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Proteomic survey of alcoholic fermentation utilizing dietary epigenetic modifiers

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A Dissertation

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Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Master of Food Innovation.

Proteomic survey of alcoholic fermentation utilizing dietary epigenetic modifiers

by

Dilja Jose

Epigenetic modifiers have the potential to alter the gene expression without changing DNA sequence. Hence, the effect of two dietary epigenetic modifiers: benzoic acid, and curcumin; on the protein expression of wine yeast was studied in the present study. The experiment was structured in such a way that specific concentrations of epigenetic modifiers was added to grape must before wine fermentation. Sampling was done at different stages of fermentation and proteomic analysis was done using MALDI-TOF and SDS-PAGE. The ethanol content, residual sugars content, and glycerol content also measured at different stages of fermentation. From the proteomic analysis it is found that, both epigenetics modifiers under study produces an altered protein expression which is entirely different from control (no added epigenetic modifiers). Many of the protein expressions was downregulated, whereas, few protein expressions were upregulated. Double dose of benzoic acid caused the maximum alterations on protein expression. These observations were evident on the MALDI-TOF spectra analysis than SDS-PAGE. Supplementation of double dose of benzoic acid resulted in significant reduction in ethanol production and subsequent decrease in sugar utilization. Benzoic acid also found to decrease the glycerol production. However, curcumin found to have no significant effect on ethanol production, sugar utilization, and glycerol production. These observed results can due to the epigenetic potential of benzoic acid and curcumin. A further study is needed to identify the effects of these epigenetic modifiers on the flavour profile of wine.

Keywords: Dietary epigenetic modifiers, alcohol, wine, proteomics, MALDI-TOF.

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Introduction

The alcohol content in some alcoholic beverages has increased, which is a widely perceived problem in recent years (Varela, et. al., 2012). Due to sensory, financial, and health related issues, and climate change, there is strong demand to produce beverages with low alcohol (Goold, Kroukamp, Williams, Paulsen, Varela, & Pretorius, 2017) (Kutyna, Varela, Henschke, Chambers, & Stanley, 2010). Hence, there were many efforts to reduce alcohol content in beverages, like manipulations in viticulture practices, pre-fermentation practices, microbial approaches, and post fermentation practices (Goold, Kroukamp, Williams, Paulsen, Varela, & Pretorius, 2017). However, there has not previously been any description of the use of epigenetic approach to curb ethanol production. Epigenetic modifications can stably alter the gene expression without any changes in the DNA sequence (Deans, & Maggert, 2015). Certain dietary chemicals like curcumin and benzoic acid have the potential to induce epigenetic modifications (Anantharaju, Reddy, Padukudru, Kumari Chitturi, Vimalambike, & Madhunapantula, 2017) (Hassan et al, 2019). Hence, our effort is to identify the effect of dietary epigenetic modifiers on ethanol production and its effect on protein expression.

1.1 Alcohol in beverages

Over the past 20 years, ethanol content in wines has increased considerably by 2% (vol/vol) (Tilloy, Ortiz-Julien, & Dequin, 2014). For instance, some wines have ethanol concentrations above 15% v/v. This is mainly due to two main factors: the impact of climate change upon the global production of grapes, and the current development in winemaking practices largely driven by consumer demand for rich and ripe fruit flavour profiles in wines, often requiring increased grape maturity (Ciani et al., 2016). Grape maturation mainly accomplished by longer hang times, result in grapes with high sugars, amino acids, phenolic compounds, and potassium; and low organic acids particularly malic acid (De Orduna, 2010). Though these grapes with long hang times can produce rich, full bodied wines with ripe fruit flavour profiles, it produces wine with high alcohol content (Kutyna, Varela, Henschke, Chambers, & Stanley, 2010).

Global climate change, on the other hand, has deeply influenced the grape composition, it accelerates metabolic rates and interferes with the accumulation of metabolites in grapes. A warmer climate results in grapes with higher sugar content, altered phenolic maturation and tannin content, and lower acidity (Kutyna, Varela, Henschke, Chambers, & Stanley, 2010)

(Ciani et al., 2016). Most importantly, high sugar concentrations result in high alcohol content in wine (Ciani et al., 2016).

To be precise, climate change and longer hang times aimed at optimizing current perception of flavour profiles, produces grapes with high sugar concentrations with a consequent higher alcohol levels in wine, (Kutyna, Varela, Henschke, Chambers, & Stanley, 2010) (Ciani et al., 2016).

1.2 Impact of high alcohol content in beverages

High sugar content in grapes and its consequent high alcohol in beverages have led to number of sensory, financial, and health associated challenges. Alcohol is the most abundant volatile compound in beverages that activates olfactory, taste, and chemesthetic receptors which ultimately result in acceptance or rejection of a beverage (Liguori, Russo, Albanese, & Di Matteo, 2018). Alcohol interacts with other components of wine, like volatile compounds and tannins resulting in sensory sensation of wine (Jordão, Vilela, & Cosme, 2015). However, at higher concentrations ethanol is known to mask sensory perception of some volatile compounds in beverages, thus altering the quality of the beverage (Kutyna, Varela, Henschke, Chambers, & Stanley, 2010). It can also interfere the viscosity of wine and perception of acidity, astringency, sweetness, flavour intensity, volatility of aroma compounds and textural properties, such as palate warmth (Liguori, Russo, Albanese, & Di Matteo, 2018). According to Styger, Prior, & Bauer, (2011), high ethanol concentrations increases the intensity of astringency of the tannins and the bitterness, roughness, and hotness of wine. High ethanol content in wine known to cause stuck and sluggish fermentations, which produces unbalanced wine with unpleasant taste (Ciani et al., 2016). Besides, excessive and frequent alcohol consumption can bring health outcomes such as liver cirrhosis, cancers, depression, alcohol dependence etc. (Liguori, Russo, Albanese, & Di Matteo, 2018) (Room, Babor, & Rehm, 2005). In some countries high taxes are levied to the beverages with high alcohol content (Kutyna, Varela, Henschke, Chambers, & Stanley, 2010).

Thus, for reasons associated with climate change, health, economics and product quality, there is a strong demand to produce a beverage with low alcohol content that retains high quality flavour and other sensory characteristics (Goold, Kroukamp, Williams, Paulsen, Varela, & Pretorius, 2017) (Kutyna, Varela, Henschke, Chambers, & Stanley, 2010).

1.3 Production of low alcohol beverages

Production of low alcohol beverage is one of the major challenges in beverage industry. Many different techniques have been developed to reduce alcohol content in beverages, mainly achieved with the collective use of viticulture practices, pre-fermentation practices, microbial approaches, and post fermentation practices (Bovo, et.al., 2016) (Goold, Kroukamp, Williams, Paulsen, Varela, & Pretorius, 2017). Many viticulture practices are practiced in order to reduce the sugar content in grapes, such as reducing leaf-area-to-fruit-mass (LA-FM) ratio, application of growth regulators, harvesting of grapes when grapes have low sugar content, and irrigation before harvest. Application of growth regulators can be an effective tool were exogenous application of growth regulators, for instance, 1-napthalene acetic acid, results in delayed ripening (Varela et al., 2015).

Pre-fermentations practices include the dilution of grape must with water or low alcohol grape juice, and the treatment of grape must to remove glucose and fructose (use of nano-filtration or addition glucose oxidase enzyme that convert glucose to gluconic acid). Though, dilution of grape must with water or low alcohol grape juice can reduce the alcohol content in resulting wine by 1% (v/v), but other parameters like, colour, tannins, sensory flavours are affected which may have a potential impact on sensory profile. The enzyme glucose oxidase (GOX) from *Aspergillus niger* can convert glucose to gluconic acid and hydrogen peroxide. Thus, application of GOX enzyme in grape juice can reduce the alcohol content in wine by 0.7% (v/v). Limitation of GOX treatment is that its activity produces hydrogen peroxide, effect of which is not yet reported. Besides, resulting gluconic acid increases the total acidity and slightly decreases the pH (Varela et al., 2015). At post-fermentative stage, the reduction of alcohol could be achieved by blending of low alcohol beverage with high alcohol beverages or by the physical removal of alcohol after fermentation (eg: reverse osmosis, evaporative perstraction, pervaporation, osmotic distillation, vaccum distillation, spinning cone technology, and supercritical CO2 extraction) (Goold, Kroukamp, Williams, Paulsen, Varela, & Pretorius, 2017). But, many pre and post fermentation practices are expensive to implement and have negative effects on the organoleptic properties of wine (Tilloy, Ortiz-Julien, & Dequin, 2014).

1.3.1 Microbial approaches to reduce alcohol content in beverages

An affordable and attractive alternative would be to use yeast that can be used to produce alcoholic beverages with less alcohol yield (Tilloy, Ortiz-Julien, & Dequin, 2014). Sadly, such yeast are not available (Biyela, Du Toit, Divol, Malherbe, & Van Rensburg, 2009). Hence, there have been many efforts to reduce alcohol content in beverages by manipulating yeast, most of

which are implemented to redirect yeast carbon metabolism into by-products other than ethanol, for instance, yeast strain producing more glycerol and less ethanol (Kutyna, et.al. 2012). In 2014, Tilloy, Ortiz-Julien, & Dequin exploited adaptive laboratory evolution strategies based on sequential increases of osmotic/saline stress to divert the carbon metabolism of commercial yeast strain towards glycerol. The resulting adapted strain produced more glycerol and less ethanol compared to original strain without accumulating undesirable compounds such as, acetate, acetaldehyde, or acetoin (Tilloy, Ortiz-Julien, & Dequin, 2014).

Potential of non-*Saccharomyces cerevisiae* strains to produce low alcohol beverage have also experimented (Ciani et al., 2016). In a study Ciani, Canonico, Oro, & Comitini, (2014) exploited the potential of non-Saccharomyces yeast in reducing alcohol content in beverages. The authors used sequential fermentation techniques, in which *Saccharomyces cerevisiae* starter culture is inoculated after the initial fermentation with non-*Saccharomyces* strains. They observed 5 to 11.5% reduction in ethanol content in sequential fermentation with *Candida stellata* (now reclassified as *Starmerella bombicola*), *Metschnikowia pulcherrima* and *Lachancea thermotolerans* as compared to control were only *Saccharomyces cerevisiae* employed.

However, genetic modification (GM) strategies have proven to be a powerful and precise tool to produce low alcohol beverage. For instance, in beer, overexpression of the GDP1 gene is known to reduce ethanol content with substantial glycerol production in *Saccharomyces cerevisiae ssp. carlsbergensis* (Nevoigt et al., 2002). GDP gene code for isozymes of glycerol 3-phosphate dehydrogenase, catalyses the conversion of dihydroxyacetone phosphate to glycerol 3-phosphate, which is subsequently dephosphorylated to glycerol by glycerol 3-phosphatase. Overexpression of *GPD gene* increases the glycerol yield by 548% depending on yeast strain used, medium, and fermentation conditions (Varela et al., 2012). Deletion of the PDC2 gene encoding pyruvate decarboxylase; modification of the glycerol transporter encoded by FPS1; impairment of alcohol dehydrogenase encoded by ADH1, ADH3, ADH4 and ADH5; and deletion of TPI1encoding triose phosphate isomerase also known to increase the glycerol yield with subsequent low alcohol yield (Goold, Kroukamp, Williams, Paulsen, Varela, & Pretorius, 2017). Moreover, in 2006, Heux, Sablayrolles, Cachon, & Dequin, demonstrated that expression of an H_2O-forming NADH oxidase lead to a marked decrease in the ethanol production in the fermentation of synthetic must under microaerobic conditions.

But, these genetic manipulations may increase the concentration of several unwanted by-products, particularly acetoin, diacetyl and acetaldehyde, which may negatively affect wine

quality (Saerens, Duong, & Nevoigt, 2010). For example, overexpression of the GDP gene leads to increased acetic acid production (Varela et al., 2012) and expression of an H_2O-forming NADH oxidase results in accumulation of acetaldehyde (Heux, Sablayrolles, Cachon, & Dequin, 2006) both of which have a negative sensorial impact on wine (Saerens, Duong, & Nevoigt, 2010). Interestingly, there were some efforts to inhibit these by-product formation, especially acetic acid. For instance, in 2006, Cambon, Monteil, Remize, Camarasa, & Dequin, studied GDP1 overexpressing in *Saccharomyces cerevisiae* commercial wine yeast strains lacking ALD6 gene which results in reduced ethanol yield with less acetic acid accumulation. But these strains have been reported to produce considerable levels of acetoin as a by-product which has low sensory threshold and aroma of rancid butter (Varela et al., 2012). However, in 2017, Cuello, Montero, Mercado, Combina, & Ciklic proposed an alternative GMO based strategy in which partial deletion of *Saccharomyces cerevisiae* PDC2 gene resulted in low ethanol production with a moderate impact on the yeast metabolism.

Another possible GMO based method to reduce ethanol production is to direct carbon metabolism towards organic acids, like gluconic acid and acids involved in the tricarboxylic acid (TCA) cycle (Varela, et.al., 2012). In 2003, Malherbe, Du Toit, Otero, Van Rensburg, & Pretorius, demonstrated that expression of the *Aspergillus niger* glucose oxidase gene (GOX gene) in *Saccharomyces cerevisiae* reduces ethanol yield with subsequent high gluconic acid production. But effect of hydrogen peroxide, a by-product of this reaction, in resulting wine is not yet reported (Varela et al., 2012).

Besides, poor public acceptance of genetically modified beverages restrict their application in beverage production (Goold, Kroukamp, Williams, Paulsen, Varela, & Pretorius, 2017). Consequently, the use of genetically modified organisms (GMOs) are not permitted in many parts of the world (Kutyna, et.al. 2012).

1.4 Potential of Epigenetics

There has not previously been any description of the use of an epigenetic approach to curb ethanol production. In fact, application of epigenetics in food industry is not yet exploited as far as our knowledge. “Epigenetics is defined as somatically heritable states of gene expression resulting from changes in chromatin structure without alterations in the DNA sequence, including DNA methylation, histone modifications, and chromatin remodelling” (Masuyama, & Hiramatsu, 2012). Epigenetic modifications open up the DNA (deoxyribonucleic acid) and make DNA accessible for transcription (Shukla et al., 2008). Thus, epigenetic modifications stably alter gene expression patterns without entailing changes in the DNA sequence (Deans, & Maggert, 2015), which may have profound effect

on phenotype. In other words, epigenetic alterations can alter phenotype without changing DNA sequence (Lillycrop, & Burdge, 2012). These changes in gene expression can be inherited during cell division (Shukla et al., 2008).

Epigenetic modifications are the result of four distinct mechanisms namely, DNA methylation, histone modifications, post transcriptional gene regulation by non-coding microRNAs (miRNAs), and RNA interference (Daniel, & Tollefsbol, 2015).

A) DNA methylation

In DNA methylation, a methyl group is added to the 5′ position of the cytosine in CpG dinucleotides to form 5-methylcytosine. This process is mainly controlled by the enzyme called DNA methyltransferase (DMNTs). Promoter regions of gene and regions of large repetitive regions of human genome are enriched with CpG dinucleotides called CpG Islands. Hence, methylation of CpG dinucleotides in the promoter region may result in gene silencing. Inversely removal of methyl group from CpG dinucleotides results in gene reactivation (Link, Balaguer, & Goel, 2010).

B) Histone modification

Unlike DNA methylation, Histone modifications may be due to methylation, acetylation, phosphorylation, ribosylation, ubiquitination, sumoylation or biotinylation, in N-terminal histone tails which result in activation or repression depending on the residue involved and the type of modification (Link, Balaguer, & Goel, 2010).

C) MicroRNA (miRNA)

MicroRNAs (miRNAs) are 19-24 nucleotides long single stranded RNAs. miRNAs can induce post transcriptional modifications by bind with the complementary sequence of target mRNA, which cause increased degradation of RNA. Although, many of these transcriptional modifications induce gene silencing, miRNA also shown to induce transcriptional activation of genes (Daniel, & Tollefsbol, 2015).

D) RNA interference

RNA interference forms antisense transcripts, which in turn, blocks the accumulation of homologous transcripts. These homologous transcripts induce the formation of heterochromatin, ultimately result in gene silencing (Daniel, & Tollefsbol, 2015).

Many of these epigenetic modifications can be induced by environmental factor. For example, oral administration of royal jelly can make female worker bee into queen. Only a minority of

honeybee's larvae designated to become queen is fed with royal jelly. Royal jelly can change the phenotype of female larvae, make them grow in size and gain reproductive capacity and becomes the queen of the colony. These change in phenotypic expression is due to the epigenetic potential of royal jelly (Godfrey, Costello, & Lillycrop, 2015).

1.5 Dietary epigenetic modifiers

Interestingly, epigenetic modifications can be induced by certain nutrients and bioactive food components. Polyphenols, flavonoids and some other bioactive food components are known to induce epigenetic modifications by controlling DNA methylation, by regulating histone modifying enzymes, by changing the availability of substrate for enzymatic reactions (Abdul, Yu, Chung, Jung, & Choi, 2017). For instance, Folate, vitamin-12, methionine, choline, and betaine can affect DNA methylation and histone methylation; genistein and tea catechin can affects DNA methyltransferases; and curcurmin can inhibit histone acetyltransferases (Choi, & Friso, 2010). Besides, many dietary polyphenols also known to have epigenetic potential: lycopene from tomatoes, phloretin from apples, hesperidin from citrus fruits, caffeic acid from coffea, isothiocyanates from broccoli, allyl mercaptan from garlic, resveratrol from grapes, EGCG (Epigallocatechin gallate) from tea, anacardic acid from cashew nuts, and coumaric acid from cinnamon (Link, Balaguer, & Goel, 2010).

1.5.1 Benzoic acid

Benzoic acid ($C_7H_6O_2$) is a weak acid in which a carboxylic group directly bonded with the benzene ring. It is used as preservative and flavouring agent in food products. It is naturally present in many plants and in animal tissues. Benzoic acid is found in many fruits, nuts, vegetables, cereals, and spices including apples (<1mg/kg), naval orange (1-2 mg/kg), banana (<1mg/kg), peach (1-2 mg/kg), watermelon (<1mg/kg), strawberries (29 mg/kg), cauliflower (<1mg/kg), cabbage (<1mg/kg), potato (<1mg/kg), onion (<1mg/kg), garlic (<1mg/kg), pumpkin (1-2 mg/kg), spinach(1-2 mg/kg), soybean (1-2 mg/kg), cashew (<1mg/kg), almond (<1mg/kg), hazelnut, peanut (1-4 mg/kg), walnuts (1-4 mg/kg), parsley (<1mg/kg), coriander (3-5 mg/kg), black pepper (3-5 mg/kg), cayenne pepper (5–10 mg/kg), mustard seed (5–10 mg/kg), cloves (15–50 mg/kg), salvia (15–50 mg/kg), thyme (15–50 mg/kg), nutmeg (15–50 mg/kg), and cinnamon (336 mg/kg). However, highest concentration of benzoic acid is found in berries (up to 500 mg/kg), ripe fruits of the *Vaccinium* species (up to 1300 mg/kg) and resin from *Styrax* trees (up to 200.000 mg/kg). Benzoic acid is also found in muscles, fluids, glands and secretions of some animals, usually at lower concentrations of less than 1mg/kg. But, milk contains comparatively high levels of benzoic acid between 2 and 5 mg/kg. On fermentation of milk, the concentration of benzoic acid increases even further up to 18.8, 25, and 36mg/L in fermented

milk of cow, goat and sheep respectively. Increase in benzoic acid concentration in fermented milk is due to the activity of lactic acid bacteria in starter culture (del Olmo, Calzada, & Nuñez, 2017).

Benzoic acid is a potential histone deacetylase (HDAC) inhibitor, which contribute to its epigenetic potential (Anantharaju, Reddy, Padukudru, Kumari Chitturi, Vimalambike, & Madhunapantula, 2017). HDAC deacetylates histones and its effect can be altered by another enzyme called histone acetyl-transferase (HAT). The balance between these two enzymes are highly regulated in cells. In presence of HDAC inhibitors like benzoic acid this balance gets disrupted due to the deacetylation of histones. Deacetylation of histones tightly wraps DNA around histones, ultimately resulting in the inhibition of gene transcription (Kim, & Bae, 2011).

1.5.2 Curcumin

Curcumin (diferuloylmethane) is a unique molecule with many biological activities including strong anti-inflammatory, antioxidant, wound healing, and anticancer effects. Curcumin, a yellow polyphenol, a major component of turmeric (*Curcuma longa*), has a great potential to act as an epigenetic modulator. Curcumin contains 80% curcuminoid complex, 17% dimethoxy-curcumin, and 3% bisdemethoxy-curcumin (Link, Balaguer, & Goel, 2010) (Hassan et al, 2019). Curcumin is known to inhibit DNA methyltransferases (DNMTs), and inhibit HDACs (Hassan et al, 2019). Role of curcumin as an epigenetic modifier in cancer prevention is well known, it prevents the DNA damage and blocks the inflammatory master molecule NF Kappa B (Shankar, Kumar, & Srivastava, 2013).

1.6 Proteomics for determining epigenetic modifications

"Proteomic analysis is defined as the analysis of the protein complement expressed by a genome". Proteomics have significant importance in many areas of research, because of its potential to determine post transcriptional modification. In comparison with genomics, proteomic studies can provide in-depth knowledge about functions of protein in cell system. Because some cellular functions are the results of synergic action of more than one protein (Khoa Pham, & Wright, 2007).

Among the methods employed to measure epigenetic changes, proteomics especially mass spectrometry (MS) - based proteomics, is proved to be a powerful tool (Han, & Garcia, 2013). MS-based proteomics are especially good in determining histone modifications and DNA-protein interactions. However, ChIP sequencing coupled with mass spectroscopy is the best known method to evaluate epigenetic modifications (Han, & Garcia, 2013).

Hence, in this experiment, we are examining the change in protein expression of *Saccharomyces cerevisiae* on presence of dietary epigenetic modifiers- curcumin and benzoic acid; and the effect of dietary epigenetic modifiers on ethanol production

2

Materials and methods

2.2 Yeast strain and culture conditions

The yeast strain used in the study was *Saccharomyces cerevisiae* (EC1118), because of its high fermentative capacity; its fast fermentation rates; and its high tolerance to fermentation conditions like pH, ethanol, and oxygen (Albergaria, H., & Arneborg, 2016). Besides, *Saccharomyces cerevisiae* is considered as model organism to study epigenetic modifications (O'Kane, & Hyland, 2019). The dietary epigenetic modifiers also were added to the Chardonnay grape juice along with yeast strain. The dietary epigenetic modifiers used in the experiment was benzoic acid and curcumin. Benzoic acid is a potent HDAC inhibitor (Anantharaju, Reddy, Padukudru, Kumari Chitturi, Vimalambike, & Madhunapantula, 2017), whereas curcumin can inhibit DNMTs, regulate miRNAs, and act as a DNA binding agent (Hassan et al, 2019).

25g of *Saccharomyces cerevisiae* (EC1118) were inoculated into 200mL of Chardonnay grape juice. The experimental setup includes 4 fermentation flasks: first fermentation flask was without any epigenetic modifiers (control); second fermentation flask had 0.025g benzoic acid added on day 1; third fermentation flask was added with double concentrations of benzoic acid: 0.025g on day 1 and 0.05g on day 2; last fermentation flask was added with 0.1g curcumin on day 1 and 0.1g curcumin on day 2. The fermentations were performed in triplicate, at 25°C, and with no agitation. Fermentations were monitored by weighing the flasks regularly (every two days at 1, 2, 4, 6, 8 and 12th day of fermentation). As the fermentation proceeds, carbon dioxide, a by-product of alcoholic fermentation releases, which may lead to mass reduction. Hence, mass loss can be used as index of fermentation rate, i.e. no weight change will signal the end of fermentation (Appiah-Nkansah, Zhang, Rooney, & Wang, 2018) (Liu, et al., 2015). Samples were withdrawn at 2-day intervals for proteomic and biochemical analysis.

2.2 Determination of viable cell count

Number of viable yeast cells were determined by direct counting under a light microscope in a hemocytometer after staining with methylene blue (Roldán, Lasanta, Caro, & Palacios, 2012). Viable cell count done for all samples collected on Day 2, Day 4, Day 6 and Day 8.

2.3 Biochemical analysis

2.3.1 Determination of ethanol content

Ethanol content was determined by direct injected gas chromatography (GC) method as described in Wang, Choong, Su, & Lee, (2003). 0.5mL of each sample solutions (control and samples with epigenetic modifiers) was transferred into 1mL caped sample vial along with 5mL of 1% internal standard solution (10g of acetonitrile in 1000mL distilled water). After mixing 0.1µl of sample directly injected into GC system with syringe. A standard solution of ethanol also ran along with sample, for which, different concentrations of 1% ethanol was used (ethanol: acetonitrile= 15:1, 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10 and 1:15). GC system generated a linear regression line graph with the GC peak area-under-curve (AUC) ratio of ethanol to acetonitrile on Y-axis and the concentration ratio of ethanol to acetonitrile on X-axis. Relative response factor (RRF or the slope of the regression line) was calculated according to the equation 1: $RRF = (AS/WS) \div (AIS/WIS)$; in which, AS= ethanol AUC, AIS = acetonitrile AUC, WS = ethanol weight (mg), WIS = acetonitrile weight (mg). Ethanol content was determined by the equation 2: $Ethanol\ (mg/mL) = (AS/AIS) \times (WIS/RRF) \times 1/V$; in which, V = sample volume (mL).

2.3.2 Determination of residual sugars

Determination of residual D-glucose and D-fructose in wine was quantified by an enzymatic test kit by vintessential laboratories. 1mL Buffer/coenzyme mix, 1.9mL distilled water and 0.1mL sample was incubated for 3minutes and read the absorbance, A1 at 340nm. 0.2mL of G6PDH/HK was added and read the absorbance A_2 after 3 minutes. Then finally 0.02mL PGI is added and read the absorbance after 10 minutes. All absorbance was read at 340nm and a standard also included in the test run. Concentration of residual sugars were measured by the equations as follows:

Concentration of D-glucose measured by the Equation 3: $Concentration\ of\ D-glucose\ (g/L) = A_G \times 0.8637 \times dilution\ factor$; where A_G was calculated by the equation 4: $A_G = (A_2 - A_1) - (blank\ A_2 - blank A_1)$.

Concentration of D-fructose measured by the Equation 5: $Concentration\ of\ D-fructose(g/L) = A_F \times 0.8694 \times dilution\ factor$; where A_F was calculated by the equation 6: $A_F = (A_3 - A_2) - (blank\ A_3 - blank\ A_2)$.

2.3.3 Determination of glycerol

Amount of glycerol produced was determined using glycerol assay kit by megazymes. For the assay, series of chemicals (provided by the kit manufacturer) added to the cuvettes along with samples, after intermittent incubation results were measured by spectroscopy. Initially, 1.90mL of distilled water, 0.10mL of sample, 0.20mL of buffer, 0.10mL of solution 2 and 0.02mL of suspension 3 were pipetted into cuvettes and read the absorbance of the solution (A_1) after 4 minutes. The nicotinamide-adenine dinucleotide (NADH), adenosine-5'-triphosphate (ATP), and phosphoenolpyruvate (PEP) were main components of solution 2; whereas, Pyruvate kinase (PK), and L-lactase dehydrogenase (L-LDH) are the main components of suspension 3. After the initial reading of solution (A_1), glycerol kinase (GK) suspension was added to the cuvettes and read the final absorbance of the solutions (A_2) after 5 minutes of incubation. All absorbance were read at 340nm.

The concentration of glycerol (g/L) produced was determined by the equation 7:

$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{glycerol}$; Where c=concentration of glycerol (g/L), V=final volume (mL), MW= molecular weight of glycerol (g/mol), ε= extinction coefficient of NADH at 340nm= 6300 ($l \times mol^{-1} \times cm^{-1}$), d= light path (cm), and v= sample volume (mL). $\Delta A_{glycerol}$ was calculated by the equation 8: $\Delta A_{glycerol} = (A_2 - A_1) - (Blank\ A_2 - Blank\ A_1)$; where A_2 = final absorbance of the solution, A_1 =initial absorbance of the solution, blank A_2 = final absorbance of blank, and blank A_1 =initial absorbance of blank.

2.3.4 Statistical analysis

For all biochemical tests significant differences were determined by analysis of variance (ANOVA), followed by the paired comparison test at a level of $P<0.05$. Data were analysed by using minitab 2018 version.

2.4 Proteomic analysis

2.4.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sampling done on day 6 is used for SDS-PAGE. For SDS-PAGE, yeast cells were extracted as described in Von Der Haar, (2007). Yeast cells were harvested from the fermentation tubes by centrifugation. Then, harvested yeast cells were resuspended in 200µl lysis buffer. Lysis buffer composed of 0.1M sodium hydroxide (NaOH), 0.05M ethylenediaminetetraaceticacid (EDTA), 2% SDS and 2% β-mercaptoethanol. Resuspended yeast cells were heated to 90^0C for 10 minutes. Then, 5 µl of 4M acetic acid was added to the lysate and vertex for 30 seconds which

was heated to 90^0C for 10 minutes. After incubation 50µl of loading buffer was added to lysate which composed of 0.25M Tris-HCl (pH6.8), 50% glycerol and 0.05% bromophenolblue. After that lysate was loaded directly into SDS-gels. Protein standards also loaded along with samples. After electrophoresis, gels were stained with Commassie Brilliant blue stain and destained.

2.4.2 Matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry (TOF MS)

Day 6 samples were used for MALDI-TOF analysis. Yeast samples were extracted and MALDI-TOF was performed (Cherie, personal communication, Date) (Moothoo-Padayachie, Kandappa, Krishna, Maier, & Govender, 2013). For the preparation of proteins, collected samples are centrifuged at 16000 rpm for 2 minutes and the supernatant was discarded. Residual pellets were re-suspended in distilled water, homogenized, and centrifuged for 16000 rpm for 2 minutes. The yeast cell pellet after centrifugation was re-suspended in 90% ethanol. The re-suspended solution was vortexed, centrifuged at 16000 rpm for 2 minutes and the supernatant was discarded. Centrifugation repeated until all residual liquid from suspension removed. The pellets after final centrifugation were air dried for 5 minutes at room temperature. For yeast protein extraction, 50µl of 70% formic acid (v/v) was added to air-dried pellets and the suspension was homogenised. Then, 50µl of 100% acetonitrile was added to suspension and homogenized by vortexing. Homogenised suspension was centrifuged at 16000rpm for 2 minutes and protein containing supernatant was separated from pellets. 1µl of supernatant was loaded onto MALDI target plate and air-dried at room temperature for 5 minutes. Then, the sample was covered with 1µl of HCCA solution (α-cyano-4-hydroxycinnamic acid, 20 µg μl^{-1}) and air-dried. Thereafter, the sample was analysed by MALDI-TOF MS.

3

Results

3.1 Biochemical analysis and Statistical analysis

As demonstrated in the table 1, *Saccharomyces cerevisiae* produced less amount of ethanol in presence of benzoic acid. Control sample which is not treated epigenetic modifiers produced 11.3mg/mL of ethanol. Sample treated with 0.025g of benzoic acid produced almost same amount of ethanol as that of control with an average of 11.7mg/mL which is 0.4mg/mL higher than that of control. Interestingly, addition of 0.05g of benzoic acid on day 2 decreased the ethanol production to 7 mg/mL which is 3.79mg/mL less than that of control. On the other hand, in presence of curcumin, yeast produced almost same amount of ethanol as that of control. Yeast cells produced less ethanol have consumed comparatively less sugars. Highest consumption of sugars was found in control samples followed by curcumin and benzoic acid (to which only 0.025g of benzoic acid was added). As compared to control enormous amount of residual sugars was found in double strength benzoic acid sample to which 0.025g (day 1) and 0.05g (day 2) of benzoic acid added. As compared to control samples, there is approximately 14g/L increase in D-glucose concentration and around 50g/L increase in D-fructose concentration. But, glycerol production showed a different trend, less glycerol is produced when yeast cells treated with benzoic acid (irrespective of amount of benzoic acid added). In fact, there is a significant reduction in amount of glycerol produced when samples are treated with benzoic acid. While curcumin produced almost same amount of glycerol as that of control (table 1).

Table 3.1 Result of biochemical analysis: table compares the mean and standard deviation of amount of ethanol (mg/mL), residual sugars (g/L), and glycerol (g/L) produced by Saccharomyces cerevisiae in presence of epigenetic modifiers and its pairwise comparison by Tukey's test.

Samples	Concentration of Ethanol (mg/mL)	Concentration of Residual sugars (g/L)		Concentration of Glycerol (g/L)
		D-glucose	D-fructose	
Control	11.3±0.0 b	0.015±0.006 b	0.32±0.044 b	0.581±0.008 a
Benzoic acid (0.025g on day 1)	11.7±0.1 a	0.041±0.024 b	1.46±0.489 b	0.220±0.008 b
Benzoic acid (0.025g on day 1+ 0.05g on day 2)	7.7±0.2 c	14.5±1.6 a	50.8±4.4 a	0.275±0.156 b
Curcumin (0.1g on day 1+ 0.1g on day 2)	11.4±0.2 ab	0.026±0.007 b	0.326±0.079 b	0.585±0.029 a

Different letters (a, b, c, or ab) indicate significant differences between samples at $P<0.05$.

3.2Proteomic analysis

3.2.1 SDS-PAGE

Due to the high number of proteins and due to the low intensity of protein expression, it is difficult to compare the protein expression by different samples. But still there is noticeable reduction in protein expression by benzoic acid as compared to control sample, even in diluted samples. Curcumin also reduced the protein expression, but not as prominent as benzoic acid.

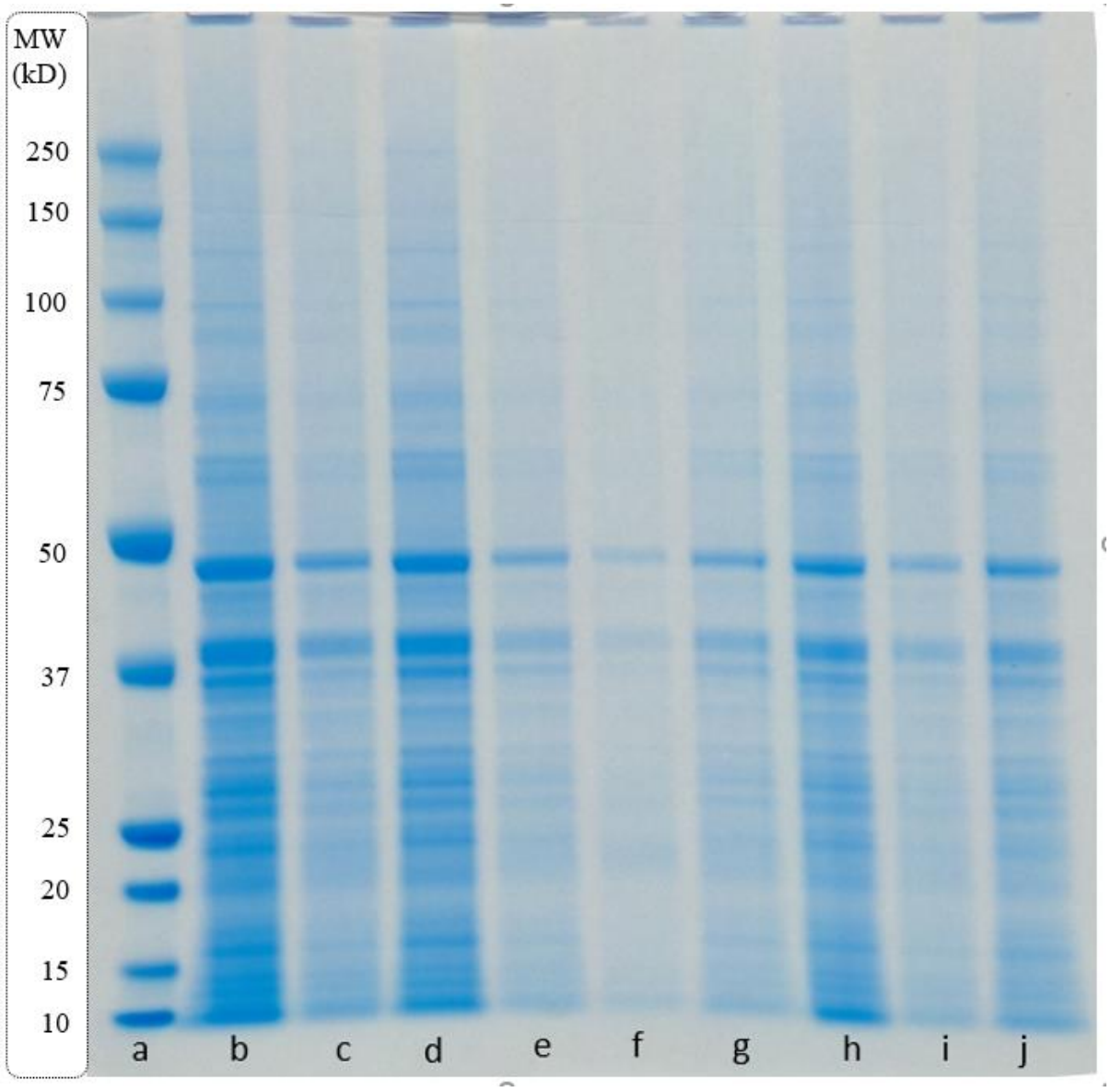


Figure 0.1 : Result of SDS-PAGE of different samples under study. MW=Molecular weight, a=protein standards, b=control sample (20µl), c= Benzoic acid- 0.025g on day 1+ 0.05g on day 2 (20µl), d= Curcumin- 0.1g on day 1+ 0.1g on day 2 (20µl), e= control sample (1 in 2 diluted-20µl), f=Benzoic acid- 0.025g on day 1+ 0.05g on day 2(1 in 2 diluted-20µl), g=Curcumin- 0.1g on day 1+ 0.1g on day 2 (1 in 2 diluted-20µl), h= control sample (5µl), i=Benzoic acid- 0.025g on day 1+ 0.05g on day 2 (5µl), j= Curcumin- 0.1g on day 1+ 0.1g on day 2 (5µl)

3.2.2 MALDI-TOF Analysis

3.2.2.1 Spectra analysis

Figure 3 presents mass spectrum of *Saccharomyces cerevisiae* showing the molecular masses of the measured proteins and the intensity of the peaks. For all samples, peaks observed are found between m/z 4000 and m/z 20000. Out of 12 prominent peaks in controls, 9 peaks have intensity more than 25% which ranges from m/z 4000-13000. Highest peak in control, between m/z 10000-11000 is found in very low intensity in curcumin and benzoic acid. Indeed, many of the prominent peaks in control are found in low intensities in benzoic acid and curcumin except the one that lies between m/z 4000-4500. Only 1 or 2 prominent peaks, which has intensity more than 25% are found in benzoic acid and curcumin. Least number of peaks found for yeast to which double dose (0.025g on day 1+ 0.05g on day 2) of benzoic acid is added.

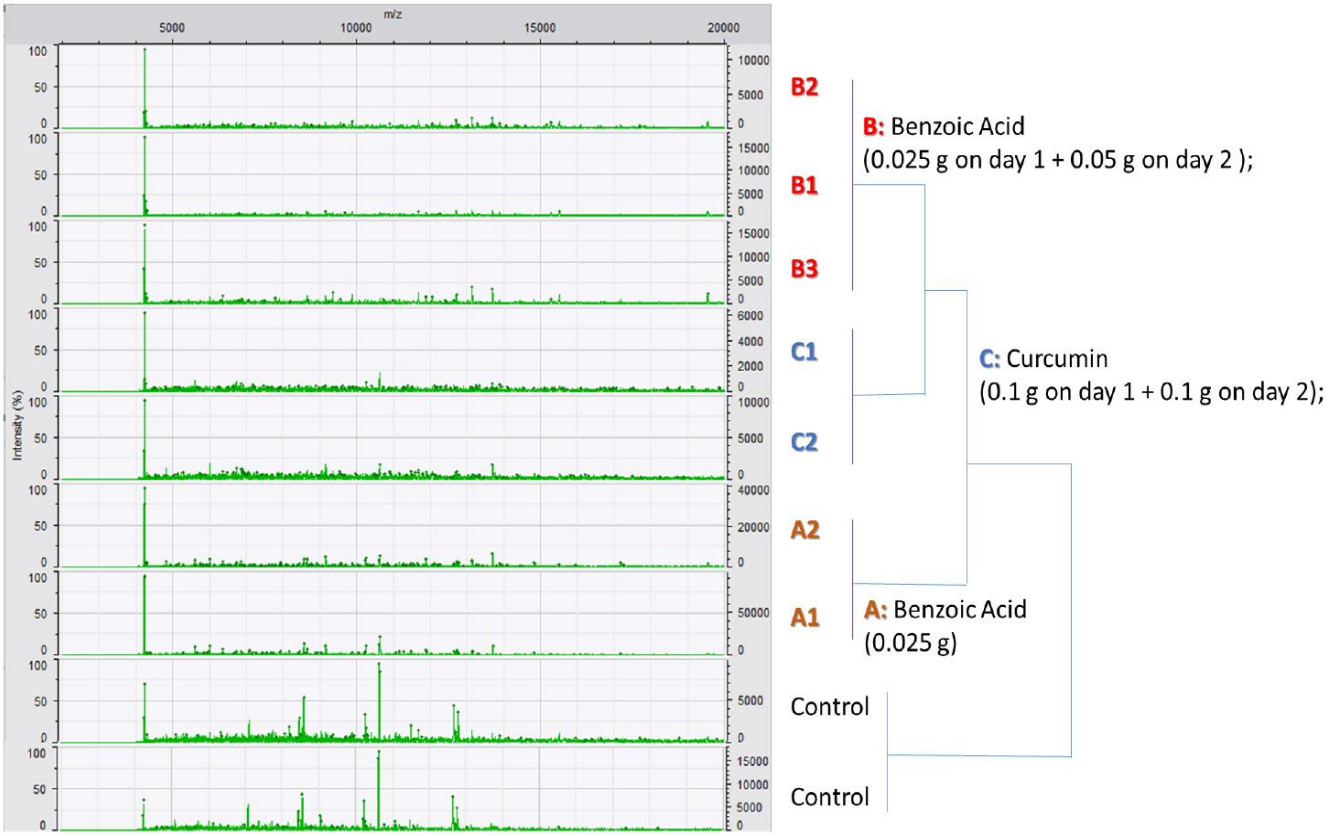
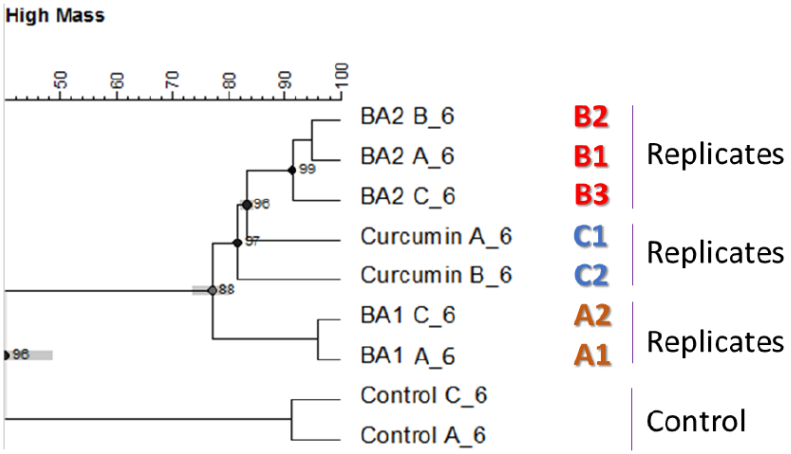


Figure 0.2: Result of spectra analysis. Figure compares the spectra of samples with or without epigenetic modifiers

3.2.2.2 Cluster analysis

The dendrogram generated by MALDI MS system, grouped control alone into one cluster, while, all other samples of curcumin and benzoic acid are grouped together into another cluster (figure 4).



A: Benzoic Acid (0.025 g) added initially; Samples collected on fermentation Day 6;
B: Benzoic Acid (0.025 g on day 1 + 0.05 g on day 2); Samples collected on Day 6;
C: Curcumin (0.1 g on day 1 + 0.1 g on day 2); Samples collected on Day 6.

Figure 0.3 : Result of cluster analysis. Figure explain the grouping of yeast samples after the treatment with epigenetic modifiers.

3.3 Cell count

All samples except samples collected on day 8 for double strength benzoic acid (Benzoic acid-0.025g on day 1+ 0.05g on day 2) had more viable cells in comparison to nonviable cells.

4

Discussion

4.1 Effect of epigenetic modifiers on biochemical properties of wine

A total concentration of 0.075g of benzoic acid was proved to reduce the amount of ethanol produced to 7mg/mL as compared to 11mg/mL in control. At the same concentration, benzoic acid also found to lessen the sugar consumption by *Saccharomyces cerevisiae* resulting in high amount of residual sugars in wine. These observations can be explained by the fermentation inhibition capacity of benzoic acid. Curcumin, on the other hand, had no influence on the production of ethanol, and glycerol; and consumption of sugars. Besides, *Saccharomyces cerevisiae* tend to utilise glucose predominantly out of all sugars (Liu, 2015). This explains the high amount of D-fructose found in comparison to D-glucose in benzoic acid treated samples

Benzoic acid is a potential inhibitor of fermentative metabolism of *Saccharomyces cerevisiae*. At lower concentrations, benzoic acid can act as a strong fermentation inhibitor and can inhibit the ethanol production by *Saccharomyces cerevisiae*, for instance, 1g/L of benzoic acid can cause 25% reduction in ethanol production (Vejarano, Morata, Loira, Gonzalez, & Suarez-Lepe, 2013). In 2011, Huang et al., demonstrated that even low concentration of benzoic acid can inhibit the fermentation capacity of *Saccharomyces cerevisiae*. They examined the potential of weak acids like benzoic acid, acetic acid, formic acid, butanoic acid and propionic acid as fermentation inhibitor of *Saccharomyces cerevisiae.* Among the weak acids under study, benzoic acid proved to be strongest fermentation inhibitor with low critical concentration of inhibition of 2 g/L. Even low concentrations of 1g/L of benzoic acid can reduce the ethanol production to approximately 25% compared to other weak acids examined. In another study by Warth, (1991) the minimum inhibitory concentration (MIC) of benzoic acid was found to be 1.4mM and anaerobic fermentation further reduced the MIC.

The mechanism by which benzoic acid inhibit the fermentation capacity of *Saccharomyces cerevisiae* is not well understood. “Decoupling theory” outlines one probable mechanism. At low pH, benzoic acid remains in the non-dissociated form and can cross cell wall barrier. But, in cytosol, due to the maintained pH of 6-7, benzoic acid dissociates and lead to acidification of the cytosol. For maintaining the pH in cytosol, protons should be pumped out of cytosol which occurs with the help of membrane bound ATPase on the expense of ATP. Thus, ATP availability for biomass synthesis becomes limited resulting in reduced fermentation capacity.

High accumulation of benzoic acid in cytoplasm may even result in cell death due to low pH in cytoplasm (Vejarano, Morata, Loira, Gonzalez, & Suarez-Lepe 2013). According to Krebs, Wiggins, Stubbs, Sols, & Bedoya, (1983), low pH also inhibits phosphofructokinase enzyme which results in accumulation of 2-hexose monophosphates of yeast glucose fermentation, suggesting inhibition of fermentation at this stage. Possible other explanation may be the intracellular accumulation of anions. Dissociation of benzoic acid generates conjugate base, which cannot diffuse back across the membrane. Thus these anions gets accumulated in the cytoplasm and their toxic effect inhibit the fermentation reactions (Hazan, Levine, & Abeliovich, 2004).

According a research placement report (Mandeep Kaur, October 20, 2019, as provided by Venkata Chelikani, January 29, 2020; Personal communication), addition of benzoic acid during fermentation produces low alcohol wine with fruitier flavour. In a series of experiments, they added benzoic acid to wine at different stages of fermentation and they observed that benzoic acid decreased the ethanol yield by 1%. In sensory analysis, they observed that decreased ethanol content induced by benzoic acid increased the fruity aroma of wine. They also found that addition of large amount of benzoic acid unfavourable for yeast growth.

But, low ethanol content in wine may negatively influence the ecological dominance of *Saccharomyces cerevisiae* (Albergaria, & Arneborg, 2016). Generally, non-*Saccharomyces* species dominates the initial stages of alcoholic fermentation, however, the middle and final stages are mainly dominated by *Saccharomyces cerevisiae* (Padilla, Zulian, Ferreres, Pastor, Esteve-Zarzoso, Beltran, & Mas, 2017). Because, many non-*Saccharomyces* species have low tolerance to ethanol, whereas *Saccharomyces cerevisiae* have comparatively high tolerance to ethanol. Indeed, niche construction via ethanol production favours the proliferation of *Saccharomyces cerevisiae* (Salvadó, Arroyo-López, Barrio, Querol, & Guillamón, 2011). Besides, ethanol known to activate plasma membrane H^+-ATPase of *Saccharomyces cerevisiae*, which helps them to maintain intracellular pH (Monteiro, & Sa-Correia, 1998). According to Goddard, (2008), along with temperature factor, ethanol can provide 7% fitness advantage to *Saccharomyces cerevisiae* over other members of community. However, according to another study by Salvadó, Arroyo-López, Barrio, Querol, & Guillamón, (2011), ethanol production does not give a clear ecological dominance to *Saccharomyces cerevisiae* over non-*Saccharomyces* yeasts like *Hanseniaspora uvarum, Torulaspora delbrueckii, Candida zemplinina, Pichia fermentans* and *Kluyveromyces marxianus* at least not until ethanol levels exceeds 9% (v/v). Hence, ethanol concentration of 7.7% (average ethanol

production by *Saccharomyces cerevisiae* in presence of double dose benzoic acid as demonstrated in table 1) may increases chance of survival of non-*Saccharomyces* species.

Wine with low ethanol content and high residual sugars is generally considered as unstable, since, they can readily refermented unless sterilised (Yokotsuka, Takayanagi, Okuda, & Yajima, 2003). Moreover, consumer acceptance of high sugar wine is a matter of concern, because high sugar wines doesn't follow the general trend towards low sugar consumption and high sugar concentrations increases perceived sweetness of wine (de Oliveira Pineli, de Aguiar, Fiusa, de Assunção Botelho, Zandonadi, & Melo, 2016).

As for glycerol, curcumin had no significant effect on glycerol production by *Saccharomyces cerevisiae* as compared to control. But, even the single dose of benzoic acid shown to decrease the glycerol production. Glycerol, a prominent compound in wine, is an osmolyte produced by *Saccharomyces cerevisiae* to counteract hyperosmotic stress (Liu, et al, 2015). High sugar stress is known to up-regulate glycolytic and pentose phosphate pathway genes resulting in increased production of glycerol (De Orduna, 2010). According to Albertyn, Hohmann, Thevelein, & Prior, (1994), osmotically stressed yeast cells produces intracellular compatible solutes to counteract osmotic stress and glycerol is the only compatible solute produced by Saccharomyces cerevisiae. In *Saccharomyces cerevisiae*, glycerol production is highly regulated with subsequent production of cellular cAMP on osmotic stress. For instance, a rapid increase in cellular cAMP production was observed when glucose is added to yeast cells grown in poorer carbon sources. An increased cellular cAMP concentration can activate protein kinase A, expression of which can influence the Msn2/4p-dependent gene induction. The binding of Msn2/4p to stress-response element (STRE, a positive promoter element) activates the transcription of target genes for stress response. Among the genes induced by osmotic stress, GPD1 gene code for glycerol-3-phosphate dehydrogenase is an enzyme involved in glycerol synthesis (Pérez-Torrado, et al, 2002). GDP 1 gene catalyses the conversion of dihydroxyacetone phosphate, a glycolytic intermediate into glycerol 3-phosphate. Glycerol-3-phosphate is dephosphorylated to glycerol by glycerol-3-phosphatase. Hence, osmotic stress induces the glycerol production (Varela et al., 2012).

However, in present study, high amount of residual sugars found in the samples where double doses of benzoic acid (Benzoic acid- 0.025g on day 1+ 0.05g on day 2) added as an epigenetic modifier, had significantly low amounts of glycerol. Interestingly, both samples of benzoic acid (Benzoic acid-0.025g on day 1, and Benzoic acid-0.025g on day 1+ 0.05g on day 2) produced almost same amounts of glycerol. Moreover, intracellular acidification induced by benzoic acid

also can increase the glycerol yield. In cytosol, benzoic acid dissociates, causing the acidification of the cytosol. According to Pérez-Torrado, et al (2002), intracellular acidification induces obvious, rapid and persistent increase in cAMP, which in turn result in activation of GPD1 gene responsible for glycerol production. But, present study demonstrated that intracellular acidification induced by benzoic acid and osmotic stress induced by high sugar concentrations were failed to increase glycerol content. In fact, there is significant decrease in glycerol. This observation may be related to the epigenetic potential of benzoic acid to alter protein expression which is discussed in the later sections.

4.1 Effect of epigenetic modifiers on protein expression

Proteomic study revealed that epigenetic stress alters the protein expression of *Saccharomyces cerevisiae*. In MALDI-TOF spectra analysis, epigenetic modifiers under study inhibited the expression of most of the proteins except the one that lies between m/z 4000-4500 (figure 1). Protein expression was minimal when double dose of benzoic acid (0.025g on day 1+ 0.05g on day 2) is applied on yeast. Though curcumin does not really affect ethanol production, sugar utilization of yeast and glycerol production; curcumin proved to alteration protein expression in yeast. These observations also reflected in cluster analysis. MALDI-TOF cluster analysis grouped the samples with epigenetic modifiers into single cluster indicating that they have similar protein expression which is different from control (without epigenetic modifiers). The alterations in protein expression induced by curcumin and benzoic acid is even visible in SDS-PAGE. However, curcumin induced alteration of protein expression is not clearly visible in SDS-PAGE. Hence, MALDI-TOF proved to be better tool for epigenetic studies as compared to SDS-PAGE.

The alteration of protein expression can be explained by the epigenetic modifying potential of benzoic acid and curcumin. Both benzoic acid and curcumin are the potential histone deacetylase (HDAC) inhibitors. [Since DNA CpG methylation is absent in yeast (O'Kane, & Hyland, 2019), ability of curcumin to inhibit methyltransferases (DNMTs) are not considered in the present study (Hassan et al, 2019).] HDACs are enzymes that removes acetyl group from histone proteins, which induces the DNA-histone binding. Hence, HDACs are associated with gene silencing by making the DNA unavailable for transcription. Effect of HDACs can be altered by another enzyme called histone acetyl-transferase (HAT). In normal cell conditions balance between these two enzymes are highly regulated (Kim, & Bae, 2011). In presence of epigenetic modifiers which have HDAC inhibiting potential (Kim, & Bae, 2011), balance between HDAC and HAT gets disturbed and resulting in protein over expression or suppression

based on the genes transcribed. Apart from histone acetylation, HDAC inhibitors have the potential to induce the production of certain transcription factors and other protein regulating transcription. Hence, HDAC inhibitors can selectively alter the gene expression which ultimately can result in altered protein expression (Xu, Parmigiani, & Marks, 2007).

The ability of HDAC inhibitors to induce alterations in gene expression is commonly exploited in cancer research. Vorinostat (suberoylanilide hydroxamic acid or SAHA) and romidepsin, two FDA approved drugs for the treatment of cutaneous T-cell lymphoma are HDAC inhibitors. Another common example where HDAC inhibitors were used for gene alteration is found in honey bees. HDAC inhibitors in royal jelly can convert non-fertile worker bees into fertile queen, though, both queen and worker bees are genetically identical. A few number of female larvae were fed with royal jelly throughout their development, rest of female population is fed with worker jelly. In royal jelly, a fatty acid, 10-hydroxy-2E-decenoic acid (10-HDA) found in higher concentrations as compared to worker jelly. The fatty acid, 10-HDA is a potent HDAC inhibitor, which will reactivate the epigenetically silenced genes. So, genetically identical female honeybee have different phenotypic expression due to the effect of HDAC inhibitor (Polsinelli, & Hongwei, 2018).

According to Xu, Parmigiani, & Marks, (2007), HDAC inhibitors induced protein alteration patterns are similar for different HDAC inhibitors except the fact they will have definite differences. This observation can be found in present study, as protein alteration pattern of benzoic acid and curcumin are similar. But, the exact mechanism by which gene expression alterations change the protein expression patterns is not very clear. A further proteomic study coupled with genomics research may resolve this problem.

5

Conclusion

Though curcumin had no substantial effect on ethanol production, sugar utilisation and glycerol production; benzoic acid found to produce low ethanol wine with comparatively low glycerol and high residual sugars. So, epigenetics can effectively reduce alcohol production in wine. But, one of the limitation for its application is the high residual sugars in the resulting wine.

However, both curcumin and benzoic acid alters the protein expression of *Saccharomyces cerevisiae*. In fact, in both cases, protein expression mainly downregulated, owing to the epigenetic modifications induced by them. A further research in this field is required to identify the altered proteins and its function in alcoholic fermentation of *Saccharomyces cerevisiae*. Moreover, the effect of dietary epigenetic modifiers on volatile compounds of wine is not yet studied. Subsequently, a further study is needed to identify the effects of these epigenetic modifiers on the flavour profile of wine. Since, it is a new method in food industry, application of dietary epigenetic modifiers in different food products also can be exploited.

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