants or from the secretions of the living parts of the plants. Honey can also be produced from the excretions of the plant sucking insects present on the plants. Honeybees then collect these secretions from the plant sources, transform it by combining various chemical secretions of their own, deposit it, dehydrate it and the store in the honeycombs to ripen and mature.

According to Agri futures, Australia (2017), Australia produce 25000 to 30000 tonnes of honey annually. Commercial honey harvest is carried along the south east coast of Queensland to South Australia including Australian Capital Territory, Tasmania and along the temperate regions of Western Australia and the Northern Territory. Approximately 70-75% of honey is produced from the nectars of native plants. Honey industry contributes to about AUD 99 million to the Australian economy. An additional of AUD 4-6 billion is generated through the honeybee pollination.

The colour, composition, flavour and aroma of the honey depends on the type of flowers, geographic regions, climatic conditions, species of honey and season of the harvest. The production of honey is influenced by the season of crop and on the region where the crop is harvested (Azeredo, Azeredo, De Souza, & Dutra, 2003; Farrar, 1937). According to the origin, honey is classified as blossom honey which is obtained from the flowers; honeydew honey is obtained from the secretions of the honeybee insects, monofloral honey obtained from the one variety of plant and multifloral honey obtained from multi variety of plants.

Honey consists of sugars (Anklam, 1998) such as fructose, glucose, sucrose and maltose (Mesallam & El-Shaarawy, 1987). Honey has high sugar content but limited nutrient's required for the fermentation to occur (Kime, McLellan, & Lee, 1991). The environmental conditions present in the honey are not suitable for the yeast growth because of the limited availability of the nutrition. The acid content of the honey is low, hence growth of the yeast can be stimulated by adding inorganic salts.

2.1.1 Composition of the honey

Honey is a food product, consists of water, sugars (Table 1) and 200 other substances (Escuredo, Míguez, Fernández-González, & Seijo, 2013). It consists of proteins, vitamins, organic acids and minerals (Finola, Lasagno, & Marioli, 2007). Pyridoxine, thiamine, niacin, riboflavin, pantothenic acid and ascorbic acid are the main vitamins found in the honey (Ciulu et al., 2011). Honey losses its vitamins during the production processes such as temperature treatment, light and storage conditions (León-Ruiz, Vera, González-Porto, & San Andrés, 2013). The chemical reactions such as fermentation, oxidation and thermal processing alter the chemical composition of the honey (Moreira, De Maria, Pietroluongo, & Trugo, 2010). Due to presence of flavonoids and phenolic acids in the honey, it acts as natural antioxidant (Alqarni, Owayss, Mahmoud, & Hannan, 2014; Bertoncelj, Doberšek, Jamnik, & Golob, 2007).

Honey is a rich source of carbohydrates and its dry matter corresponds to approximately 79.7g/100g (Olaitan, Adeleke, & Iyabo, 2007). It has got wide glycaemic index between 32 to 85 (Bogdanov et al., 2008).The major carbohydrate sugars present are fructose (38.2g/100g), glucose (31.3g/100g), sucrose (0.7g/100g) (Bogdanov, 2011). According to the Codex Alimentarius Commission (Codex Alimentatius Commission, 2001), the total glucose and

fructose content should be around 60% (w/w) in the raw honey. The concentration of fructose and glucose depends on the source of origin of the nectar (Anklam, 1998).

Table1: Average composition of the honey from different floral sources.

Content	Blossom honey avg g/100gm	Honeydew honey avg g/100gm
Water content	17.2	16.3
Fructose	38.2	31.8
Glucose	31.3	26.1
Sucrose	0.7	0.5
Other disaccharides	5.0	4.0
Melezitose	<0.1	4.0
Erlose	0.8	1.0
Other oligosaccharides	3.6	13.1
Total sugar	79.7	80.5
Minerals	0.2	0.9
Amino acids, proteins	0.3	0.6
Acids	0.5	1.1
pH	3.9	5.2

Source: (Bogdanov et al., 2008)

The average water content of the honey is 17.2g/100g (Bogdanov, 2011; Ramalhosa, Gomes, Pereira, Dias, & Estevinho, 2011). It depends on the climatic conditions, maturity of the honey hive as well as on the collection and storage conditions (Finola et al., 2007). The water content of the honey influences the physical properties such as viscosity and surface tension (Olaitan et al., 2007). The moisture content helps in predicting the shell life and prevents honey from auto fermentation by the existing microbes (Ramalhosa et al., 2011).

The mineral composition of the honey 0.2g/100g. The principal mineral present in the honey is potassium (about two-thirds) and depends on the geographic location, climatic conditions and biological species involved in the production (Alvarez-Suarez, Tulipani, Romandini, Bertoli, & Battino, 2010). The honey produced from the light blossom have lower mineral content compared to the honey produced from the honeydew and multifloral honey (Bogdanov, Haldimann, Luginbühl, & Gallmann, 2007).

The organic acids present in the honey are gluconic acid, pyruvic acid, malic acid, succinic acid and fumaric acid (Bogdanov, 2011). The acid content of the honey is 0.5g/100g of the dry matter (Bogdanov et al., 2008). The increased acidity is due to the presence of the osmophilic yeast and related high osmotic pressures generated due to high sugar content. The natural honey is acidic in nature with an average pH value of 3.9 (Iurlina & Fritz, 2005).

The amino acids and protein content of the honey accounts for 0.3g/100g (Alvarez-Suarez et al., 2010; Iurlina & Fritz, 2005). Proline is the main amino acid present in the honey. Enzymes such as invertase, diastase, glucose oxidase and catalase are also present in the honey (Anklam, 1998). Invertase and diastase are important in assessing the quality of the honey. Amino acids, proteins and other nucleic acids are the nitrogenous sources present in the honey. Th nitrogen contribution of the honey is derived from alkaloids, chlorophyll derivatives, amino acids and

amines (Al-Mamary, Al-Meeri, & Al-Habori, 2002). The nitrogen content of the honey is 0.04 g/100g (Ramalhosa et al., 2011).

The volatile compounds present in the honey are responsible for the flavour of the honey (Finola et al., 2007). The major volatile compounds present in the honey belongs to 7 main groups namely ketones, acids, aldehydes, alcohols, esters, hydrocarbons and cyclic compounds. The main phenolic compounds present in the honey are flavonoids and phenolic acids. The flavonoids concentration is 0.46g/100g and phenolic acids concentration is 0.10g/100g. Dark coloured honey have high phenolic acids content compared to the light coloured honey (Bogdanov, 2011). Monofloral honey have higher aroma compounds compared to the multifloral honey (Kaškonienė & Venskutonis, 2010).

The colour of the honey is related with the phenolic compounds, flavonoids and mineral contents (Alvarez-Suarez et al., 2010; Bertoncelj et al., 2007). Honey with high mineral content are darker and taste stronger compared to the lighter honey (González-Miret, Terrab, Hernanz, Fernández-Recamales, & Heredia, 2005). The colour of the honey depends on the processing conditions and period of storage (age) (Olaitan et al., 2007). The colour ranges from white - water to dark amber (Bertoncelj et al., 2007). The degree of colour of the honey depends upon the Maillard reactions and also on the chemical interactions among the polyphenolic compounds (Bertoncelj et al., 2007). Maillard reaction causes changes in the aromatic profiles and flavours while reducing the sugars present in the honey in the presence of heat.

2.1.2. Common microbes present in the honey

The microbes present in the honey originate from the harvested environment. These microbes present usually reside in the digestive tract of the honey bees (Snowdon & Cliver, 1996). The microbes act as initial source of the honey contamination. It has been estimated that 27% of microbes present in the honey belongs to gram positive, 70% belong to gram negative and 2%

are yeast. The commonly found gram positive microbes are *Bacillus*, *Bacteridium*, *Streptococcus* and *Clostridium* species and common gram negative bacteria are *Achromobacter, Citrobacter, Enterobacter, Erwinia, Esheriachia coli, Flavobacterium, Klebsiella proteus* and *Pseudomonas* (Olaitan et al., 2007). The chemical compounds present in the honey influence the growth rate of the microbes. Factors such as pH, water content and amount sugars present in the honey play a vital role on the growth of the microbes (Al-Waili, Salom, Al-Ghamdi, & Ansari, 2012). The yeast population present in the honey is below 100 colony forming units per gram (Finola et al., 2007). Osmophilic yeast tends to grow in the acidic medium causing fermentation to occur at moderate moisture and temperature conditions (Furuta & Okimoto, 1978).

2.1.3. Health benefits of Honey

The health benefits of the honey includes anti-inflammatory, antitumor, immunomodulatory and probiotic effect (Veroljub, Ivan, & Visnja, 2012). Honey also has got the potential antimicrobial and antioxidant properties (Gomes, Dias, Moreira, Rodrigues, & Estevinho, 2010; Nagai, Inoue, Kanamori, Suzuki, & Nagashima, 2006). Hydrogen peroxide, methylglyoxal (MGO), defensin-1 present in the honey, have the antibacterial effects (Kwakman et al., 2010). Hydrogen peroxide is produced enzymatically in the honey secretion. It is produced as a sterilising agent during the ripening of the honey (Molan, 1997). Honey is used in the treatment of diabetic ulcers under close observation of blood glucose levels (Cadogan, 2008). Honey can be used as cost effective treatment methods in primary and secondary surgical care (Belcher, 2012). Treating the wound areas with honey increased the rate of granulation, decreased putrid smell and increased speed of recovery (Knipping, Grünewald, & Hirt, 2012). The antioxidant potential of the dark honey is greater compared to the light honey (Estevinho, Pereira, Moreira, Dias, & Pereira, 2008).

2.2. Honey mead

Honey mead is the oldest fermented drink. Its evidence was dated back to the archaeological period of 7000 B.C. The known evidence of mead preparation was found in Rig Veda during 1700-1100 B.C. (Gupta & Sharma, 2009). In china the mead was prepared in the pottery jars during the Neolithic period (McGovern et al., 2004). The Shang dynasty were the first rulers to use the fermented drinks in the ancient china (McGovern et al., 2004). Honey Mead was considered as "THE DRINK OF THE GODS "during the roman empire (Dane, 2000). The economic contribution of honey mead was present in many countries worldwide. Depending upon the raw materials used and fermentation conditions, the alcoholic content of the honey mead varies between 7 to 22%.

The tradition of consuming alcoholic beverages is practiced all over the world during different occasions such as wedding, naming ceremonies (Bahiru, Mehari, & Ashenafi, 2001), burials and even at dispute settlements (Steinkraus, Cullen, Pederson, Nellis, & Gavvitt, 1983). The indigenous alcoholic fermented drinks are prepared in variety of ways and differ from country to country. These fermented drinks were used as medicines by the ancient tribes of the African continent by adding the plant derivates (Okafor, 1977). The traditional alcoholic beverages served as a source of calories and even functioned as good intake of vitamin B complex (Steinkraus et al., 1983). The products derived from honey are cloudy, coloured with residues of the substrates and fermenting microbes such as bacteria, yeast etc (Bahiru et al., 2001). In the northern European countries such as Denmark and Sweden, consumed mead in larger quantities (Digby, 1910).

Alcoholic beverages are named differently according to the source of the origin. Beer is made from fermentation of cereals and fermented drinks from fruits are called as wines (Bahiru et al., 2001). Sack Mead is made by adding more portions of honey to water, Metheglin is made by

adding spices and herbs to honey, Melomel is made with fruit juice and Hyppocras is a spiced pyment (Acton, Aston, & Duncan, 1965).

Mead was prepared by mixing water, honey and fermentation by micro-organisms. The quality of mead obtained depends on the proportions of the honey, water and added supplements. The honey mead was produced by mixing honey and water respectively in different ratios (1:0.5, 1:1, 1:2, 1:3) (Iglesias et al., 2014). Other researchers even obtained mead by mixing honey and water in 1:1, 1:2, 1:3 proportions. To maintain the pH of the honey must, citric acid or tartaric acids were added as they act as buffers (Gogol & Tuszynski, 1996). Honey mead fermentation takes days to several months to complete the fermentation (Navrátil, Šturdík, & Gemeiner, 2001).

The acidity of the honey-must increases rapidly in the initial stages of fermentation. Acetic acid and succinic acids are the main organic acids formed during the early stages of fermentation (Sroka & Tuszyński, 2007). Formation of succinic acid during the fermentation is one of the main reason for the slow-down of fermentation which is dependent on the yeast strain and availability of nitrogen (Fleet, 1993).

2.3. Factors affecting the fermentation of Honey

2.3.1. Saccharomyces cerevisiae

Saccharomyces cerevisiae is the preferred yeast for the production of wine, beer, champagne, mead and other alcoholic beverages (Ramalhosa et al., 2011). This yeast metabolises glucose and fructose and produce ethanol and carbon dioxide. These microbes have undergone adoptive evolution over the decades to adopt to the new stressful environments (Ibstedt et al., 2014).

Yeast used in the brewing industries undergoes stress forces (Gibson, Lawrence, Leclaire, Powell, & Smart, 2007). These stress forces are namely general stress force response and heat shock stress force response. General stress forces are encountered by the *Saccharomyces* , when fermenting medium temperature is high above 35°c, at increased ethanol concentration, at increased osmolarity, at reactive oxidative forces and during lack of sufficient nutrition (Laluce, Tognolli, de Oliveira, Souza, & Morais, 2009; Martinez-Pastor, Marchler, Schuller, Marchler-Bauer, & Ruis; Ruis & Schüller, 1995; P. Verbelen et al., 2009). During fermentation, when the carbon and nutrition related factors are increased or decreased, changes in the ethanol concentration causes the yeast cells to undergo stress (Casey, Magnus, & Ingledew, 1984). The behaviour of the yeast cells is affected when the ethanol concentration ranges between 15 (v/v) to 20% (v/v) , and further growth of yeast cells is stopped (Pereira, Dias, Andrade, Ramalhosa, & Estevinho, 2009). The yeast cells able to adapt to adverse stress related forces (Martinez-Pastor et al.).

During the early stages of fermentation, oxygen is necessary for the generating the biomass of the saccharomyces and to maintain the essential environmental conditions for the fermentation to proceed (Smart, 2017). Oxygen is also required for the synthesis of lipids, sterols, unsaturated fatty acids and to maintain the integrity of the yeast cell membrane (Briggs, Boulton, Brookes, & Stevens, 2004; Lorenz & Parks, 1991). Increased exposure of the yeast cells to oxygen, causes yeast cells to grow exponentially at increased rate of ethanol production (Briggs et al., 2004). The high gravity of the solute in wineries at increased ethanol concentration leads to increase in the osmotic pressure affecting the viability and growth pattern of the yeast cells (D'Amore, 1992).

Saccharomyces cerevisiae can adapt to the osmotic stress conditions by counterbalancing the stress forces. The osmotic stress forces are controlled by the intracellular glycerol synthesis (Nevoigt & Stahl, 1997). A redox balance is created by the yeast cells during the times of stress

environments by reduced ethanol synthesis and increased oxidation of acetate (van Dijken & Scheffers, 1986). Increased usage of energy currency by the fermenting yeast cells was because of the conversion of acetic ions into acetyl-CoA. Low Buffers present in the honey must can also lead to incomplete fermentation (Maugenet, 1964). Because of this, refermentation is encountered by the mead industries. It was caused by the yeast itself, or by the previously synthesised acetic acid and lactic acids present in the fermentation tanks. This can alter the aroma and the organoleptic character of the final product (O'Connor-Cox & Ingledew, 1991).

During fermentation of the honey must, saccharomyces utilises preferred carbon sources such monosaccharides and disaccharides (Rodríguez & Gancedo, 1999). These sugars are transported by the facilitated diffusion across the membrane with the assistance of glucose and galactose transporters (Lagunas, 1993).

The growth of the yeast in the fermentation tanks depends on the availability of nutrients to the growing yeast colonies. In the presence of appropriate nitrogen sources, the yeast cells and proteins expand in exponential manner (Lucero, Moreno, & Lagunas, 2002). In the absence of nitrogen sources in the fermentation tanks, yeast cell multiplied in about 6 hours but the protein turnover is not noticed (Lucero et al., 2002). This happened due to the carbohydrate accumulation (Lagunas & Ruiz, 1988). The changes are caused because of the degradation of the glucose transporters by maltose (Needleman et al., 1984). In subtle the cellular content changes and transport activities observed are due to the previously synthesized transporter system by maltose.

The toxic effect of the ethanol produced by the yeast strains act as inhibitor of the microbial growth population (Santos et al., 2008). This decreases the population of the viable cells and block the glucose (hexoses) transport system (Lewis, Elkon, McGee, Higbee, & Gasch, 2010). The energy required by the yeasts is taken from the diffusion of ions across the cytoplasm.

The energy sources present in the fermentation tanks gets depleted in the exponential phase. With the start of stationary phase, the yeast cells do not require the energy. The carbohydrates such as trehalose gets accumulated during the nutrition deficient fermenting environments including at times of osmotic stresses and heat shock (Nissen, Schulze, Nielsen, & Villadsen, 1997). The yeasts utilise the newly synthesised trehalose and continue to produce ethanol at lower fermentation time.

2.3.2. Evolution of non-Saccharomyces microbes as fermenting agents.

The selection of yeast for the alcoholic fermentation is important to obtain a desired fermented beverage. For over a decade, *Saccharomyces* has been the most preferred microbe for the mead. In the recent years, the prevalence of the non-*Saccharomyces* microbes is increasing and gaining attention by the beverage industries for their distinguished characters. They are able to contribute to the better aroma and flavour as well as able to synthesise the desired compounds (Holt, Mukherjee, Lievens, Verstrepen, & Thevelein, 2018).

Candida stellate, Hanseniaspora uvarum, Zygosaccharomyces bisporus, Issatchenkia orientalis are some of the non-*Saccharomyces* yeasts which are capable of fermenting sugars and produce ethanol. These microbes are able to increase their colonies during the fermentation and are able to alter the flavour of the meads (Hierro, González, Mas, & Guillamón, 2006).

Starmerella bacillaris can ferment sugars but the yield of the ethanol in the final product is less. At 9% of ethanol concentration in the fermentation medium and 48 h of incubation time, this microbe can grow independently. All the isolates of this species were able to synthesise 9.5% (v/v) of ethanol (Englezos et al., 2015). AWRI1199 strain of *Pichia fermentans* was able to produce 0.04g of ethanol for 10.5% of the sugars of consumed (Contreras et al., 2015).

A study conducted by (Escribano-Viana et al., 2018) concluded non *Saccharomyces* microbes such as *T.delbrueckii, L.thermotolerans M.pulcherrima* are able to synthesise the higher

amounts of 2-phenyl ethanol,1 butanol and methionol compared to the *Saccharomyces cerevisiae*. All these microbes were able to produce the aromatic concentration of below 200mg/L. The amount of ethanol synthesised are higher compared to *Saccharomyces* cerevisiae. The amount of ethanol synthesised by non-*Saccharomyces* microbes was *T.delbrueckii* 25mg/L, *L.thermotolerans* 22.5mg/L *M.pulcherrima* 13.5mg/L, whereas *Saccharomyces cerevisiae* was able to synthesise only 11.0mg/L of ethanol.

Another study conducted by (Zhang et al., 2017) concluded that *Zymomonas mobilis*, an anaerobic bacteria was able to produce ethanol at lower nitrogen usage. This microbe was also able to produce 16gm/L of ethanol when immobilised by PVA (poly vinyl alcohol) under continuous fermentation mode at 30°C. *Zymomonas mobilis* can grow at higher ethanol concentration.

2.3.3. Microbial load

The number of yeast cells added to fermentation tanks can affect the fermentation kinetics (Pereira, Mendes-Ferreira, Oliveira, Estevinho, & Mendes-Faia, 2013).The time taken to complete the fermentation can be reduced by increasing the microbial load (Verbelen et al., 2009). A study conducted by (Pereira et al., 2013) on *Saccharomyces* strains QA 23 and ICV D47 revealed, maximum biomass of the yeast *Saccharomyces* was achieved at 10^8 colony forming units (CFU) for both the strains. The growth of the ICV D47 decreased with the increase in the microbial load of the cells (Carrau, Medina, Fariña, Boido, & Dellacassa, 2010). Both the strains QA23 and ICV D47, when inoculated into the fermentation tanks with inoculum size of 10^7 CFUs /ml took 72hr and at 4×10^7 CFUs /ml took 48 hours to reach the stage of fermentation. The final ethanol synthesised by ICV D47 varied between 9.70 (1.5×10^5 CFUs/mL) to 10.37% (1.6×10^8 CFUs/mL). The residual nitrogen varied between

29.17 and 42.0 mg/L for both strains of *Saccharomyces* and this did not have any effect on the microbial load.

In another study conducted by Carrau et al (2010) with an inoculum size of 10^6 of yeast strains M522 AND KU1, resulted in a decrease in the yield of the aromatic compounds. There is no clear evidence published so far about the correlation between the inoculum size, aroma compounds. However, inoculum size had effect on the synthesis of higher alcohols and ethyl acetate (Erten, Tanguler, Cabaroglu, & Canbas, 2006; Mateo, Jiménez, Pastor, & Huerta, 2001). The concentration of the alcohol increased when the inoculum size increase from 1×10^6 to 1×10^7 cells/ml (Erten et al., 2006). Iso butanol increased from 40.97 to 60.65mg/ L, 2-Methyl-1-butanol increased from 44.83 to 55.16 mg/ L, 3-Methyl-1-butanol increased from 178.96 to 255.70 mg/ L whereas n-propanol decreased to 14.88 from 15.25 mg/ L.

Esters give fruity flavour to the honey mead. The increase in the inoculum size from 1×10^4 to 1×10^7 decreased ethyl acetate from 79.23 to 40.01 mg/ L. This did not affect the overall fruity flavour of the final product. When the concentration of the esters increased overall over 200 mg/ L, this affected the quality of the final fermented beverage and consumer satisfaction. The change in the esters concentration incurred due to the other existing non *Saccharomyces* yeast in the fermentation tanks (Ciani, Beco, & Comitini, 2006).

2.4.3. Nitrogen

Nitrogen is essential for the growth of yeast during the process of fermentation (Ferreira Monteiro & Bisson, 1992). The agreement of the European legislation for the wine must supplementation states nitrogen is required as 1g/L which is the legal limit. This can be in the form of 300 mg/L diammonium phosphate or ammonium sulphate (Mendes-Ferreira et al., 2010). The rate of fermentation depends on the availability of nitrogen (Mendes-Ferreira, Mendes-Faia, & Leão, 2004). The required satisfactory amount of nitrogen for the fermentation

to proceed is estimated to be 120-140 mg N/L. This depends on the primary composition of the honey must and yeast strains used for fermentation (Mendes-Ferreira et al., 2004). Under experimental conditions, 140 mg N/L, is necessary for the fermentation to complete by saccharomyces strains (Beltran, Esteve-Zarzoso, Rozès, Mas, & Guillamón, 2005; Kemsawasd, Viana, Ardö, & Arneborg, 2015).

Ammonium salts as a source of nitrogen to the yeast and are preferred by the wineries largely to overcome the problems related to fermentation of the honey must (Nahvi, Emtiazi, & Alkabi, 2002). Utilisation of the added nitrogen by the *Saccharomyces* is dependent on the specific strain (Manginot, Roustan, & Sablayrolles, 1998). This can be explained by using strain QA23 which has minimum nitrogen requirement 60 mg N/L, where as in the same experiment the controls were supplemented with 147 mg N/L (Beltran et al., 2005) . Addition of nitrogen after the initiation of the fermentation, had no effect on the biomass of the yeast cells.

Addition of nitrogen to the fermentation tank at the beginning of fermentation led to give out maximum of 1.4 g/L per hour of carbon dioxide. This was observed when *Saccharomyces* strain CY3079 was supplemented with mixture of concentration of amino acids 70mg N/L and at temperature 24°C (Seguinot et al., 2018). But 0.9g /L per hour of carbon dioxide was given out, when the nitrogen supplement was added at the beginning of the stationary phase of the fermentation. The biomass of the yeast cell population did not effect with the timing of the addition of nitrogen (Bell & Henschke, 2005). According to the study conducted by (Sablayrolles, Dubois, Manginot, Roustan, & Barre, 1996), they observed kinetics of the fermentation were better when nitrogen supplement was added at the start of stationary phase. The yeast metabolism improved without effecting yeast cell membrane lipid composition (Tesniere, Delobel, Pradal, & Blondin, 2013).

Addition of nitrogen derivatives during the fermentation of honey-must intensified the ethanol concentration from 3.0 to 6.3% with strain PYCC 4072 (Mendes-Ferreira et al., 2004). An inverse relationship was noticed between higher alcohols and initial nitrogen concentration present in the medium (Guitart, Orte, Ferreira, Peña, & Cacho, 1999; Rapp & Versini, 1991). When research was conducted on *Saccharomyces cerevisiae* PYCC 4072, increasing the initial nitrogen concentration from 16.5 to 805.0mg/L resulted in change in the final ethanol concentration from 0.5 to 11.0% (v/v) at the temperature of 20° C and with pH of 3.7 (A. Mendes-Ferreira et al., 2004).

2.3.5. pH

Saccharomyces cerevisiae grows at the pH range of 2.50 and 8.50 (Carmelo, Bogaerts, & Sa-Correia, 1996). These microbes are acidophilic in nature. The pH required for the growth of *Saccharomyces* depends upon the growth medium and environmental conditions such as oxygen and temperature (Narendranath & Power, 2005). The initial pH required for the growth of yeast was 4.00 to 5.00 (Buzas, Dallmann, & Szajani, 1989). Reducing the pH of the fermenting medium to 4.0 can prevent the growth of unwanted microorganisms such as lactobacillus (Narendranath & Power, 2005). When the pH of the fermenting medium falls less than 4.0, the yeast strains undergoes osmotic stress because of the increased concentration of the solute (sugars) surrounding the yeast cell membrane (Cardona, Carrasco, Pérez-Ortín, lí del Olmo, & Aranda, 2007). The metabolic activities of the fermenting yeast can be inhibited at the initial pH lower than 3.0 (Vine, Harkness, & Linton, 2012). The maximum ethanol concentration of 13% was obtained with *Saccharomyces* strain BY4742 at pH of 4.5 (Lin et al., 2012). The synthesis of ethanol progressed without any cross contamination at temperature of 30°C and pH 2.6-5.0 (Gupta & Sharma, 2009).

2.3.6. Temperature

Temperature is one factor that affect the kinetics of the fermentation, rate of fermentation and the quality of the mead produced. It has impact on the secondary metabolites formed during fermentation as well (Lafon-Lafourcade, 1983). Increase in the temperature above the threshold can lead to the formation of toxic substances, by effecting membrane composition of the *Saccharomyces* cell wall (Bisson, 1999; Casey & Ingledew, 1986). The transport of the metabolites is controlled by the yeast membrane in the presence of temperature, which had direct impact on the metabolism of the yeast (Valero, Millan, Mauricio, & Ortega, 1998). Any modification in the composition of the yeast membrane can enable the microbes to adapt to the challenging physical conditions (Hazel & Williams, 1990). The membrane fatty acyl composition of the yeast changes at lower temperatures resulting in more unsaturation of the honey-must (Watson, 1987).

The suitable temperatures for the fermentation of honey mead varied between 20 to 30°C. The optimal temperature for the *Saccharomyces* to ferment adequately ranges between 20 to 28°C. The fermentation rate decreased at the temperature less than 15°C and above 30°C. The temperature of the fermentation medium also effected the consumption of the sugars such as glucose and fructose (Berthels, Cordero Otero, Bauer, Thevelein, & Pretorius, 2004). The temperatures above 28°C contributed to the increased glucose concentrations to 1.8 g/L and glycerol to 7.8 g/L (Gomes et al., 2013). An experiment conducted on yeast strain PB2002 at temperature of 24°C was able to produce 10.2% of ethanol with commercial nutrient containing DAP at the concentration of 0.88 g/L (Gomes et al., 2013).

At lower temperatures the growth of the yeast slowed down and further took more time to reach the maximum colony size formation (Torija et al., 2003a). Volatile compounds increased in their concentrations at low temperature but this was not the same with all the strains of

Saccharomyces (Torija et al., 2003a). The amount of 2-phenylethanol produced varied between the *S.cerevisiae* and *S.bayanus* strains at the same temperature. The shorter chain length of *S.bayanus* is also making it more sensitive to ethanol at lower temperatures leading to slow down fermentation.

According to study conducted by (Molina, Swiegers, Varela, Pretorius, & Agosin, 2007) sugar consumption was taken into consideration to monitor the alcohols synthesized at different temperatures. At 28°C the glycerol concentration was higher compared to fermentation at 15°C. The rate of fermentation at 28°C was twice faster than at 18°C. The total concentration of the volatile compounds formed were higher at 18°C compared to 28°C. The concentration of the ethyl esters is fourfold higher at 18°C compared to 28°C. The total acidity produced was also higher at 18°C compared to acidity produced at 28°C. The odour activity value is the potential sensory aroma contribution to the final product. These volatile compounds are mostly synthesised at exponential growth phase during the fermentation at both temperatures. The organic acids formed during the fermentation of wines at both 18°C and 28°C are within the ranges of the content of organic acid of wine 795 to 850 mg/L (Lambrechts & Pretorius, 2000).

2.3.7. Oxygen

Oxygen is involved in the metabolic pathway of ergosterol. Oxygen is utilised in the lipid deficient conditions by the yeast. Oxygen required for the *Saccharomyces* was 7 mg/L at oenological conditions. This required amount of oxygen enables to continue fermentation without any arrest or struck in the fermentation. With increase in the oxygen consumption by the yeast cells, total biomass increases. The consumption rate of oxygen by different strains of the yeast was particularly very similar. Addition of oxygen at the end of the growth phase during the fermentation is more effective than at beginning of the fermentation. By the end of growth phase, free and stored up lipids (sterols) are utilised by the yeast cell completely. Early addition

of oxygen to the fermenting yeast causes oxygen to be used by polyphenol oxidases. Required oxygen additions causes the yeast to completely utilise the available nitrogen present in the musts preparations (Casalta, Cervi, Sablayrolles, & Salmon, 2012). In the absence of the solid sterols and oxygen, solid and liquid interactions does not occur, the yeast does not get the required nutrients leading to struck fermentation and sometimes takes longer duration to complete fermentation (Casalta, Vernhet, Sablayrolles, Tesnière, & Salmon, 2016; Tesniere et al., 2013).

At industrial alcohol production, pre cultures of *Saccharomyces* strains are able to undergo exponential generations of cell growth, even in the absence of ergosterols, due to reserved sterol pool (Fornairon-Bonnefond, Aguera, Deytieux, Sablayrolles, & Salmon, 2003). This corelates that ergosterol concentration is required for at least half the number of generations which is contributed by the ergosterol assimilation. In the presence of excess oxygen supply to the yeast, the cellular sterol content decreases because of the stronger oxidation of ergosterol by the NADPH -dependent non respiratory oxygen consumption (NOC) pathway (Fornairon-Bonnefond et al., 2003). The excess oxygen taken by *Saccharomyces* undergoes oxidation by partial functioning of the uncoupled cytochrome P450 (Rosenfeld, Beauvoit, Rigoulet, & Salmon, 2002).

2.3.8. Sterols

During fermentation of honey-musts, solid particles supply certain nutrients to the yeast cells. These nutrients include lipid components such as phospholipids, sterols, sphingolipids and glycolipids; which play a vital role in maintaining and protecting the integrity of the cell membrane. The viability of the yeast cell depends on the lipid composition (Ribereau-Gayon, Lafon-Lafourcade, & Bertrand, 1975). The solid particles in the honey-musts contribute to the phytosterols synthesis during the fermentation (Luparia, Soubeyrand, Bergès, Julien, &

Salmon, 2004). The phytosterols provide the musts with oxygen and fatty acids in nutrient deficient conditions (Andreasen & Stier, 1953; Cabanis & Flanzy, 1998). A minimum of 2-4mg of phytosterols/L are required at the beginning of the fermentation of honey to produce ethanol and are required for the yeast to acquire their maximum growth potential (Deytieux, Mussard, Biron, & Salmon, 2005).

The phytosterols prevent the slowdown of fermentation and enables the *Saccharomyces* to overcome the ethanol toxicity. Yeast cell take up the sterols from the endoplasmic reticulum both in the presence and in the absence of oxygen. In the presence of oxygen, yeast cells synthesise their own sterols, ergosterol in the endoplasmic reticulum and transfer to the plasma membranes. In the absence of oxygen, free sterols from the environment gets esterified and stored in the form of lipid droplets in the plasma membrane. Sterols in the yeast cell membranes are responsible for maintaining the integrity. They serve to maintain the membrane permeability and fluidity (Daum, Lees, Bard, & Dickson, 1998). Sterols helps in the metabolism under anaerobic conditions as well as oxygen consumption (Rosenfeld, Beauvoit, Blondin, & Salmon, 2003). They are up taken by the cell membranes exogenously (Daum et al., 1998). In the absence of oxygen, sterols and fatty acids synthesis decreased. Further the growth of the yeast cells is also inhibited leading to low cell viability and slow down the rate of fermentation (Fornairon-Bonnefond, Demaretz, Rosenfeld, & Salmon, 2002; Jahnke & Klein, 1983).

2.4. Fermentation aids used in honey mead preparation

Evidence from the literature suggests, addition of supplements to the honey fermentation improves the rates of fermentation and yield as well. The fermentation time can be decreased and quality of the ethanol can be achieved by supplementing the honey must preparations with

supplements such as DAP, pollen, hydrocolloids, royal jellies and various other derivatives of bio elements and vitamins.

2.4.1. Diammonium phosphate

Salts of ammonium are commonly used by the mead or wine makers, to improve the fermentation times. Various chemical forms of ammonia are used by wine makers to supply nutrients (nitrogen) to the yeast cells. Nitrogen improves biomass of the yeast, reduces the duration of fermentation and prevents the formations of hydrogen sulphide (Bell & Henschke, 2005). It contributes to the formation of flavour and aroma of the final product (Mar Vilanova, Siebert, Varela, Pretorius, & Henschke, 2012). The growing yeast cells are able to utilise the DAP added at temperature of 22°C, making them utilise all added nutrient (Pérez, Assof, Bolcato, Sari, & Fanzone, 2018). At lower temperatures less than 15°C, the yeast cell membrane losses the fluidity and added DAP remains unutilised by the yeast cells (Beltran, Rozes, Mas, & Guillamon, 2007). Addition of DAP to the honey-must reduce the fermentation time from 240 hours to 96 hours with saccharomyces strains QA23 and ICV D47 (Pereira, Mendes-Ferreira, Oliveira, Estevinho, & Mendes-Faia, 2015b).

2.4.2. Minerals and vitamins

The fermentation of the honey can be improved by adding the minerals and vitamins (Gibson, 2011). The growing yeast cells during the fermentation requires mesoinositol, pantothenic acid and biotin. Addition of these vitamins can decrease the stressful environments during the process of fermentation (Gibson, 2011). In particular vitamin biotin helps to improve the colony forming units of the *Saccharomyces* (Alfenore et al., 2002). Adding the vitamins after the beginning of the fermentation increased the ethanol concentration production from 18 % to 34 % (Alfenore et al., 2002).

A study conducted by Pereira, Mendes-Ferreira, Estevinho, & Mendes-Faia, (2015a) using *Saccharomyces* strains QA23 AND ICV D47 showed that when 100mg/l of inositol, 2mg/l of pyridoxine and nicotinic acid are added to honey-must , there is no significant effect on the fermentation. This study also proved that the yeast strains took about 24 hours to adopt to the fermenting environments. Both the strains remained in the stationary phase of fermentation after 48 hours and this affected the growth profile of the strains used.

Calcium and magnesium are proved to have beneficial effect on the ethanol production (Birch & Walker, 2000; T. L. Nissen, Kielland-Brandt, Nielsen, & Villadsen, 2000). Addition of magnesium to the fermenting medium enabled the yeast cells to overcome heat stress response and improved the ethanol production by maintaining the cell membrane integrity (Birch & Walker, 2000). Supplementation the hone- must fermentation with minerals and vitamins did not influence the fermentation kinetics. Dark coloured honey from multiflora origin appeared to overcome these requirements. The amount of ethanol synthesised from honey with mineral and vitamin supplementation was 11.13 % and that in the control with any supplementation was able to synthesise 10.60 %.

2.4.3. Hydrocolloids

Hydrocolloids are polymers which are used for thickening and stabilising the food products. They are made up of carboxylic acids mainly and are able to cause dissociation of the chemical compounds (Pegg, 2012). Gum Arabic is accepted gum in the wine industry as it prevents the precipitation of substances (Sroka & Satora, 2017). This gum is made up of six carbohydrate moieties and single protein in its chemical structure (Idris, Williams, & Phillips, 1998). They have capacity to increase fermentation kinetics of the yeast in honey by creating high turbidity force (Boivin, Feuillat, Alexandre, & Charpentier, 1998). The reason behind this is, yeast cells are flocculated and released carbon dioxide help in creating the capillary pores in the fermenting

medium. This causes the yeast cells to interact with the particles and nutrients present in the fermentation medium effectively.

A study conducted by Sroka & Satora (2017) concluded, honey mead supplemented with hydrocolloids such as with 0.2g gum Arabic produced 13% (v/v) of ethanol and with 0.2g karaya gum produced 13.9% (v/v) of ethanol. But without any hydrocolloid supplementation, only 10.6% (v/v) of ethanol was produced. In the same experiment, the authors also reported, that volatile acidity decreased from 1.12 g/L (control) to 0.95 g/L with 0.2g gum Arabic. This is because of the improved resistance to the yeast cell to increased toxicity of the ethanol.

2.4.4. Pollen

Pollen from the plants is a good source of fermentation aid. Pollen from the flowers is an important source of proteins, lipids, minerals and vitamins E and C, carotenoids and flavonoids (Roldán, van Muiswinkel, Lasanta, & Caro, 2008; Rzepecka-Stojko, Pilawa, Ramos, & Stojko, 2012). The amino acid composition has significant impact on the metabolic process of the yeast. Amino acids act as precursors in the process of fermentation caused by the yeast (Bouseta, Scheirman, & Collin, 1996; Vilanova et al., 2007). Proline, aspartic acid, phenyalanine, lysine, leucine and glutamic acid account for 16% of the pollen (Paramás, Bárez, Marcos, García-Villanova, & Sánchez, 2006). Evidence suggest that pollen has important therapeutic and antioxidant properties (Leja, Mareczek, Wyżgolik, Klepacz-Baniak, & Czekońska, 2007).

As pollen consists of salts of potassium and calcium, decrease in the acidity of the honey was observed (Ouchemoukh, Louaileche, & Schweitzer, 2007; Silva, Videira, Monteiro, Valentão, & Andrade, 2009). The microbial load of the honey mead can be stabilized with the addition of 40 to 50g/L pollen (Roldán, van Muiswinkel, Lasanta, Palacios, & Caro, 2011). Due to the presence of linoleic acid and linolenic acid in greater amounts in pollen, yeast cells metabolism

increased resulting in improved fermentation kinetics (Sroka & Tuszyński, 2007; Xu, Sun, Dong, & Zhang, 2009).

A study conducted by Roldán et al. (2011) revealed that addition of pollen at the concentration of 10 to 50 g/L increased the fermentation rate, decreased the fermentation time, increased the ethanol yield from 36 to 44%. These authors also stated addition of pollen enhanced aroma of the honey mead by increasing the levels of phenyacetaldehyde. The significant changes in relation to colour, taste quality and acceptability were enhanced in the pollen added honey meads. The increased fermentation rate was correlated to the increased turbidity (Bisson & Butzke, 2000). The improved fermentation kinetics were also corelated with increased turbidity from 137 (NTU) with 10 g/L pollen to more than 10000 (NTU) with 50 g/L pollen (Bisson & Butzke, 2000; M Vilanova et al., 2007). Alcoholic compounds like isoamyl alcohols and methanol content increased in the mead because of the pollen contribution to the honey-must (Roldán et al., 2008). The aromatic profile also improved with the addition pollen (Vidrih & Hribar, 2007)

2.5. Ethanol -the end product of fermentation

Ethanol is the main end products of the fermentation. Ethanol is inhibitory to the fermenting micro-organisms and is one of the major stress creating factor (Bisson, 1999). Ethanol diffuses freely through plasma membrane of the yeast. It can damage the membrane proteins, phospholipid layer and structures related with the intracellular enzymes. This results in the increase passive proton influx due to the increased membrane permeability (Hallsworth, 1998). Ethanol has capacity for both glucose and fructose utilisation, but not the affinity for the transport system of glucose (Leão & Van Uden, 1982). Glucose and fructose are transported by the same carrier (hexose transport system). At the end of fermentation the concentration of

glucose and fructose should be low in order to avoid auto fermentation and prevent off flavour of the final product (Pereira et al., 2013).

2.5.1. Ethyl carbamate toxicity

Ethyl carbamate is formed during fermentation of alcoholic beverages. The International Agency of Research on Cancer in 1974 classified ethyl carbamate as a possible human carcinogen (Cadranel et al., 1993). The increase in the level of added nitrogen supply more than the required causes the formation of ethyl carbamate. The nitrogen in the form of fertilizer supplied to the crop during the period of harvesting also has impact on the ethanol synthesised (Ough, Stevens, & Almy, 1989). Ethyl carbamate is formed from the yeast metabolite carbamyl phosphate (Lacroute, Pierard, Grenson, & Wiame, 1965). This is leading to the contamination of the synthesised alcohols due to increased microbial load (Monteiro, Trousdale, & Bisson, 1989; Ough et al., 1989).

Ethyl carbamate is formed as a result of reactions between urea and ethanol. Urea is one of the precursors for the formation of Ethyl carbamate during the fermentation of alcoholic beverages. Ethyl carbamate is formed due to the enzymatic reaction between the yeast and lactic acid bacteria present in the fermentation medium (Hofman-Bang, 1999). Lactic acid bacteria are able to produce Ethyl carbamate by converting amino acid arginine into citrulline during fermentation (Jiao, Dong, & Chen, 2014).Yeast is able to convert amino acid arginine into urea (Benucci, Fiorelli, Lombardelli, Liburdi, & Esti, 2017). Other factor responsible for formation of Ethyl carbamate are temperature , pH and environmental conditions of the microorganisms (Zhao et al., 2013).

2.5.2. Quality standards of alcoholic beverages

Alcoholic beverages are consumed worldwide on different occasions. It has been reported that 42.8% of alcohol is consumed in form alcoholic beverages such as beer, wine, fortified wines

and mead. Ethanol and water are the two main components of the alcoholic beverages. The alcohol quality refers to taste, flavour and colour. The quality of alcohol consumed has impact on the public health. Safety and quality standards requirements have been established by the various countries and international organisations such as Codex Alimentarius Commission (CAC), European Union (EU), Food standards of Australia and New Zealand (FSANZ). The specific gravity of alcohol in retention to water should be 0.79067 in a vacuum at 20°C. The maximum limit of toxic carcinogens should not exceed 1.0 mg /L and maximum limit for methanol should not exceed 8.0 mg/L (Codex Alimentarius Commission, Programme, & Organization, 2001). The food ingredients or additives in the alcoholic beverages should not exceed 10 mg/Kg.

2.6. Quality parameter of honey mead

2.6.1. Aroma

Aroma of the final product is important factor for marketing the honey mead. It has major influence on the customers acceptability and gaining economic value (King, Osidacz, Curtin, Bastian, & Francis, 2011). The consumers acceptability helps the winemakers to identity the desired sensory qualities and produce desired mead by altering the methods of production and addition of supplements (Lattey, Bramley, & Francis, 2010). The aroma of mead depends on the origin of the honey and nutrients available to yeast in the fermentation. Certain nitrogen resources are reported as precursors of the volatile compounds produced by the *S.cerevisae* (Fairbairn, McKinnon, Musarurwa, Ferreira, & Bauer, 2017). The volatile compounds present in the honey mead contribute to the formation of aroma. Based on the geographic location of the honey crop harvest, aroma of the mead differs (Manyi-Loh, Ndip, & Clarke, 2011).

Odour activity values (OAVs) describes the aroma of the final product. OAVs can be determined by dividing the concentration of each synthesised volatile compound by its threshold perception. When OAV is greater than or equal to 1, such compounds contribute to the aroma of the final product. Certain volatile compounds and their OAV listed in the Table 2.

Table:2 Odour activity values (OAV) and odour descriptor of different volatile compounds produced by different strains of *Saccharomyces.*

Compound	strain Mt-R1B	strain Fm-R	Odour descriptor	Odour threshold(µg/l)
Acetaldehyde	11.3	22.5	fresh, green leaves	500[a]
1-Propanol	0	0	alcohol, pungent	830000[b]
Isobuthyl acetate	0	0	sweet, fruity	350[c]
1-Octanol	1.0	1.3	lemon, wax, coconut	120[d]
Ethyl acetate	2.4	3.0	pineapple, balsamic	12000[abd]

Source: [a](Pereira et al., 2013),[b](Carrau et al., 2008),[c](Barbosa, Falco, Mendes-Faia, & Mendes-Ferreira, 2009),[d](Roldán et al., 2008).

A study conducted by Pereira, Mendes-Ferreira, Oliveira, Estevinho, & Mendes-Faia (2015c) on strain QA23 and ICV D47, with DAP a nitrogen supplement produced 27 volatile compounds. The compounds produced belongs to alcohols, esters, carbonyl compounds, volatile fatty acids and phenols. Alcohols and esters were major compounds produced. Both compounds contributed to the fruity flavours. Certain compounds such as acetaldehyde synthesis is strain specific and not depend on the nutrition. 3-Methyl-1-butanol, Ethyl acetate,

Octanoic acid did not have any differences with QA23 and ICV D47 and with DAP as supplement. Fatty acids contribute to give rancid and cheese like flavour to the final products whereas phenols are well known for their off flavours. Fatty acids and phenols produced in high concentrations with DAP as supplement in comparison to the controls.

Another study conducted by Chen, Wu, Lo, & Wu (2013) concluded that inoculating the fermentation tanks with one variety of strain can often contribute to unexpected and contaminated flavours. These authors also found that inoculating with multiple strains of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* would contribute to the favourable aroma. Such results are were also concluded by (Eglinton et al., 2000). They showed that 3-methyl-1-butanol is responsible for burnt and whiskey odour, Ethyl butyrate for acid food flavour and Ethyl acetate for nail polish flavour.

2.6.1.1. Alcohols

Alcohols are important volatile compounds synthesised as secondary metabolites by the *Saccharomyces cerevisiae* (Mendes-Ferreira et al., 2010; Roldán et al., 2011). Most synthesised alcohol in mead is 3-methyl-1-butanol. It is synthesised in the concentration range of 90 to 350 mg/L (Roldán et al., 2011).

2.6.1.2. Esters

Chemical interactions between fatty acids, esters and higher alcohols results in formation of esters. These are dominantly present in the meads and contribute to fruity flavour (Mendes-Ferreira et al., 2004). The concentration of esters also depends upon on the amount of nitrogen added to the fermentation tanks (Roldán et al., 2011).

2.6.1.3. Fatty acids

Volatile fatty acids are short chain, medium chain and long chain fatty acids. These are synthesised from the β-oxidation of the fatty compounds (Vilanova et al., 2007). Acetic acids is the predominant compound synthesised during the fermentation of alcoholic beverages. The accepted concentration of the fatty acids are in the range of 0.2 to 0.6 g/L (Swiegers, Bartowsky, Henschke, & Pretorius, 2005). Depending upon the geographic location honey harvest, the concentration of the fatty acids changes and ranges between 0.2 to 1.1 g/L (Wintersteen, Andrae, & Engeseth, 2005). The concentration of the fatty acids in the mead depend upon the added nitrogen and yeast strain (Roldán et al., 2011).

2.6.1.4. Carbonyl compounds

The predominant compound of this group is acetaldehyde produced from the sugars. Multi floral honey produce high amounts of acetaldehyde compared to other types of honey. The synthesis of acetaldehyde is dependent on the specific strain used and on the concentration of the nitrogen used in the fermentation. The amount of acetaldehyde produced ranges from 0.5 to 700mg/l (Liu & Pilone, 2000).

2.6.1.5. Volatile phenols

Volatile phenols compounds are synthesised in minor quantities and contribute to give off flavour to the final product. The off flavours include band aid and barnyard. The production of these compounds are not dependent upon the yeast strain used. The dominant compounds include Ethylphenols and Vinylphenols (Pereira et al., 2015a).

2.6.2. Colour

The colour of the honey mead influences the taste of the honey mead. In general, light coloured honey meads taste acidic and give hay like flavour whereas darker honey meads taste like rubber and give off flavours (Kime et al., 1991). The colour of the honey mead also depends on the heat and type of heat used to pasteurise the honey. Honey samples heated for longer durations imparted dark colour whereas lighter colour to limited heating or no heating (Gupta & Sharma, 2009).

2.6.3. Flavour

Flavour is the most important characteristic of honey mead. Flavour can be assessed based on aroma of the final product and its taste components. These taste components are incorporated segments of the acidity, sweetness, strength of the beverage and astringency. Flavour refers to the odour and taste of the mead, but aroma is related with the volatile compounds. The metabolites formed at the end of fermentation are the principle flavour contributing compounds. The flavour of the mead is mainly produced by alcohols and esters during the fermentation (Mar Vilanova, Genisheva, Masa, & Oliveira, 2010). The total concentration of the higher alcohols and esters should be 300 mg/L. This concentration acts as limit of sensory threshold. A concentration above this gives the final product off flavour and below this gives a mouth feel and pleasantness (Vidrih & Hribar, 2007).

According to the study conducted by (Gomes et al., 2015) concluded that the ethanol concentration in the honey mead had not shown any effect on the flavour and on the characteristic appreciation such as colour, aroma and mouth feel. The sweetness of the mead is favoured by the consumers. The association between the sweet mead and flavour was well accepted (r=0.79).

3. Materials and methods

3.1. Chemicals and raw materials

Dry yeast strains, *Saccharomyces cerevisiae* Lalvin EC1118 and *Saccharomyces bayanus* Lalvin 71B were purchased from Ibrew, Brisbane, Australia. Honey was supplied by Australia Natural Biotechnology PTY Ltd, Perth, Western Australia.

3.2. Preparation of the honey-must

For each experiment, honey (665g) (weighed by using from PSC precision weighing scale, NSW, Australia) was diluted with 370 g of sterile water and mixed to form a homogeneous solution by using a glass rod stirrer. Three different honey-musts were tested, one with no nitrogen supplement and two honey-musts containing nitrogen supplement from different sources, di-ammonium phosphate (DAP) 2g, food grade (Ibrew, Brisbane, Australia)), and Lupin (56.5g) (Bulk foods, Sydney, Australia) respectively. The nitrogen supplement was added at this point as described in Pereira *et al* (Pereira et al., 2015b). These honey-musts mixtures were then pasteurized at 65°C, heated using a hot plate stirrer (Barnard thermolyne, Crown scientific, NSW, Australia) for 10 minutes and then allowed to cool to 24°C. The temperature of the honey-must during pasteurization was checked using thermometer (FRIO-TEMP thermometer, Sigma-Aldrich, Castle Hill, Australia). Various treatment combinations are present in Appendix 1.

3.3. Fermentation conditions

For each experiment, 15 samples were prepared by measuring 60 g of the pasteurized honey-must into a 200 ml conical flask. The dry yeast (1g or 2g) was then added to the honey-must solution according to the manufacturer's instructions at 24°C. The flasks were covered using aluminium foil and placed in the incubator (Laboratory oven from LABEC laboratory equipment pty Ltd, Australia) at temperature of 23°C. Three (3) samples were taken immediately. After 24, 48, 72 and 96 hr intervals, three (3) samples were collected. Samples were immediately placed in a -20^{o}C freezer (Thermoline Scientific, NSW, Australia). During fermentation, the temperature of the incubator was checked every 12 hours. The temperature was recorded using the digital data logger (Universal Thermocouple, Victoria, Australia).

3.4. Total Acidity

Total acidity was determined by validated standard method OIV-MA-F1-05, method type 4 (International Organisation of Vine and Wine, 2011).

The total acidity of the honey mead is the sum of all the titratable acidities, when titrated to pH 7 against a standard alkaline solution without carbon dioxide. The principle of this method is to titrate with bromothymol blue as indicator with end point colour standard change to blue green.

Into the conical flask, 10g of the fermented honey mead sample was taken, and 10g of sterile water were added. Carbon dioxide present in the sample prepared is eliminated. Sodium hydroxide (Anhydrous, Sigma-Aldrich, Castle Hill, Australia**)** (0.1M) was added from burette while stirring continuously until the standard blue green colour appeared. Bromothymol (ACS reagent ≥ 99%, Sigma-Aldrich, Castle Hill, Australia**)** was used as indicator. The experiment

was repeated twice with all the honey mead samples. Standardisation of the sodium hydroxide solution was performed for every experiment.

$$\text{Total Acidity} = \frac{\text{Concentration of NaOH} \times \text{Volume of NaOH} \times 1000}{\text{Volume of the sample}}$$

3.5. pH

The pH of the fermented samples was determined by digital pH meter (IPS Rowne Scientific, Australia). The pH meter was calibrated by using known standard buffer solutions with pH of 4 and 7 and then cleaned with sterile water. A sample of the unfiltered fermented solution was placed on the pH meter using Pasteur pipette and the pH was recorded immediately.

3.6. Brix

Brix of the fermented samples was determined by using Brix refractometer (Brix refractometer from Bellingham + Stanley Ltd, Germany). A sample of unfiltered fermented solution was placed on the Brix by using the Pasteur pipette and the value was recorded.

3.7. Determination of Ethanol content

Alcoholic fermentation takes place in the aqueous solutions containing sugars and other related compounds. Direct injection of such fermented samples into the GC-FID is possible (Wang, Choong, Su, & Lee, 2003). However due to the potential of damaging the equipment and column, this method is not usually prefered. In order to overcome these damaging causes, extraction methods have been developed to analyse the ethanol content using GC-FID.

Extraction of the organic compounds from the water required careful consideration of the chemical and internal standards (Hewavitharana, 2009). The main features of the organic solvents used as extracts should include but not limited to moderate volatility, solubility in water and also have high partition coefficients for both the organic compound and internal standard. The retention time for the analyte and internal standards should be different and distinct from each other to give a realistic quantification of the organic material.

Ethanol content was determined by GC-FID. Gas chromatography with Flame Ionised Detector from Agilent technologies 7890A, GC system was used for the analysis. It is equipped with split/spitless injector. The capillary column is 30m×0.320mm and 0.25micron film thickness. The temperature of the inlet was 210°C, pressure 5.8094psi, total flow 58mL/min, septum purge flow 4mL/min. The split ratio 35:1. The temperature program had an initial oven temperature of 75°C, hold time for 2 min, and ramped to 80°C (final temperature) at 1^{o}C/min. Total run time was 7 min. The detector parameters were heater temperature 300°C, H_2 flow 30mL/min, air flow 300mL/min, N_2 makeup flow 10mL/min. The carrier gas was hydrogen with a flow rate of 1 mL/min. The concentration of the ethanol after the detector response for each injection of the analyte was determined with Aligent chem station software version B.1.05.11059.0332.

3.7.1. Extraction Method A

Standard solutions of ethanol in chloroform (ACS reagent ≥ 99%, Sigma-Aldrich, Castle Hill, Australia**,)** were prepared in the range 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0% w/w. 1-Pentanol (ACS reagent ≥ 99%, Sigma-Aldrich, Castle Hill, Australia**)** (0.1g) was added to each solution as internal standard. 2 µL of this solution was analysed by GC-FID as described in (3.7) to determine the amount of ethanol extracted from the original aqueous ethanol solution.

3.7.2. Extraction method B

An aqueous ethanol solution of 2.5% (w/w) was prepared. Five (5g) of this solution was added into a 50 ml falcon tube (Thermo fischer scientific, Australia) followed by 2 g Na_2SO_4 anhydrous salt (Sigma-Aldrich, Castle Hill, Australia**)** and 10 g of chloroform. The tube was given a shake for 10 minutes and centrifuged (Centrifuge from ThermoFisher scientific, Australia) for 10 minutes at 8000 rpm. Two (2g) of the bottom layer of the solution was extracted using paster pipette and placed into 2 ml vial. 1-Pentanol (0.1g) was added as internal standard and the sample was shaken. 2 µL of this solution was analysed by GC-FID as described in (3.7) to determine the amount of ethanol extracted from the original aqueous ethanol solution. Aqueous ethanol solutions were also prepared with a concentration range 5.0% (w/w) to 15% (w/w). A plot of extracted ethanol (% w/w) with concentration of ethanol in the original aqueous ethanol solution did not yield a linear curve indicating that the extraction efficiency was changing with ethanol concentration.

3.7.3. Calculation of extraction efficiency

A second 5 gm aliquot of the 2.5% (w/w) aqueous ethanol solution was taken. A spike of 0.05 gm of ethanol (ACS reagent ≥ 99%, Chem Supply Pty limited, Gillman, South Australia, Australia) was added to this aliquot and this resultant solution was extracted as described in (3.7.2) and the extracted amount of ethanol was determined as described in (3.7).

The concentration of ethanol determined from the analysis was significantly outside the experiment uncertainty when compared to the concentration of the prepared aqueous ethanol solution of 2.5% w/w. The reason for this was attributed to the variations generated to the low amount of ethanol extracted into the chloroform and the variations in the volumes of the layers after extraction.

3.7.4. Extraction method C

The extraction efficiency determination was repeated as described in (3.7.3) except 2g NaCl anhydrous salt (Sigma-Aldrich, Castle Hill, Australia**)** was used in place of the 2g Na_2SO_4. The concentration of ethanol determined from the analysis was significantly outside the experiment uncertainty when compared to the concentration of the prepared aqueous ethanol solution of 2.5% w/w.

3.7.5. Extraction method D

Standard solutions of ethanol (Chem Supply Pty limited, South Australia) in ethyl acetate (ACS reagent ≥ 99, Sigma-Aldrich, Castle Hill, Australia) were prepared in the range 0.5, 1.0, 2.0, 3., 4.0 and 5.0% w/w. 1-Pentanol (0.1 gm) was added to each solution as internal standard. The analysis was performed with an injection volume of 2 µL as described in (3.7).

3.7.6. Calculation of extraction efficiency

An aqueous ethanol solution of 2.5% (w/w) was prepared. Five (5g) of this solution was added into a falcon tube, followed by 2 g NaCl. The solute was shaken and then 10 g of ethyl acetate was added. The tube is given a brisk shake for 10 minutes and centrifuged for 10 minutes at 8000 rpm. Two (2g) of the bottom layer of the solution was extracted using paster pipette and placed into 2 ml vial. 1-Pentanol (0.1g) was added as internal standard and the sample was shaken. 2 µL of this solution was analysed by GC-FID as described in (3.7) to determine the amount of ethanol extracted from the original aqueous ethanol solution.

A second 5 gm aliquot of the 2.5% (w/w) aqueous ethanol solution was taken. A spike of 0.05 gm of ethanol was added to this aliquot and this resultant solution was extracted with ethyl acetate as described in (3.7.6) and the extracted amount of ethanol was determined as described in (3.7). The concentration of ethanol determined from the analysis was significantly outside

the experiment uncertainty when compared to the concentration of the prepared aqueous ethanol solution of 2.5% w/w.

3.7.7. Direct measurement of ethanol in aqueous solution

Debebe et al. (2017) reported the direct analysis of ethanol by GC-FID in aqueous solutions. A standard solution of ethanol in water at specific ethanol concentrations in the range of 0 – 3 % (w/w) were prepared. 1-Butanol (ACS reagent ≥ 99, Sigma-Aldrich, Castle Hill, Australia) was added as internal standard as 1-pentanol does not have enough solubility in these aqueous solutions.

A stock solution of four (4g), 5% w/w ethanol in water, was prepared. The following samples of this stock solution were measured, 0.2, 0.4, 0.6, 0.8 and 1.0 g. Sterilised water was added to each of these samples to yield a total mass of 2.0 g. The concentration of ethanol in each of these samples was 0.5, 1.0, 1.5, 2.0, and 2.5% w/w respectively. Each of these solutions was analysed in the following manner.

The prepared sample solution of 1.5 g was placed into a separate vial, then 0.1 g of 1-Butanol as internal standard was added and sterilised water was then added to yield a final mass of 2 g. The solution was then shaken for 10 seconds. The analysis was performed with an injection volume of 3 µL. This analysis was repeated several times on different days and showed excellent reproducibility and repeatability.

3.7.8. Analysis of Fermentation solution

The fermentation solution of two (2g) was filtered through a bio nylon filter diameter 25mm and pore size 0.22µm (Sigma Aldrich, Castle Hill, Australia). This filtered solution was used directly or was diluted as follows (as determined by FTIR analysis) 0.5 g of the filtered solution was added to a clean vial and 1.5 g of sterilized water is added. 1.5 g of the filtered or diluted

solution was placed into a clean vial, then 0.1 g of 1-butanol as internal standard was added and sterilised water was then added to yield a final mass of 2.0 g. The solution was then shaken for 10 seconds. The concentration of ethanol was analysed by GC-FID as described in (3.7) with an injection of 3µL of sample.

3.7.9. FT-IR analysis of the fermented samples

FT-IR/FT-NR spectrometer. All the spectral measurements were carried by using Perkin Elmer FT-IR/FT-NR spectrometer. The instrument was equipped with ATR corrector. Spectrum 400 and software version V6.3.4.0164 from Perkin Elmer were used to control the spectrometer as well to analyse the results.

A method of preliminary determination of the ethanol concentration was developed using FT-IR standard aqueous ethanol solution to determine the required dilution. Coldea et al. (2013) reported a method for determination of ethanol concentration by FT-IR. Aqueous ethanol solution of 5, 10 and 15% w/w were prepared. The FT-IR spectrum of these solutions, as well as a pure water sample, was recorded in the range of 4000-600 cm^{-1}. These four spectra have the characteristic vibration frequency at 1045 cm^{-1} and was overlayed in a single figure and height of this peak was manually measured from these spectra. This frequency is generally attributed to the C-O stretching vibration for ethanol. The spectral frequency of 1045 cm^{-1} was selected because of the sensitivity and specificity for the ethanol (Coldea, Socaciu, Fetea, POP, & Florea, 2013).

The solution was placed onto the ATP crystal using a Pasteur pipette. ATR correction was applied to the spectrum. The transmittance of the 1045 cm^{-1} peak of ethanol was measured. The height of each transmittance peak was measured by using a ruler. A horizontal line was drawn between the two shoulders of the peak. A vertical line was drawn from the top of the peak down to this horizontal line.

The filtered fermentation solutions were analysed in a similar manner. Interference from peaks for organic compounds like sugars in the honey and final honey mead reduced the accuracy of this technique for ethanol concentration. This technique was used to obtain an approximate ethanol concentration and to decide whether dilution was required to obtain an ethanol concentration within the linear range of the GC-FID.

3.8. Analysis of Aroma components of Honey Mead by GC-MS

The fermentation solution (Honey Mead) collected on day 4 (96h) was filtered with a bio nylon filter diameter 25mm and pore size 0.22µm (Sigma Aldrich, Castle Hill, Australia). A 4.5 g aliquot of this filtered solution was added to a 20 ml pear shaped flask. A 25 g aliquot of a 5.14% w/w butyl acetate in ethanol solution was added to the flask. The flask was stirred and heated at 35^{o}C for 60 minutes.

GC-MS. GC-MS analysis was performed on a Shimadzu GC-2010 Plus GC coupled to a GCMS-QP2010 Mass Spectrometer. The capillary column was a SH-Rxi-5Sil MS 30mx0.25mmID with 0.25 micron film thickness. The temperature of inlet port was 250^{o}C, septum flow rate of 3mL/min. the split ratio was 5:1. The column carrier gas was helium and flow rate was 1.0 mL/min. The interface temperature was 280^{o} C and the ion source was 200^{o} C. The following temperature program was used: initial temperature 35°C, hold time 6 min; raised to 250^{o} C at 10^{o} C/min; then raised to 270^{o} C at 50^{o} C/min, hold min 10 min.

A 50/30um DVB/CAR/PDMS SPME fibre (Sulpelco analytical) was heated at 270^{o} C in the inlet port of a Shimazdu GC-MS for 1 hour. The SPME fibre was then inserted into the flask and allowed to stand for 45 minutes to allow the aroma components to adsorb onto the fibre. The flask was stirred and heated at 35^{o} C during this time. The SPME fibre was then desorbed

at 250^{o} C in the inlet port of the GC-MS and the components were separated and analysed by GC-MS as described**.**

A standard solution of compounds listed in Table 3 was prepared in ethanol at the stated concentrations. This solution was diluted with water to yield an ethanol concentration of 10.09% w/w. The final concentration of all compounds in this solution is shown in Table 3. This standard solution was analysed using the same method for the fermentation solution as described.

3.9. Statistical analysis

All the data were collected in three replicates and recorded in Microsoft Excel sheet Windows 10. The statistical analysis was carried out for amount of ethanol produced, total acidity, pH and °Brix only. One-way of variance ANOVA was conducted and differences between the group of means were compared by Fisher's individual error rate test using Minitab version 16. The statistical significance level of acceptance was set at $\alpha = 0.05$. If the p-value is $< \alpha$ there is no significant difference, and if the p-value is $> \alpha$ there is a significant difference. All values were expressed as means ± standard deviation.

Table 3. Prepared concentration and final concentration in mg/kg of aroma compounds in standard solution used to determine aroma concentrations by SPME of Honey Mead.

Compound	Prepared concentration in ethanol solution (g/kg)	Final concentration in standard solution (g/kg)
methanol	1921	193.1
iso-propanol	2347	236
1-propanol	2327	234
acetic acid	8130	817.4
ethyl acetate	619	62.3
iso-butanol	319	32
iso-amyl alcohol	2268	228
2-methyl-1-butanol	1644	165.3
iso-amyl acetate	104	10.4
phenyl ethyl alcohol	1634	164.3

4. Results and Discussion

The progress of the fermentation of the honey into honey mead was monitored by a variety of parameters including ethanol content, pH, sugar content, and titratable acidity. Aroma composition was also measured on the honey mead after 4 days.

Sugar content was determined by the standard method by measuring the °Brix value of the filtered fermentation solution using a refractometer. pH was measured using a calibrated pH meter. Titratable acidity was measured by the titration of a 50 g aliquot of the filtered fermentation solution with standardised NaOH. The equivalence point of the titration was change of colour to blue green with bromothymol blue.

4.1. Ethanol content

The fermented honey-must samples were analysed to determine the accurate concentration of the ethanol present. Ethanol concentration in the honey mead was determined by GC-FID. The original methodology was to extract the ethanol from the aqueous honey mead solution with an organic solvent using the established methods of determining organic compounds in aqueous solutions.

Chloroform was initially used as the extraction solvent. Standard solutions of aqueous ethanol were prepared and extracted with chloroform. The concentration of ethanol in the chloroform extract was determined using GC-FID with 1-pentanol as the internal standard. A plot of ethanol concentration in chloroform extract against original ethanol concentration in the aqueous solution is shown (Figure 1). A plot of normalised area of ethanol with concentration of ethanol yielded a linear graph over this concentration (r^2 = 0.88). If the extraction efficiency of this extraction method was constant over the range of starting ethanol concentrations, this plot

should be a linear curve. The plot did not show a strong relationship, consistent with extraction efficiency changing with ethanol concentration in the aqueous solution.

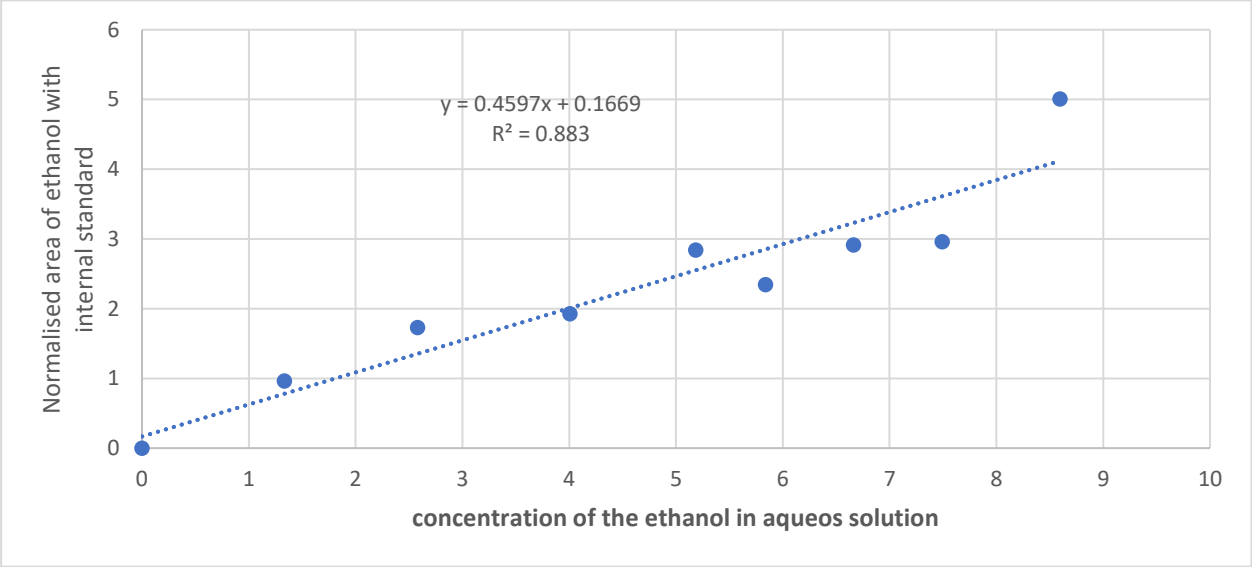

Figure 1. Plot of normalised peak area of ethanol concentration in aqueous solution with internal standard.

The next method studied was the addition of a spiking control experiment to determine the specific extraction efficiency of the technique at the aqueous ethanol concentration. The amount of ethanol in the chloroform extract was determined as described in (3.4.4.) for two solutions, an aliquot of the known standard of aqueous ethanol and the same aliquot which was spiked with a small amount of ethanol. The calculated aqueous ethanol concentration from this method was significantly in error compared to the actual concentration in known standard of aqueous ethanol. Also this technique lacked reproducibility. It was concluded that the amount of ethanol being extracted by the solvent, chloroform, was too small and the inherent uncertainty of the technique was causing the inaccuracy and unreliability.

The extraction procedure was varied to attempt to increase the extraction efficiency. Different solvent ratios, addition of salts such as NaCl to the aqueous solution did not sufficiently increase the extraction. Ethyl acetate was also tried as an extraction solvent but this alteration did

significantly increase the extracted amount of ethanol. It was concluded that the extraction method was not accurate enough as ethanol was not completely extracted from the solvent. A plot of normalised area of ethanol with concentration of ethanol yielded a linear graph over this concentration $r^2 = 0.91$ (Figure 2).

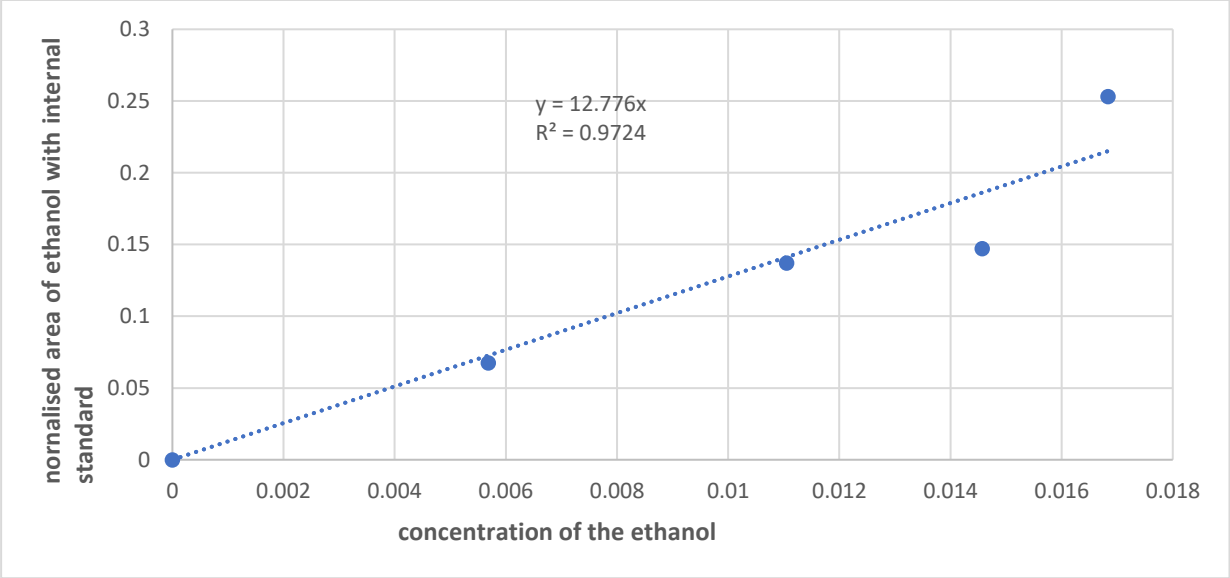

Figure 2. Plot of normalised peak area of ethanol concentration in aqueous solution with internal standard.

The ethanol concentration was determined by an adaption of method of Debebe's (2017). A normalised plot of the response area of the ethanol peak against the ethanol concentration yielded a linear curve up to a concentration of 2.7% w/w as shown in the (Figure 3) (y = 1239x-0.0015, r^2=0.98). The peak areas were averaged over triplicate injection. This analysis was repeated on different days and was able to reproduce the results in each experiment.

All the fermented samples were analysed in the similar manner for ethanol content with 1-butanol as internal standard. Fermented honey mead was diluted as required to obtain the ethanol content within the linear range of GC-FID value (0 - 2.7 % w/w). The required dilution of the fermented homey mead samples was determined by analysing the ethanol content using FT-IR.

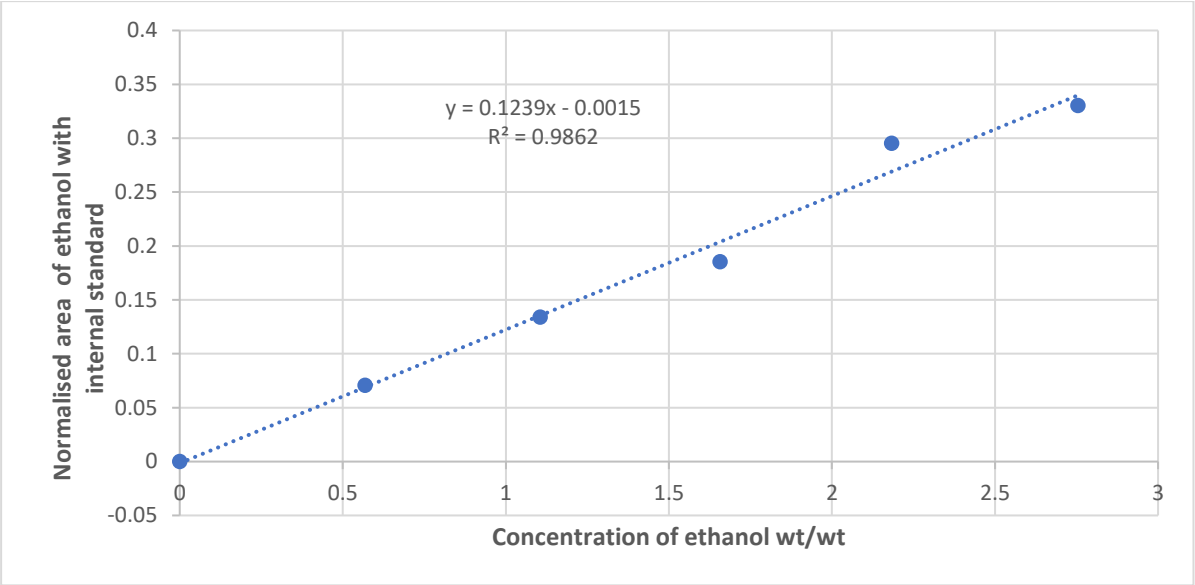

Figure 3. Plot of normalised peak area of ethanol versus ethanol concentration in aqueous ethanol standards.

4.1.1. Analysis of ethanol by using FT-1R Spectrometry

The direct GC-FID analysis of the ethanol in aqueous solution was only possible in the concentration range below 3.0% w/w. As the honey mead yielded from the fermentation had ethanol concentrations up to 15% w/w, dilution of these sample was often required. The typical transmission of the wavelength with water, DAP and 10% ethanol was shown in the (Figure 4). The transmission heights were plotted against the ethanol concentration and this gave a linear curve as shown in the (Figure 5) ($y=0.458x-0.01$, $r^2=0.99$).

Other species, such as longer chain alcohols and esters were present in the fermentation solution and these species yielded absorption close to this peak and so the accuracy of this analysis has uncertainty for the fermentation solutions and so was only used to determine a rough estimate value of the ethanol concentration.

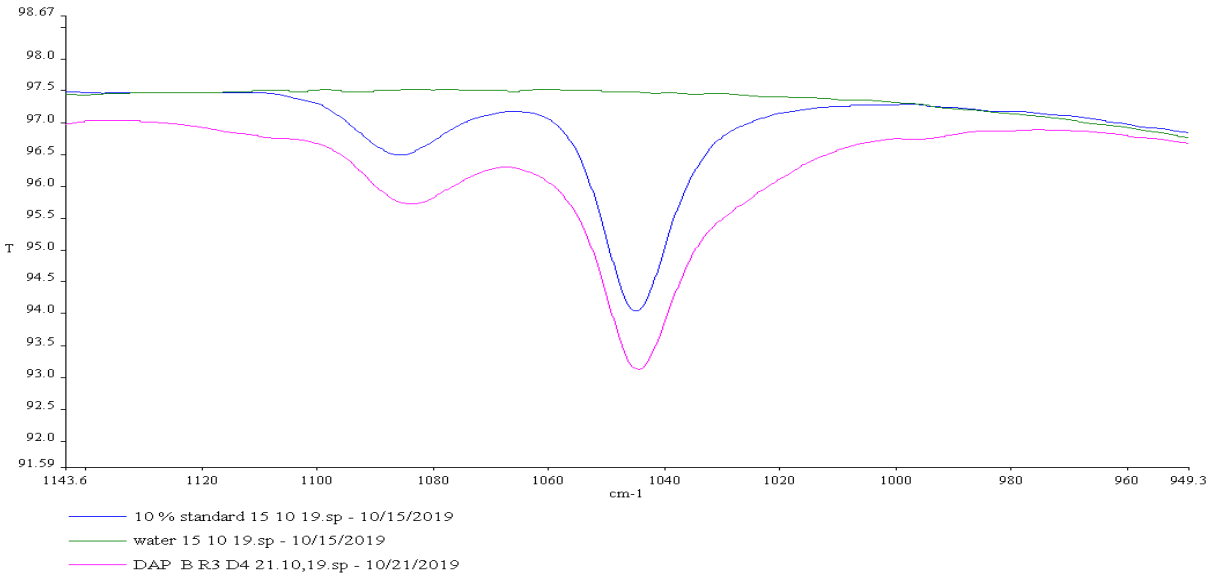

Figure 4. Plot area of ethanol against the transmission wavelength with water, DAP and 10 % ethanol as standard.

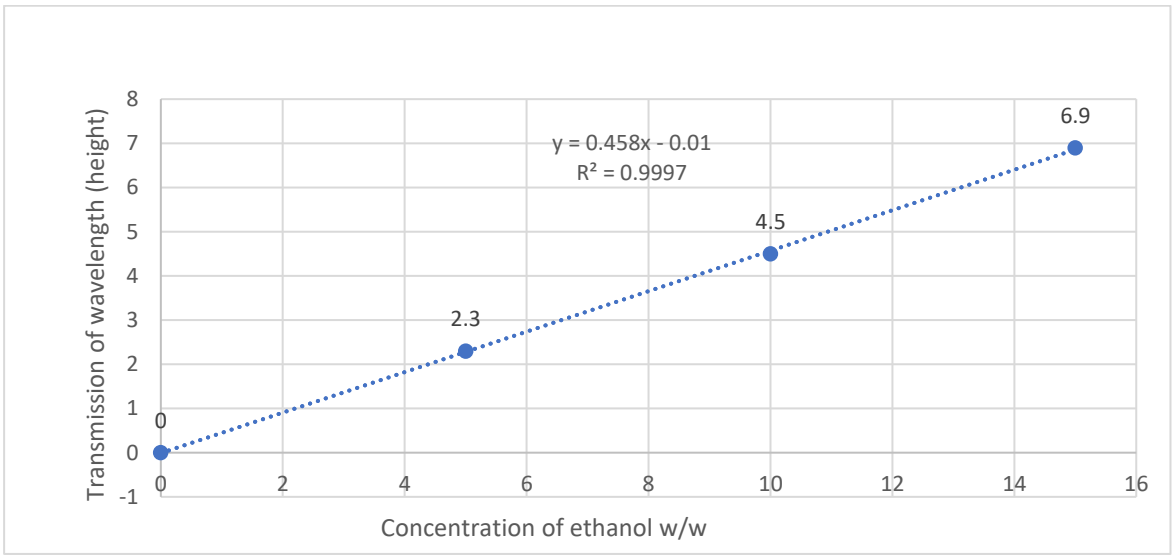

Figure 5. Plot area of ethanol against the transmission wavelength.

4.1.2. Analysis of the ethanol in the fermented samples by GC-FID

As described in (4.1.2), a rough estimate of the concentration was determined from the FT-IR analysis of the fermentation sample. An aliquot of the fermentation solution was then taken and diluted into the range of 0-3.0% w/w ethanol. The final mass of this diluted sample was 1.50g. 1-Butanol was added as the internal standard and the solution was diluted with 0.4 g of sterile water. This sample was analysed by GC-FID as described in (3.4.4).

4.2. Effect of type of yeast strains without nutritional supplement on the ethanol production

Saccharomyces strains are widely used by the mead and wine industries to produce variety of fermented alcoholic beverages. Selection of appropriate yeast strains for the fermentation of honey to produce high quality honey mead is an important criterion.

With no added nutritional supplement, the amount of ethanol formed after 24 hours was higher for the 2 g inoculation compared to 1 g inoculation for both microorganisms (Table 4). The fermentation with 1 g inoculation of both microorganisms appeared to plateau at 8 – 9% w/w of ethanol whereas the fermentation with 2 g inoculation of EC1118 continued to rise in ethanol concentration throughout the fermentation to 10.5 % w/w at 96h (Fig 6). With 2 g inoculation of 71B, the amount of ethanol gradually increased till 72 h and then remained constant between 72 to 96 h (Fig 7).

Without any nutritional additive, the of amount ethanol produced by 1g inoculation of EC1118 ranged from 4.29 to 8.82 %, whereas with 2g inoculation the amount of ethanol produced ranged from 7.12 to 10.50 %. The yeast cells of EC1118 appeared to be quickly adapting to the fermenting environment as the initial amount of ethanol produced after 24 h -was 4.29 and 7.12

for 1g and 2g respectively. The targeted amount of ethanol (aim of the experiment 8-12%) was produced at 48h by 1g inoculation of EC1118 and at 72h by 2g inoculation of EC1118.

Table 4. Effect of type of yeast and no nutritional supplement on the ethanol produced.

Yeast type	Mass of yeast (g)	Nutritional supplement	Ethanol % 0 h	Ethanol % 24 h	Ethanol % 48h	Ethanol % 72h	Ethanol % 96h
EC1118 (Control)	1	NIL	0	4.29(0.3)^{b}	8.43(1.21)^{a}	8.82(0.77)^{ab}	8.39(0.5)^{c}
EC1118	2	NIL	0	7.12(0.66)^{a}	7.34(0.31)^{a}	9.42(0.27)^{a}	10.50(0.33)^{a}
71B	1	NIL	0	3.89(0.05)^{b}	8.3(0.34)^{a}	9.45(0.54)^{a}	9.23(0.22)^{b}
71B	2	NIL	0	7.63(0.06)^{a}	7.47(0.39)^{a}	8.20(0.32)^{b}	8.11(0.47)^{c}

Mean (SD) in columns with different superscript are significantly different ($p<0.05$)

With no additional nutrition, the maximum percentage of ethanol produced by 1g inoculation of 71B was 9.45 % at 72h. The maximum percentage of ethanol produced by 2g inoculation of 71B was 8.2 % at 72h. The desired amount of ethanol (aim of the experiment 8-12%) was produced at 48h by 1g inoculation of 71B and at 72h by 2g inoculation of 71B. The yeast cells of 71B also showed a quick adaptation to the fermenting environment. The amount of ethanol produced by 2g inoculation of 71B was initially higher than 1g inoculation, but after 72h the amount of ethanol produced by 1g inoculation was higher than 2g inoculation of 71B.

The amount of ethanol produced by EC1118 and 71B yeast strains did not show any dependence on the mass of inoculation at 48h fermentation. The amount of ethanol concentration of 2g of EC1118 did not plateau like 1g of EC1118 and continued to increase from 24 to 96h (Fig 6). Th amount of ethanol production by 1g and 2g inoculation of 71B showed a decline at 96h

compared to ethanol produced at 72h (Fig 7). The plateauing of the ethanol concentration could be due to depletion of the nutritional energy sources and decrease in the population of the viable cells. The accumulated energy resources get depleted more quickly because of the increased density of the yeast cell population in the fermentation media. This can thus reduce the fermentation time (Carrau et al., 2010).

There is a direct relationship between the quantity of biomass of the yeast cells present in the fermentation medium and the amount of ethanol produced (Verbelen et al., 2009b). Rao et al. (2004) reported with increased inoculation of the yeast, the fermentation time was decreased. In our study, the rate of fermentation process also appeared to be proportional to the mass of the yeast because at initial 24h, the amount of ethanol produced by 2g inoculation of both the microorganisms is twice the amount of ethanol produced by 1g inoculation. A previous study concluded that yeast strains ICV D47 and QA47 are able to produce 10-11% w/w of ethanol after 96 h of fermentation of honey with water and reduced the fermentation time by 34% with the inoculation size of 10^6 CFU/ml (Pereira, Mendes-Ferreira, Oliveira, Estevinho, & Mendes-Faia, 2014). Both the strains EC1118 (2g) and 71B(1g) used in this study fermented the honey without any nutritional supplements and produced 10.5% (96h) and 9.45% (72h) respectively. Various strains of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* showed differences in their fermentation behaviour in terms of ethanol production at different fermentation times (Roldán et al., 2011).

With the increase in the inoculum size, the amount of ethanol produced was nearly doubled at the initial 24h. But later the amount of ethanol produced did not show any dependence with increased inoculum size. Verbelen et al. (2009b) reported, as the fermentation progresses the young cell population gets decreased. They also reported that the rate of fermentation is yeast strain specific and also depends on the oxygen requirements of the yeast strain.

The decreased amount of ethanol production with increased inoculation was due to the increased yeast cell to cell contact. This inhibited the amount of the ethanol production (Nissen, Nielsen, & Arneborg, 2003).

The results from this experiment showed microorganisms can ferment honey and produce, ethanol without nitrogen-based additives. Similar results were also observed by Mendes-Ferreira et al. (2010). These authors found in the absence of the nitrogen supplement; the ethanol production occurs at slower rate of fermentation compared to the ethanol production with nitrogen supplement.

4.3. Effect of type of yeast on the total acidity

There was no significant differences on the amount and type of yeast on the total acidity at 24, 48 and72h of fermentation. At 96h of fermentation, the total acidity produced by EC1118 (1g) and 71B(2g) samples showed lower values than the other samples (Table 5).

The total acidity is an important factor as it regulates the quality parameters of the final mead. As the fermentation progressed, the total acidity was increased. The increase in the acidity was due to the formation of organic acids such as acetic acid and succinic acid. But the increase in the total acidity is contributed by mainly by succinic acid (Sroka & Tuszyński, 2007). Succinic acid contributes to the salty and bitter flavour. Acetic acid produced during fermentation contribute to sour and vinegar like smell and taste (Benito, Ortiz, Sánchez, Sarabia, & Iñiguez, 1999). The amount of the sugars present in the honey affects the total acidity. Fermentation with high sugar concentrations, results in increased level of acetic acid (Sroka & Tuszyński, 2007). The increased acetic acid has inhibitory effect on the fermentation due to the changes in the internal pH of the yeast cell membrane.

Table 5. Effect of type of yeast on the total acidity (g/L).

Yeast type	Mass of yeast (g)	Nutritional supplement	Total acidity (g/L) 0 h	Total acidity (g/L) 24 h	Total acidity (g/L) 48 h	Total acidity (g/L)72 h	Total acidity (g/L) 96 h
EC1118 (control)	1	NIL	3.21(0.11)[a]	3.67(0.18)[a]	4.44(0.49)[a]	4.75(0.06)[a]	4.27(0.42)[ab]
EC1118	2	NIL	3.34(0.11)[a]	3.94(0.21)[a]	4.37(0.6)[a]	4.19(0.6)[a]	4.51(0.15)[a]
71B	1	NIL	3.53(0.74)[a]	4.39((0.55)[a]	4.40(0.4)[a]	4.15(0.43)[a]	4.74(0.11)[a]
71B	2	NIL	3.61(0.09)[a]	4.11(0.67)[a]	4.14(0.66)[a]	4.69(0.13)[a]	3.87(0.27)[b]

Mean (SD) in columns with different superscript are significantly different ($p<0.05$)

4.4. Effect of type of yeast on the pH

The trend of the pH increased during the ethanol production without any nutritional supplementation throughout 96h fermentation time. Similar trends were observed for both 1g and 2g inoculation of EC1118 and 71B (Table 6).

During the fermentation of alcoholic beverages, the pH trend to decrease and total acidity increases. Significant changes were observed with 1g inoculation of 71B at 24, 48 and 72h of fermentation time. The yeast strain EC1118 did not show any significant changes with the mass of inoculation. The pH of commercial honey mead is around 4.0. The pH of honey mead produced in this study also produced similar pH. The total acidity effects the aroma and flavour of the final mead.

Table 6. Effect of type of yeast on the pH.

Yeast type	Mass of yeast (g)	Nutritional supplement	pH 0h	pH 24h	pH 48h	pH 72h	pH 96h
EC1118 (control)	1	NIL	3.75(0.01)^{a}	4.14(0.02)^{a}	4.28(0.07)^{a}	4.30(0.02)^{b}	4.70(0.12)^{a}
EC1118	2	NIL	3.76(0.03)^{a}	4.18(0.02)^{a}	4.29(0.02)^{a}	4.57(0.1)^{a}	4.77(0.03)^{a}
71B	1	NIL	3.68(0.03)^{a}	4.07(0.03)^{b}	4.15(0.02)^{b}	4.27(0.07)^{b}	4.75(0.04)^{a}
71B	2	NIL	3.74(0.02)^{a}	4.19(0.04)^{a}	4.38(0.08)^{a}	4.53(0.03)^{a}	4.75(0.02)^{a}

Mean (SD) in columns with different superscript are significantly different ($p<0.05$)

4.5. Effect of type of yeast on the °Brix during fermentation

With both the yeast strains EC1118 and 71B, there were slight variations in the °Brix value (Table 7). The °Brix value showed no significant differences with type and mass of inoculation or without nutritional supplementation at 48 and 72h of fermentation times.

Table 7. Effect of type of yeast on the °Brix during fermentation.

Yeast type	Mass of yeast (g)	Nutritional supplement	°Brix 0h	°Brix 24h	°Brix 48h	°Brix 72h	°Brix 96h
EC1118 (control)	1	NIL	23.43(0.38)^{a}	10.45(0.45)^{b}	10.49(0.13)^{a}	10.69(0.16)^{a}	10.5(0.16)^{bc}
EC1118	2	NIL	23.48(0.21)^{a}	10.34(0.08)^{b}	10.61(0.28)^{a}	10.75(0.17)^{a}	10.73(0.2)^{ab}
71B	1	NIL	23.43(0.23)^{a}	10.71(0.21)^{a}	10.48(0.28)^{a}	10.6(0.25)^{a}	10.4(0.12)^{c}
71B	2	NIL	23.54(0.19)^{a}	10.77(0.03)^{a}	10.68(0.12)^{a}	10.78(0.09)^{a}	10.87(0.04)^{a}

Mean (SD) in columns with different superscript are significantly different ($p<0.05$)

The °Brix value indicates the total soluble materials. The °Brix value reduced from approximately 23 to 10 within 24 h and then did not show any variation with the yeast type and mass of inoculations. The rate of utilisation of the total soluble materials decrease the °Brix formation and increases the fermentation efficiency over the period of days to years (Kocher, Phutela, & Gill, 2011). But in this study, the solubles are consumed the yeast at a rapid rate and the concentration of the sugars (total solubles present in honey are mainly sugars) decreased to more than 50% after initial 24h.

4.6. Effect of type of yeast on the ethanol production with DAP

Saccharomyces is the globally used microorganism for its potential capability to ferment sugars into ethanol. Fermenting yeast cells require nutrition. The yeast depends upon the nitrogen

source to undergo fermentation. Hence, the growth of the yeast is dependent upon the available nitrogen in the fermenting medium.

In this study, yeast strains EC1118 and 71B are used to evaluate the amount of ethanol with DAP. Significant changes were observed with the type and mass of the yeast used with DAP supplementation (Table 8). Fermentation of the honey with DAP as nutritional supplement resulted in the increase in the amount of ethanol production by the *Saccharomyces* yeast strains.

The percentage of ethanol produced by 1g inoculation of EC1118 with DAP as nutritional supplement was ranged from 7.59 to 8.91 %, whereas the percentage of ethanol produced by 2g inoculation of EC1118 with DAP was ranged from 8.56 to 10.11 %. The ethanol production followed an increasing trend pattern from start of fermentation to upto 72h (Fig 6). The ethanol production at 96h was less than the ethanol produced at 24h by both 1g and 2g inoculation of EC1118. The ethanol produced with both 1g and 2g inoculation of EC1118 followed a similar trend as the control. The ethanol produced by 1g inoculation of EC1118 with DAP showed a plateau approximately around 8% of ethanol concentration between 48 to 72h. This was similar to the control, which also showed plateauing around 8% of ethanol concentration after 48 h. In case of 2g inoculation of EC1118, plateauing took place around 10% of ethanol concentration between 48 and 72 h. At 96h of fermentation time, the amount of ethanol produced by both 1g and 2g inoculation of EC1118 showed a decline. This may be an indication that the growth of yeast cells, number of living cells in the fermentation medium and the amount of ethanol produced, all three factors are interlinked with each other. Similar reported was also observed by Bazua & Wilke (1977). These authors observed that Saccharomyces yeast strain ATCC No.4126 showed a correlation between growth (mass) and the amount of ethanol produced. The desired 8-12% amount of ethanol was produced by 1g and 2g inoculation of EC1118 within 48h and 24h respectively.

Table 8. Effect of type of yeast with DAP as nutritional supplement on the ethanol production.

Yeast type	Mass of yeast (g)	Nutritional supplement	Ethanol % 24h	Ethanol % 24h	Ethanol % 48h	Ethanol % 72h	Ethanol % 96h
EC1118 (Control)	1	NIL	0	4.29(0.3)^{d}	8.43(1.21)^{c}	8.82(0.77)^{c}	8.39(0.5)^{b}
EC1118	1	DAP	0	7.82(0.34)^{b}	8.84(0.21)^{bc}	8.91(0.83)^{c}	7.59(0.26)^{b}
EC1118	2	DAP	0	8.56(0.35)^{ab}	10.03(0.61)^{ab}	10.11(0.44)^{b}	8.51(0.47)^{b}
71B	1	DAP	0	6.45(0.75)^{c}	8.65(0.48)^{bc}	8.59(0.28)^{c}	9.18(0.28)^{b}
71B	2	DAP	0	8.95(0.75)^{a}	10.86(0.92)^{a}	12.47(0.32)^{a}	12.79(0.52)^{a}

Mean (SD) in columns with different superscript are significantly different ($p<0.05$)

On addition of DAP to the fermentation medium, the yeast cells start to utilise the nutrients required for their growth and survival. The initial available nitrogen sources are increased at the start of fermentation on addition of DAP. Due to availability of the adequate nutrient sources to the yeast, the newly dividing yeast cells utilised the available nitrogen in the fermenting medium and produced more amounts of ethanol. This could be the reason for such high ethanol levels at initial 24h. The decrease in the ethanol production by the yeast strain EC1118 at 96h may be due to the following reason. The first one being inhibition of the yeast growth by ethanol itself and second reason is due to decrease in the yeast cell biomass (Brown, Oliver, Harrison, & Righelato, 1981; Viegas, Rosa, Sá-Correia, & Novais, 1989). The viable cell population was decreased as yeast cells died because of the toxic metabolite accumulation.

With 1g and 2g of 71B, the amount of ethanol produced at 24h with DAP supplementation was 6.45 and 8.95 % respectively. The maximum amount of ethanol produced by 1g inoculation of 71B was 9.18%, whereas with 2g inoculation, the maximum amount of ethanol produced was 12.79 %. The amount of ethanol produced by 71B followed an increasing trend (Fig 7). The yeast strain 71B 1g showed a plateau between 48h to 72h and at 96h increased again. Whereas with 2g inoculation of 71B, the plateauing took place at 72h and there appeared no increase at 96h. Both 1g and 2g inoculation of 71B did not follow the trend of the control. The desired amount of ethanol (aim of the experiment) was produced by 1g inoculation of 71B at 48h (8.65%) and by 2g inoculation at 24h (8.95%).

The availability of the required growth nutrients to yeast cells at start of fermentation, improved the ethanol production and reduced the fermentation time. The nitrogen supplements are selectively taken up by the fermenting yeast cells during the early growth phase (Henschke, 1993). 2g of 71B was able to produce the amount of ethanol more than 2g of EC1118. The differences in the concentration of the ethanol produced is because of the distinctive biological character of the individual yeast strains (Carrau et al., 2010). It can be concluded that 71B yeast strain is ethanol resistant as ethanol production was greater than 10%. From the literature, it was found dark coloured honey produced the highest ethanol concentration around 9% (Pereira et al., 2015c). This occurred after 48h with the addition of supplements such as DAP. Similar results were also documented by Ilha, Sant Anna, Torres, Porto, & Meinert (2000). They reported dark coloured honey with nitrogen-based supplements produced ethanol at faster fermentation times. Similar results were also observed in this study.

With DAP as nutritional supplementation, the amount of ethanol production increased and fermentation duration decreased (Mar Vilanova et al., 2012). Yeast cells divided rapidly during the initial stages of the fermentation and produce more ethanol (O'Connor-Cox, Paik, & Ingledew, 1991). They also reported that with available initial nitrogen, the yeast cells increased

in magnitude and ethanol production followed a linear curve. The yeast requires 140mg N/L of nitrogen. Below this threshold of nitrogen, the rate of fermentation decreases.

4.7. Effect of type of yeast on the total acidity with DAP

The total acidity increased as the fermentation proceeded over the time period. There was an initial significant rise in the first 24 hours to approximately from < 3 to < 4 g/L in the total acidity. Slight fluctuations in this trend were observed over the following days. There were no significant changes between the 1g and 2g inoculations of EC1118 and 71B with DAP at 24, 48, 72 and 96h of fermentation time. But at 96h of fermentation time, the total acidity significantly decreased with 2g inoculation of 71B only. The addition of nutritional supplementation DAP appeared to yield a slightly higher total acidity at 0h compared to the fermentation with no nutritional supplement. The effect on total acidity (g/L) over the time period for 1 g and 2g inoculation of the strains EC1118 and 71B are shown in the Table 9.

Initially the total acidity increased due to secreted organic acids by the fermenting yeast cells (Sroka & Tuszyński, 2007). The yeast cells produced acids such as acetic acid and succinic acids during the initial stages of the fermentation. Hence there is a raise in the total acidity produced after initial 24 h. The total acidity did not show any dependence on the type and mass of inoculation in comparison with the control. The quantity of the acids synthesised during the fermentation depends upon the concentration of the sugars present in the honey and also on the nutrient sources available to the yeast (Fleet, 1993). When the concentration of the sugars in the fermentation was more than 80 % (sugars present in the honey), yeast cells undergo osmotic stress reactions. This can led to increase in the acidity and decrease the ethanol production (Erasmus, van der Merwe, & van Vuuren, 2003). The increase in the acidity is strain specific and depends on the yeast nutritional requirements.

Table 9. Effect of type of yeast on total acidity with DAP.

Yeast type	Mass of yeast (g)	Nutritional supplement	Total acidity (g/L) 0h	Total acidity (g/L) 24h	Total acidity (g/L) 48h	Total acidity (g/L)72h	Total acidity (g/L) 96h
EC1118 (control)	1	NIL	3.21(0.11)^{b}	3.67(0.18)^{a}	4.44(0.49)^{a}	4.75(0.06)^{a}	4.27(0.42)^{ab}
EC1118	1	DAP	3.77(0.08)^{a}	4.06(0.55)^{a}	4.66(0.24)^{a}	4.46(0.78)^{a}	4.53(0.14)^{a}
EC1118	2	DAP	3.84(0.12)^{a}	4.37(0.56)^{a}	4.21(0.77)^{a}	4.58(0.08)^{a}	4.80(0.48)^{a}
71B	1	DAP	3.86(0.87)^{a}	4.09(0.85)^{a}	4.0(0.61)^{a}	3.83(0.34)^{a}	4.63(0.21)^{a}
71B	2	DAP	3.86(0.81)^{a}	4.47(0.36)^{a}	4.37(0.62)^{a}	4.36(0.95)^{a}	3.72(0.03)^{b}

Mean (SD) in columns with different superscript are significantly different ($p<0.05$)

4.8. Effect of type of yeast on the pH with DAP

The pH gradually increased during fermentation of the honey. The pH values appeared to vary, showing no dependence on the mass and type of yeast inoculation or on type of added nutrition. No significant changes were observed in the pH formed at 24 and 48h of fermentation time (Table 10).

Table 10. Effect of type of yeast on pH with DAP.

Yeast type	Mass of yeast (g)	Nutritional supplement	pH 0h	pH 24h	pH 48h	pH 72h	pH 96h
EC1118 (control)	1	NIL	3.75(0.01)^{a}	4.14(0.02)^{a}	4.28(0.07)^{a}	4.3(0.02)^{b}	4.7(0.12)^{b}
EC1118	1	DAP	3.76(0.05)^{a}	4.15(0.03)^{a}	4.31(0.05)^{a}	4.51(0.02)^{a}	4.70(0.02)^{b}
EC1118	2	DAP	3.77(0.01)^{a}	4.17(0.06)^{a}	4.29(0.04)^{a}	4.58(0.03)^{a}	4.83(0.08)^{ab}
71B	1	DAP	3.87(0.14)^{a}	4.18(0.03)^{a}	4.23(0.03)^{a}	4.37(0.02)^{b}	4.88(0.08)^{a}
71B	2	DAP	3.91(0.11)^{a}	4.16(0.07)^{a}	4.25(0.02)^{a}	4.59(0.05)^{a}	4.83(0.03)^{ab}

Mean (SD) in columns with different superscript are significantly different ($p<0.05$)

With addition of DAP, the potassium concentration is increased. Boulton (1980) suggested that increase in the Ph in his experiments was due to the precipitation of potassium during fermentation. In our experiments, the increase in the Ph was observed both in the presence and in the absence of DAP. This clearly explains that increase in the pH was not solely due to the exchange of the hydrogen ions with potassium. The pH of the honey mead trend to increase due to the more weaker acids synthesis taking place during fermentation.

4.9. Effect of type of yeast on the °Brix with DAP

With both the yeast strains EC1118 and 71B, there were slight changes in the °Brix value only after the initial decrease at 24h (Table 11). The °Brix value showed no dependence with type and mass of yeast inoculation or with nutritional supplementation.

Table 11. Effect of type of yeast on °Brix with DAP.

Yeast type	Mass of yeast (g)	Nutritional supplement	°Brix 0h	°Brix 24h	°Brix 48h	° Brix 72h	°Brix 96h
EC1118 (control)	1	NIL	23.43(0.38)^{a}	10.45(0.45)^{b}	10.49(0.13)^{b}	10.69(0.16)^{ab}	10.50(0.16)^{b}
EC1118	1	DAP	23.41(0.24)^{a}	10.69(0.13)^{a}	10.72(0.15)^{a}	10.79(0.09)^{a}	10.70(0.20)^{ab}
EC1118	2	DAP	23.47(0.22)^{a}	10.77(0.07)^{a}	10.74(0.14)^{a}	10.63(0.08)^{b}	10.80(0.04)^{a}
71B	1	DAP	23.39(0.75)^{a}	10.71(0.13)^{a}	10.64(0.14)^{a}	10.73(0.18)^{ab}	10.82(0.1)^{a}
71B	2	DAP	23.45(0.39)^{a}	10.66(0.17)^{ab}	10.78(0.09)^{a}	10.85(0.09)^{a}	10.82(0.1)^{a}

Mean (SD) in columns with different superscript are significantly different ($p<0.05$)

After initial 24h of fermentation the total soluble materials reduced to more than 50%. The °Brix formed with addition of DAP was similar to the °Brix formed with no supplementation. No changes in the °Brix value was reported by Mendes-Ferreira et al. (2010) with DAP and without any supplementation with UCD522 yeast strain. The °Brix formed remained constant at 22.2 in their experiment. Such findings was not observed in this study.

4.10. Effect of type of yeast with added lupin on the ethanol production

In this experiment, lupin was used as source of nitrogen to the fermentation. Lupin belongs to genus Lupinus. The quantity of protein and potassium present in 100g of lupin are 36g and

1.01g respectively. The ethanol produced with lupin by 1g and 2g inoculation of EC1118 and 71B are shown in Table 12.

Table 12. Effect of type of yeast with lupin on the ethanol production.

Yeast type	Mass of yeast (g)	Nutritional supplement	Ethanol % 0 h	Ethanol % 24h	Ethanol % 48h	Ethanol % 72h	Ethanol % 96h
EC1118 (Control)	1	NIL	0	4.29(0.3)^{d}	8.43(1.21)^{ab}	8.82(0.77)^{a}	8.39(0.5)^{ab}
EC1118	1	LUPIN	0	4.77(0.17)^{d}	7.45(0.42)^{bc}	7.33(0.23)^{bc}	7.91(0.24)^{b}
EC1118	2	LUPIN	0	6.62(0.86)^{b}	7.26(0.17)^{bc}	8.43(0.77)^{ab}	7.4(0.38)^{b}
71B	1	LUPIN	0	9.67(0.23)^{a}	9.15(0.27)^{a}	7.17(0.16)^{c}	8.7(0.41)^{a}
71B	2	LUPIN	0	5.70(0.33)^{c}	6.38(0.55)^{c}	7.88(0.80)^{abc}	7.65(0.47)^{bc}

Mean (SD) in columns with different superscript are significantly different ($p<0.05$)

The amount of ethanol produced by 1g and 2g of EC1118 at 24h with lupin as nutritional supplement was 4.77 and 6.62 % respectively. The amount of ethanol production with 1g inoculation of EC1118 followed an increasing trend on the following days, whereas 2g inoculation showed a decline in the amount of ethanol produced at 96h (Fig 6). The amount of ethanol production with 2g inoculation of EC1118 increased up to 72h and later showed a decline at 96h of fermentation time.

The amount of ethanol produced by 1g and 2g of 71B at 24h with lupin as nutritional supplement was 9.67 and 5.70 % respectively. 71B yeast strain showed rapid adaptation to the new fermenting environment more quickly than EC1118. The ethanol produced with 71B was much higher than EC1118. There were significant changes observed with 1g and 2g inoculation

of 71B. 1g and 2g inoculation of 71B did not follow similar trends (Fig 7). The biphasic pattern of the amount of ethanol production is caused due the higher alcohols production on addition of nitrogen based supplementation (Carrau et al., 2008). This biphasic pattern was shown by both 2g inoculation of EC1118 and 71B.

The addition of lupin, in general, had a negative effect on the production of ethanol, yielding amounts of ethanol lower than the experiments with no additional nutritional supplement. This was consistent with the nitrogen of the protein in lupin being unassimilable to the yeast cells. The fermenting yeast cells can utilise the freely available nitrogen present in the fermentation medium to enhance the fermentation kinetics and amount of ethanol production. Lupin used in this study is good source of protein. However, proteins are not free amino acids and must be broken down to be available to the yeast cells as nutrition. Saccharomyces yeast strains selectively uptake the free nitrogen available in the fermentation media. The free assimilable nitrogen sources such as ammonia are preferred over the other forms of nitrogen like amino acids proline, threonine and protein rich sources (Marks, van der Merwe, & van Vuuren, 2003). The transport carriers present in the yeast cell membrane suppresses the uptake of the other forms of nitrogen sources. This phenomenon is called as Nitrogen catabolite repression (NCR) (Cooper, 1982).

This was the opposite effect observed for DAP as the nutritional supplement. The nitrogen in the ammonium ions of DAP is readily assimilable to the yeast cells, hence the addition of DAP was observed to increase the ethanol production.

However, these results suggested that lupin had a further effect as ethanol production decreased compared to the absence of lupin. The lupin may be inactivating the sugar transporter enzymes present in the yeast cells as reported previously (Lucero et al., 2002). Another suggestion was that lupin was poisoning the yeast. This need further research studies to be undertaken. Our

results contradicted the results documented with 20g soya flour on the fermentation of wheat mashes (Bafrncová, Sláviková, Pátková, & Dömény, 1999). Soya flour has a similar protein content to lupin. The authors reported an increase in the rate of ethanol production, an increase in the overall amount ethanol formed, and a decrease in the biomass of the fermenting yeast. The decrease in the biomass may not have been taken place in our experiments, as the ethanol production continued to increase up to 96h. This prediction was based on that mass of the fermenting yeast cells and ethanol produced are dependent upon each other (Casey, Magnus, & Ingledew, 1983). The decrease in the ethanol production by 2g inoculation of EC1118 might be due to the toxicity generated with the high gravity sugars and changes in the fermenting medium (Torija, Rozès, Poblet, Guillamón, & Mas, 2003b).

4.11. Effect of type of yeast with lupin on the total acidity

With 1g and 2g inoculation of EC1118 and 71B, significant changes were observed at 24, 48 and 72h of fermentation time. But no significant changes were observed at 96h of fermentation time (Table 13).

Table 13. Effect of type of yeast with lupin on total acidity (g/L) during fermentation.

Yeast type	Mass of yeast (g)	Nutritional supplement	Total acidity (g/L) 0 h	Total acidity (g/L) 24h	Total acidity (g/L) 48h	Total acidity (g/L) 72h	Total acidity (g/L) 96h
EC1118 (control)	1	NIL	3.21(0.11)^{b}	3.67(0.18)^{b}	4.44(0.49)^{a}	4.75(0.06)^{a}	4.27(0.42)^{a}
EC1118	1	LUPIN	3.34(.08)^{a}	4.24(0.65)^{ab}	3.65(0.19)^{b}	4.36(0.53)^{ab}	4.53(0.14)^{a}
EC1118	2	LUPIN	3.24(0.34)^{ab}	4.07(0.05)^{ab}	4.13(0.4)^{ab}	3.89(0.54)^{b}	4.54(0.14)^{a}
71B	1	LUPIN	3.11(0.18)^{b}	4.2(0.38)^{ab}	4.08(0.52)^{ab}	4.41(0.17)^{ab}	4.63(0.21)^{a}
71B	2	LUPIN	3.37(0.17)^{a}	4.92(0.75)^{a}	4.49(0.23)^{a}	4.31(0.38)^{ab}	4.07(0.62)^{a}

Mean (SD) in columns with different superscript are significantly different ($p<0.05$)

The total acidity raised to greater than 4g/L after the initial 24h and then showed slight fluctuations. The total acidity formed did not any dependence on the type and mass of the inoculation of the yeast EC1118 and 71B with the added lupin.

4.12. Effect of type of yeast with lupin on the pH

With 1g and 2g inoculation of EC1118 and 71B, significant changes were noticed in the pH at 72 and 96h of fermentation time. The type and mass of inoculation of EC1118 and 71B has no significant changes on the pH up to 48h. Throughout the four days, the pH formed followed an increasing trend. Such increasing trend were also observed with no supplementation and with DAP as supplementation. The pH formed by EC1118 and 71B are shown (Table 14).

Table 14. Effect of type of yeast with lupin on pH.

Yeast type	Mass of yeast (g)	Nutritional supplement	pH 0h	pH 24h	pH 48h	pH 72h	pH 96h
EC1118 (control)	1	NIL	3.75(0.01)^{a}	4.14(0.02)^{b}	4.28(0.07)^{a}	4.3(0.02)^{d}	4.7(0.12)^{b}
EC1118	1	LUPIN	3.64(0.24)^{a}	4.14(0.04)^{ab}	4.27(0.04)^{a}	4.57(0.02)^{ab}	4.88(0.08)^{a}
EC1118	2	LUPIN	3.57(0.11)^{a}	4.16(0.05)^{ab}	4.31(0.07)^{a}	4.59(0.05)^{a}	4.83(0.03)^{ab}
71B	1	LUPIN	3.68(0.71)^{a}	4.18(0.09)^{ab}	4.37(0.06)^{a}	4.46(0.05)^{c}	4.7(0.05)^{b}
71B	2	LUPIN	3.75(0.39)^{a}	423(0.04)^{a}	4.31(0.04)^{a}	4.52(0.02)^{bc}	4.81(0.05)^{ab}

Mean (SD) in columns with different superscript are significantly different ($p<0.05$)

4.13. Effect of type of yeast with lupin on the °Brix

The °Brix formed with 1g and 2g inoculation of EC1118 and 71B showed no significant differences after 24h to 96h time period (Table 15).

Table 15. Effect of type of yeast with lupin on °Brix.

Yeast type	Mass of yeast (g)	Nutritional supplement	°Brix 0h	°Brix 24h	°Brix 48h	°Brix 72h	°Brix 96
EC1118 (control)	1	NIL	23.43(0.38)^{a}	10.45(0.45)^{b}	10.49(0.13)^{b}	10.69(0.16)^{b}	10.50(0.16)^{b}
EC1118	1	LUPIN	23.37(0.19)^{a}	10.72(0.12)^{a}	10.62(0.06)^{b}	10.72(0.1)^{a}	10.70(0.2)^{a}
EC1118	2	LUPIN	23.34(0.31)^{a}	10.74(0.08)^{a}	10.79(0.03)^{ab}	10.78(0.05)^{a}	10.79(0.04)^{a}
71B	1	LUPIN	23.41(0.28)^{a}	10.69(0.12)^{a}	10.72(0.2)^{ab}	10.73(0.18)^{a}	10.72(0.14)^{a}
71B	2	LUPIN	23.39(0.33)^{a}	10.80(0.10)^{a}	10.87(0.04)^{a}	10.85(0.09)^{a}	10.7(0.09)^{a}

Mean (SD) in columns with different superscript are significantly different ($p<0.05$)

The amount of total soluble materials reduced by more than 50%. The reduction in the °Brix value was quite similar to that of the control. The type of yeast and mass of inoculation with added lupin did not show any effect on the observed °Brix value.

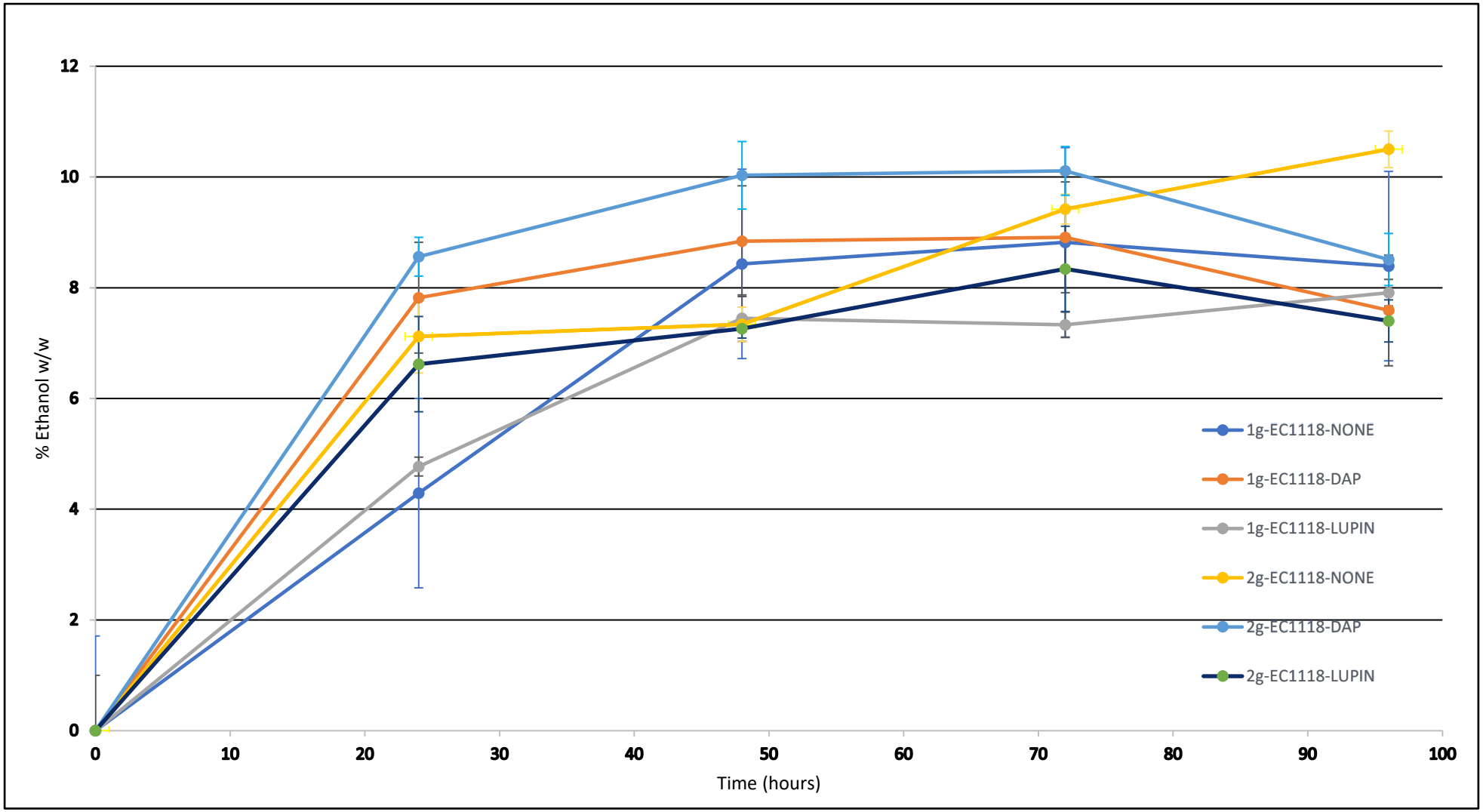

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Figure 6. The ethanol concentration in fermentation solution with progress on time using yeast strain EC1118.

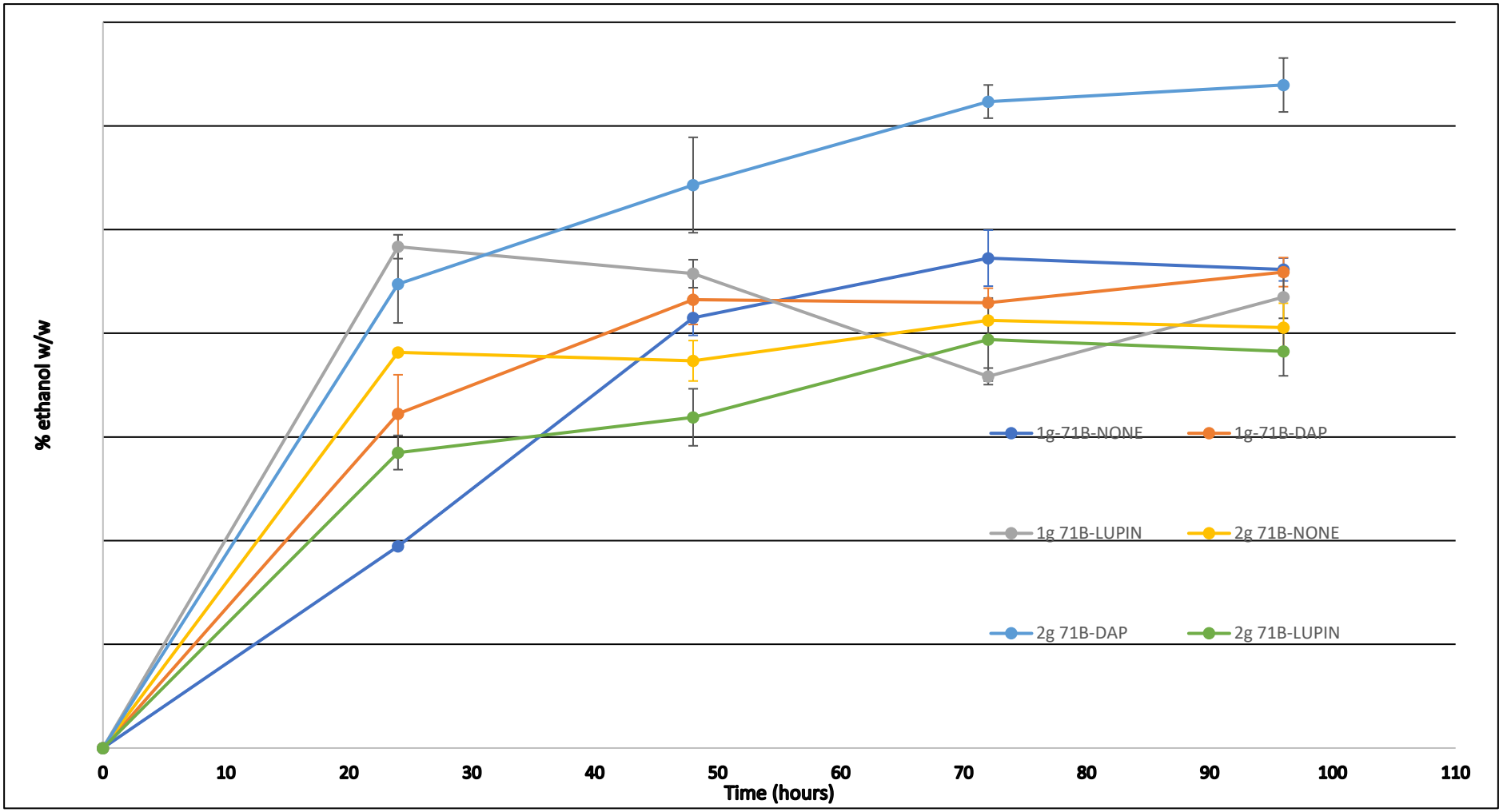

Figure 7. The ethanol concentration in fermentation solution with progress on time using yeast strain 71B

4.14 Analysis of Aroma compounds in Honey Mead

Headspace analysis was performed on the Honey Mead samples that were fermented for 96 hours (4 days). The aroma compounds in the headspace were adsorbed onto a DVB/CAR/PDMS SPME fibre and identified and measured by GC-MS. The relative concentration of these compounds are shown in the Table 16.

The aroma compounds were alcohols, esters, acetal derivatives of acetaldehyde and acetic acid. These are compounds previously reported in the aroma of honey mead (Carrau et al., 2008; Mendes-Ferreira, Barbosa, Falco, Leão, & Mendes-Faia, 2009; Šmogrovičová, Nádaský, Tandlich, Wilhelmi, & Cambray, 2012). Iso-propanol was also observed on all the samples but co-eluted with ethanol and so could not be quantified. Many other compounds were observed however their intensity was too low for accurate identification. There is little variation in the relative concentration of the aroma compounds between honey mead formed from the yeasts, EC1118 and 71B. Similarly, there is no significant alternation from the different inoculations or the absence of type of added nutrition. The absolute concentration was determined for a selected number of compounds. These concentrations are shown in the Table 17 (mg/L).

Table 16. Relative amounts of identified aroma components after day 4 on fermentation. Areas were normalised to the area of each component recorded for fermentation using EC1118 with 2 g inoculation with added lupin.

			acetic acid	methanol	1-propanol	iso-butanol	iso-amyl alcohol	2-methyl-1-butanol	phenyl ethyl alcohol	ethyl acetate	iso-amyl acetate	2-methyl-1-butyl acetate	ethyl propanoate	ethyl butanoate	ethyl pentanoate	ethyl hexanoate	ethyl octanoate	ethyl decanoate	acetal	ethyl amyl acetal
EC1118	1g	NN	0.5	1.4	0.8	1.1	1.3	1.3	1.1	1.2	2.1	1.2	0.7	1.7	0.7	1.6	1.3	1.6	1.7	3.4
		DAP	0.7	2.2	1.5	3.4	1.9	1.8	3.0	3.8	3.1	2.3	2.6	3.8	2.6	1.7	2.0	1.7	2.8	3.1
		Lupin	0.9	1.1	0.8	1.2	0.9	1.1	0.9	1.1	1.0	1.1	0.9	0.9	1.2	0.8	0.9	1.2	1.0	0.8
	2g	NN	0.6	1.4	0.7	1.3	1.4	1.2	0.9	1.3	1.9	1.3	0.8	1.5	0.8	1.3	1.6	1.5	1.8	3.4
		DAP	0.9	2.3	1.8	3.9	1.7	1.7	2.9	3.3	2.9	2.1	2.8	4.0	2.6	2.1	1.7	1.9	2.3	3.3
		Lupin	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
71B	1g	NN	0.9	1.3	a	1.8	1.5	1.3	0.9	1.0	0.9	0.9	1.2	1.1	0.8	1.4	2.8	1.8	2.4	4.0
		DAP	0.9	2.0	a	2.7	1.2	1.5	2.3	3.9	1.1	1.5	1.2	1.2	1.1	1.0	1.2	1.5	1.8	3.2
		Lupin	1.1	1.6	a	0.4	1.2	1.1	2.1	0.7	0.9	1.0	1.1	1.0	1.0	0.5	1.1	1.1	2.3	1.4
	2g	NN	0.8	1.5	a	1.7	1.5	1.3	0.9	0.9	1.1	0.8	1.2	1.2	0.8	1.6	2.5	1.5	2.0	3.8
		DAP	0.8	2.3	a	2.8	1.1	1.5	2.4	3.3	1.3	1.3	1.1	1.1	1.0	0.9	1.4	1.6	2.2	3.6
		Lupin	1.0	1.5	a	0.4	1.1	1.1	1.8	0.8	1.0	1.2	1.1	0.9	0.9	0.5	1.1	1.3	2.0	1.5

a: not observed

Table 17. Concentrations of identified aroma components in fermentation solution after day 4. Concentration are in mg/kg (ppm).

			acetic acid	methanol	1-propanol	iso-butanol	iso-amyl alcohol	2-methyl-1-butanol	phenyl ethyl alcohol	ethyl acetate	iso-amyl acetate
EC1118	1g	NN	993	116	32	20	263	102	16	34	3.1
		DAP	1308	183	60	62	390	139	45	106	4.7
		Lupin	1641	92	33	21	189	88	14	32	1.5
	2g	NN	1147	112	30	23	292	89	14	36	2.9
		DAP	1631	192	75	69	349	131	44	92	4.3
		Lupin	1833	83	41	18	202	78	15	28	1.5
71B	1g	NN	1577	109	0	33	304	101	14	28	1.4
		DAP	1627	167	0	49	252	114	34	108	1.7
		Lupin	1966	131	0	7.6	247	83	31	19	1.4
	2g	NN	1494	128	0	31	310	97	14	25	1.6
		DAP	1525	194	0	50	215	117	37	93	2.0
		Lupin	1801	125	0	6.6	216	87	28	23	1.5

Acetic acid had the highest concentration other than ethanol in the aroma of these honey mead samples. Concentration of acetic acid ranged from 1 – 2 g/kg which is typical of honey mead samples (A. Mendes-Ferreira et al., 2010).

5. Conclusions

The yeast strains had a positive impact on the alcohol content of honey mead. The magnitude of this impact depended on the type of yeast and the inoculation amount as well as the presence or absence of nutritional supplements. The largest quantity of ethanol was yielded in the fermentation of the honey preparation supplemented with DAP which produced 12% ethanol with the yeast strain 71B after 72h of fermentation. All fermentation experiments yielded the target ethanol content range of 8 – 12% ethanol with 48 – 72 h of fermentation. This was a significant reduction in the time, generally required for the fermentation of honey must to reach this target. As expected, the inoculum size resulted in improvement of the amount of ethanol produced but only within the first 24 h. After 24 h the amount of ethanol content showed no dependence on the mass of the inoculum. This was true for both the yeast strains EC1118 and 71B and without and with added nutritional supplement. With addition of DAP as nutritional supplement, yeast strain EC1118 and 71B with 1g inoculation produced the targeted amount of ethanol at 48h, whereas with 2g inoculation of EC1118 and 71B, the desired amount of ethanol was produced at 24h. Addition of DAP reduced the fermentation time and overall yielded increased amounts of ethanol . Without any added nutrition, the maximum of 10% ethanol was produced by 2g inoculation of EC1118 at 96h and 8% ethanol was produced by 1g inoculation of EC1118 at 48h while 1g inoculation of 71B produced maximum of 9% of ethanol at 72h and with 2g inoculation of 71B, a maximum of 8% of ethanol was produced at 72h. Although high in protein (nitrogen supplement), the addition of lupin inhibited the amount of ethanol production in the honey mead. Fermentation experiments with lupin only yielded ethanol content at the low end (8%) of the target range. However, this was achieved within 48 – 72 h which was still a reduction in the fermentation time of honey must reported in the literature.

6. Suggestions for future studies

- Perform sensory analysis on honey mead samples.
- Conduct studies to evaluate the effects of various yeast and bacterial strains and in their combinations without and with nutritional supplementation.
- Study the growth patterns of the microbes throughout the fermentation process and measure the amount of residual nitrogen.
- Develop honey mead derivatives considering the growing interest of consumers in gourmet products, including vinegars, mead could be used as raw materials for various food and beverages.

7. References

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8. Appendix

APPENDIX A. Various treatment combinations

NUMBER	REPLICATE	YEAST TYPE	Fermentation TIME	DAP gm	LUPIN gm	% Ethanol	Total Acidity	pH	Brix
1	R1	EC1118	24	0	0	4.64	3.81	4.12	10.44
2	R2	EC1118	24	0	0	4.18	3.46	4.17	10.47
3	R3	EC1118	24	0	0	4.07	3.76	4.13	10.46
4	R1	EC1118	48	0	0	8.77	3.88	4.22	10.44
5	R2	EC1118	48	0	0	9.44	4.64	4.27	10.39
6	R3	EC1118	48	0	0	7.08	4.81	4.37	10.64
7	R1	EC1118	72	0	0	9.70	4.77	4.29	10.84
8	R2	EC1118	72	0	0	8.52	4.69	4.34	10.74
9	R3	EC1118	72	0	0	8.25	4.81	4.29	10.51
10	R1	EC1118	96	0	0	7.86	4.69	4.56	10.65
11	R2	EC1118	96	0	0	8.46	4.28	4.79	10.54
12	R3	EC1118	96	0	0	8.86	3.84	4.77	10.33
13	R1	71B	24	0	0	3.94	3.76	4.07	10.84
14	R2	71B	24	0	0	3.90	4.81	4.11	10.47
15	R3	71B	24	0	0	3.84	4.62	4.05	10.84
16	R1	71B	48	0	0	8.25	4.87	4.13	10.74
17	R2	71B	48	0	0	7.99	4.19	4.14	10.17
18	R3	71B	48	0	0	8.67	4.16	4.18	10.54
19	R1	71B	72	0	0	9.92	4.66	4.21	10.64
20	R2	71B	72	0	0	9.59	3.94	4.35	10.33

21	R3	71B	72	0	0	8.85	3.87	4.25	10.84
22	R1	71B	96	0	0	9.08	4.67	4.77	1029
23	R2	71B	96	0	0	9.14	4.68	4.71	10.54
24	R3	71B	96	0	0	7.49	4.87	4.79	10.38
25	R1	EC1118	24	1G	0	8.19	3.66	4.11	10.84
26	R2	EC1118	24	1G	0	7.50	3.84	4.18	10.67
27	R3	EC1118	24	1G	0	7.77	4.69	4.17	10.57
28	R1	EC1118	48	1G	0	8.90	4.81	4.29	10.84
29	R2	EC1118	48	1G	0	9.03	4.38	4.27	10.77
30	R3	EC1118	48	1G	0	8.61	4.8	4.37	10.54
32	R1	EC1118	72	1G	0	9.88	4.97	4.51	10.68
32	R2	EC1118	72	1G	0	8.39	3.56	4.49	10.84
33	R3	EC1118	72	1G	0	8.48	4.85	4.54	10.86
34	R1	EC1118	96	1G	0	9.30	3.67	4.78	10.81
35	R2	EC1118	96	1G	0	7.69	4.81	4.77	10.94
36	R3	EC1118	96	1G	0	7.80	3.69	4.81	1066
37	R1	71B	24	1G	0	5.59	3.15	4.15	10.56
38	R2	71B	24	1G	0	6.81	4.32	4.19	10.83
39	R3	71B	24	1G	0	6.95	4.82	4.21	10.75
40	R1	71B	48	1G	0	10.10	4.67	4.22	10.57
41	R2	71B	48	1G	0	8.94	3.46	4.28	10.54
42	R3	71B	48	1G	0	8.93	3.87	4.21	10.81
43	R1	71B	72	1G	0	8.90	4.16	4.37	10.94
44	R2	71B	72	1G	0	8.34	3.87	4.33	10.72
45	R3	71B	72	1G	0	8.55	3.48	4.41	10.69
46	R1	71B	96	1G	0	9.38	4.67	4.44	10.8
47	R2	71B	96	1G	0	9.32	4.19	4.49	10.74
48	R3	71B	96	1G	0	8.86	3.66	4.61	10.94
49	R1	EC1118	24	0	1G	4.60	3.49	4.17	10.59

50	R2	EC1118	24	0	1G	4.77	4.57	4.1	10.83
51	R3	EC1118	24	0	1G	4.95	4.66	4.17	10.76
52	R1	EC1118	48	0	1G	7.93	3.84	4.23	10.68
53	R2	EC1118	48	0	1G	8.33	3.67	4.29	10.55
54	R3	EC1118	48	0	1G	7.11	3.46	4.31	10.64
55	R1	EC1118	72	0	1G	7.06	3.75	4.56	10.69
56	R2	EC1118	72	0	1G	7.45	4.6	4.55	10.64
57	R3	EC1118	72	0	1G	7.50	4.73	4.6	10.84
58	R1	EC1118	96	0	1G	7.63	4.67	4.88	10.94
59	R2	EC1118	96	0	1G	8.05	4.55	4.81	10.57
60	R3	EC1118	96	0	1G	8.05	4.38	4.97	10.61
61	R1	71B	24	0	1G	9.59	4.6	4.16	10.59
62	R2	71B	24	0	1G	9.94	4.18	4.11	10.66
63	R3	71B	24	0	1G	9.49	3.84	4.29	10.84
64	R1	71B	48	0	1G	9.12	3.76	4.31	10.95
65	R2	71B	48	0	1G	7.52	3.81	4.38	10.67
66	R3	71B	48	0	1G	9.45	4.67	4.44	10.55
67	R1	71B	72	0	1G	7.06	4.61	4.41	10.94
68	R2	71B	72	0	1G	7.36	4.29	4.51	10.67
69	R3	71B	72	0	1G	8.10	4.33	4.48	10.59
70	R1	71B	96	0	1G	8.23	4.67	4.77	10.88
71	R2	71B	96	0	1G	8.99	4.81	4.67	10.67
72	R3	71B	96	0	1G	8.90	4.39	4.68	10.61
73	R1	EC1118	24	0	0	7.48	3.76	4.18	10.28
74	R2	EC1118	24	0	0	7.55	3.9	4.21	10.32
75	R3	EC1118	24	0	0	6.36	4.18	4.17	10.44
76	R1	EC1118	48	0	0	7.03	4.87	4.31	10.64
77	R2	EC1118	48	0	0	7.66	4.39	4.29	10.32
78	R3	EC1118	48	0	0	7.35	3.86	4.27	10.89

79	R1	EC1118	72	0	0	9.73	3.72	4.46	10.88
80	R2	EC1118	72	0	0	9.19	3.99	4.67	10.83
81	R3	EC1118	72	0	0	7.36	4.87	4.59	10.56
82	R1	EC1118	96	0	0	10.75	4.37	4.78	10.66
83	R2	EC1118	96	0	0	10.65	4.51	4.74	10.58
84	R3	EC1118	96	0	0	7.13	4.67	4.81	10.97
85	R1	71B	24	0	0	7.63	4.88	4.19	10.75
86	R2	71B	24	0	0	7.70	3.6	4.15	10.81
87	R3	71B	24	0	0	7.57	3.87	4.24	10.76
88	R1	71B	48	0	0	7.05	4.87	4.37	10.82
89	R2	71B	48	0	0	8.55	3.99	4.31	10.57
90	R3	71B	48	0	0	9.82	3.57	4.47	10.67
91	R1	71B	72	0	0	8.00	4.84	4.51	10.84
92	R2	71B	72	0	0	8.03	4.67	4.57	10.67
93	R3	71B	72	0	0	8.58	4.57	4.51	10.84
94	R1	71B	96	0	0	7.59	4.18	4.73	10.83
95	R2	71B	96	0	0	4.53	3.77	4.78	10.91
96	R3	71B	96	0	0	8.53	3.67	4.75	10.87
97	R1	EC1118	24	2G	0	8.61	4.86	4.11	10.7
98	R2	EC1118	24	2G	0	8.89	4.5	4.19	10.78
99	R3	EC1118	24	2G	0	8.19	3.76	4.23	10.84
100	R1	EC1118	48	2G	0	9.34	3.88	4.35	10.76
101	R2	EC1118	48	2G	0	10.28	3.67	4.28	10.88
102	R3	EC1118	48	2G	0	10.50	5.1	4.26	10.59
103	R1	EC1118	72	2G	0	10.54	4.67	4.64	10.73
104	R2	EC1118	72	2G	0	9.65	4.51	4.59	10.59
105	R3	EC1118	72	2G	0	10.16	4.58	4.51	10.57
106	R1	EC1118	96	2G	0	8.40	4.67	4.88	10.84
107	R2	EC1118	96	2G	0	9.03	4.39	4.73	10.83

108	R3	EC1118	96	2G	0	8.11	5.34	4.88	10.75
109	R1	71B	24	2G	0	9.82	4.87	4.08	10.49
110	R2	71B	24	2G	0	8.56	4.16	4.17	10.84
111	R3	71B	24	2G	0	8.47	4.38	4.23	10.67
112	R1	71B	48	2G	0	11.93	4.56	4.26	10.83
113	R2	71B	48	2G	0	10.34	4.88	4.22	10.67
114	R3	71B	48	2G	0	10.34	3.67	4.27	10.84
115	R1	71B	72	2G	0	13.33	3.67	4.54	10.83
116	R2	71B	72	2G	0	12.25	5.46	4.59	10.88
117	R3	71B	72	2G	0	12.84	3.97	4.61	10.87
118	R1	71B	96	2G	0	13.23	3.75	4.87	10.94
119	R2	71B	96	2G	0	12.21	3.74	4.82	10.76
120	R3	71B	96	2G	0	12.93	3.68	4.81	10.77
121	R1	EC1118	24	0	2G	7.62	3.88	4.16	10.67
122	R2	EC1118	24	0	2G	6.15	3.76	4.17	10.84
123	R3	EC1118	24	0	2G	6.10	4.59	4.17	10.71
124	R1	EC1118	48	0	2G	9.44	4.57	4.23	10.76
125	R2	EC1118	48	0	2G	8.10	4.08	4.33	10.83
126	R3	EC1118	48	0	2G	7.26	3.76	4.37	10.78
127	R1	EC1118	72	0	2G	7.55	3.48	4.64	10.83
128	R2	EC1118	72	0	2G	8.83	3.7	4.61	10.72
129	R3	EC1118	72	0	2G	8.93	4.51	4.53	10.81
130	R1	EC1118	96	0	2G	7.67	4.67	4.81	10.76
131	R2	EC1118	96	0	2G	6.96	4.58	4.83	10.84
132	R3	EC1118	96	0	2G	7.57	4.39	4.87	10.79
133	R1	71B	24	0	2G	5.36	4.88	4.24	10.9
134	R2	71B	24	0	2G	6.04	5.7	4.27	10.69
135	R3	71B	24	0	2G	5.70	4.19	4.19	10.83
136	R1	71B	48	0	2G	7.02	4.23	4.3	10.87

137	R2	71B	48	0	2G	6.02	4.57	4.37	10.92
138	R3	71B	48	0	2G	6.11	4.68	4.28	10.83
139	R1	71B	72	0	2G	7.07	4.51	4.54	10.86
140	R2	71B	72	0	2G	7.90	4.55	4.53	10.94
141	R3	71B	72	0	2G	8.68	3.87	4.49	10.76
142	R1	71B	96	0	2G	7.26	3.43	4.8	10.81
143	R2	71B	96	0	2G	7.51	4.67	4.87	10.64
144	R3	71B	96	0	2G	8.19	4.11	4.76	10.67

Appendix B: Mean values of all the parameters for the yeast (EC1118) over the time period.

Mass of yeast	Type of nutrition	Parameters	0h	24h	48h	72h	96h
1g	NN	Ethanol	0	4.29(0.30)	8.43(1.21)	8.82(0.77)	8.39(0.50)
		T.Acidity	3.21(0.11)	3.67(0.18)	4.44(0.49)	4.75(0.06)	4.27(0.42)
		pH	3.75(0.01)	4.14(0.02)	4.28(0.07)	4.30(0.02)	4.70(0.12)
		Brix	23.43(0.38)	10.45(0.45)	10.49(0.13)	10.69(0.16)	10.50(0.16)
	DAP	Ethanol	0	7.82(0.34)	8.84(0.21)	8.91(0.83)	7.59(0.26)
		T.Acidity	3.77(0.08)	4.06(0.55)	4.66(0.24)	4.46(0.78)	4.53(0.14)
		pH	3.76(0.05)	4.15(0.03)	4.31(0.05)	4.51(0.02)	4.70(0.02)
		Brix	23.41(0.24)	10.69(0.13)	10.72(0.15)	10.79(0.09)	10.80(0.14)
	LUPIN	Ethanol	0	4.77(0.17)	7.45(0.42)	7.33(0.23)	7.91(0.24)
		T.Acidity	3.34(0.08)	4.24(0.65)	3.65(0.19)	4.36(0.53)	4.53(0.14)
		pH	3.64(0.24)	4.14(0.04)	4.27(0.04)	4.57(0.02)	4.88(0.08)
		Brix	23.37(0.19)	10.72(0.12)	10.62(0.06)	10.72(0.10)	10.70(0.20)
2g	NN	Ethanol	0	7.12(0.66)	7.34(0.31)	9.42(0.27)	10.50(0.33)
		T.Acidity	3.34(0.11)	3.94(0.21)	4.37(0.60)	4.19(0.60)	4.51(0.15)
		pH	3.76(0.03)	4.18(0.02)	4.29(0.02)	4.57(0.10)	4.77(0.03)
		Brix	23.48(0.21)	10.34(0.08)	10.61(0.28)	10.75(0.17)	10.73(0.20)
	DAP	Ethanol	0	8.56(0.35)	10.03(0.61)	10.11(0.44)	8.51(0.47)
		T.Acidity	3.84(0.12)	4.37(0.56)	4.21(0.77)	4.58(0.08)	4.80(0.48)
		pH	3.77(0.01)	4.17(0.06)	4.29(0.04)	4.58(0.03)	4.83(0.08)
		Brix	23.47(0.22)	10.77(0.07)	10.74(0.14)	10.63(0.08)	10.80(0.04)
	LUPIN	Ethanol	0	6.62(0.86)	7.26(0.17)	8.43(0.77)	7.40(0.38)
		T.Acidity	3.24(0.34)	4.07(0.05)	4.13(0.40)	3.89(0.54)	4.54(0.14)
		pH	3.57(0.11)	4.16(0.05)	4.31(0.07)	4.59(0.05)	4.83(0.03)
		Brix	23.34(0.31)	10.74(0.08)	10.79(0.03)	10.78(0.05)	10.79(0.04)

Appendix C: Mean values of all the parameters for the yeast (71B) over the time period.

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Mass of yeast	Type of nutrition	Parameters	0h	24h	48h	72h	96h
1g	NN	Ethanol	0	3.89(0.05)	8.30(0.34)	9.45(0.54)	9.23(0.22)
		T.Acidity	3.53(0.74)	4.39(0.55)	4.40(0.40)	4.15(0.43)	4.74(0.11)
		pH	3.68(0.03)	4.07(0.03)	4.15(0.02)	4.27(0.07)	4.75(0.04)
		Brix	23.43(0.23)	10.71(0.21)	10.48(0.28)	10.60(0.25)	10.40(0.12)
	DAP	Ethanol	0	6.45(0.75)	8.65(0.48)	8.59(0.28)	9.18(0.28)
		T.Acidity	3.86(0.87)	4.09(0.85)	4.00(0.61)	3.83(0.34)	4.63(0.21)
		pH	3.87(0.14)	4.18(0.03)	4.23(0.03)	4.37(0.02)	4.88(0.08)
		Brix	23.39(0.75)	10.71(0.13)	10.64(0.14)	10.73(0.18)	10.82(0.10)
	LUPIN	Ethanol	0	9.67(0.23)	9.15(0.27)	7.17(0.16)	8.70(0.41)
		T.Acidity	3.11(0.18)	4.20(0.38)	4.08(0.52)	4.41(0.17)	4.63(0.21)
		pH	3.68(0.71)	4.18(0.09)	4.37(0.06)	4.46(0.05)	4.70(0.05)
		Brix	23.41(0.28)	10.69(0.12)	10.72(0.20)	10.73(0.18)	10.72(0.14)
2g	NN	Ethanol	0	7.63(0.06)	7.47(0.39)	8.20(0.32)	8.11(0.47)
		T.Acidity	3.61(0.09)	4.11(0.67)	4.14(0.66)	4.69(0.13)	3.87(0.27)
		pH	3.74(0.02)	4.19(0.04)	4.38(0.08)	4.53(0.03)	4.75(0.02)
		Brix	23.54(0.19)	10.77(0.03)	10.68(0.12)	10.78(0.09)	10.87(0.04)
	DAP	Ethanol	0	8.95(0.75)	10.86(0.92)	12.47(0.32)	12.79(0.52)
		T.Acidity	3.86(0.81)	4.47(0.36)	4.37(0.62)	4.36(0.95)	3.72(0.03)
		pH	3.91(0.11)	4.16(0.07)	4.25(0.02)	4.59(0.05)	4.83(0.03)
		Brix	23.45(0.39)	10.66(0.17)	10.78(0.09)	10.85(0.09)	10.82(0.10)
	LUPIN	Ethanol	0	5.70(0.33)	6.38(0.55)	7.88(0.80)	7.65(0.47)
		T.Acidity	3.37(0.17)	4.92(0.75)	4.49(0.23)	4.31(0.38)	4.07(0.62)
		pH	3.75(0.39)	4.23(0.04)	4.31(0.04)	4.52(0.02)	4.81(0.05)
		Brix	23.39(0.33)	10.80(0.10)	10.87(0.04)	10.85(0.09)	10.70(0.09)