UNIVERSITI PUTRA MALAYSIA

PROPERTIES OF SOURDOUGH STARTERS FOR BREADMAKING

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ABSTRACT

Sourdough fermentation consumes only water and flour to initiate spontaneous biochemical reactions of wild lactic acid bacteria (LAB) and yeast for leavening, acidification and shelf life extension of bakery products, especially bread. The quality of sourdough is usually determined by physicochemical properties, rheological properties, sensory characteristics and microbial properties. Since sourdough is still not well known in local market and most researches reported on sourdoughs are not climatised to weather in our region, this research was conducted with aims to measure and predict physicochemical properties as well as identify beneficial bacteria of sourdough starters. Sourdough starters were made using wheat, sorghum and rye flours for comparison. 50 grams of flour and 50 grams of distilled water were mixed for each flour type for a 10-day-long fermentation at room temperature of 30.5 $\pm$ 2.5°C. Each of them was refreshed daily by discarding 50 grams for the first refresh and 100 g for subsequent refresh thereafter adding flour and water of 50 grams each after taking samples for analyses. Daily analyses were conducted for measuring sourdough starter's leavening height, pH, total titratable acidity (TTA) and concentration of fermentable sugars. A microbial test was conducted at the end of fermentation. The analysed properties of sourdough starters are interconnected and interdependent. Through the fermentation period, pH of sourdough starters decreased while TTA increased. Wheat sourdough starter achieved the lowest pH (3.23) and highest TTA (14.8 ml) at the end of fermentation, followed by sorghum and rye. Sorghum sourdough starter consisted of the least concentration of sugars, which ranged between 0 and 0.936 g/100g sample for all sugars types and exhibited almost similar trend of sugar concentration changes as wheat and rye sourdough samples due to three-phase microbial succession. As the

greatest height increment among all samples, a leavening height of 82.91% was shown by wheat sourdough starter on day 2 of fermentation. Statistical models were fitted for sourdough starter properties estimation during the fermentation period. Wheat sourdough starter had the lowest microbial diversity, with only one significant class of *Bacilli* (90.2%) while the rest was mostly unclassified. *L. brevis*, *L. paraplantarum*, *L. helveticus*, *Enterococcus* and *Streptococcus* are some important bacteria detected in sourdough starters. Based on the properties studied, the sourdough starters developed in this research had the potential for bread volume increment in breadmaking process as well as shelf life extension in breads. The consumption of sourdough bread aids in enhancing body health too.

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CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

Sourdough fermentation is one of the most ancient method in baking which only requires the mixture of flour and water, involves spontaneous biochemical reaction by lactic acid bacteria (LAB) as well as yeast for leavening and acidification of bread (Lhomme et al., 2015). Due to its inconsistent and time-consuming properties together with the introduction of industrial yeast in the industry since the 19th century, the utilization of this baking method did not grow (Patel et al., 2011). However, the sourdough fermentation method is slowly gaining back its popularity among the artisan bakers worldwide as it provides many benefits for the bakers, especially the excellent leavening properties for all kinds of breads. The market value of sourdough products is also raising because of enhanced aroma, flavour and texture. Consumers are attracted by the healthy sourdough which has its shelf life extended without addition of artificial preservatives (Hui & Sherkat, 2005; Gobbetti et al., 2014; Lhomme et al., 2015; Patel et al., 2011)

Due to geographical and cultural factors, the varieties of sourdough are also large in number as different types of flour are preferred in different parts of the world. For instance, wheat flour is used widely in Mediterranean area like Italy,

Greece, Spain and Egypt, whereas some European countries including Germany, Sweden, Russia and Portugal prefer using rye flour (Hui & Sherkat, 2005). In order to encourage more practice of sourdough fermentation, the properties of different types of sourdough have to be identified so that the fermentation process can be carried out under control to ensure production of sourdough with consistently high quality.

For sourdough, the standard is usually determined by physicochemical properties, rheological properties, sensory characteristics, microbial properties, etc. Among all, physicochemical properties especially pH and total titratable acidity (TTA) which both describe acidity of sourdough were studied the most. Banu et al. (2011) concluded pH and TTA values of rye sourdough ranged between 3.42 and 4.15, and 12.27 ml and 17 ml respectively for different types of flour and fermentation temperatures. There were studies that investigated concentration of organic acids, ethanol and soluble sugars in sourdough too. Besides the properties mentioned, Hanis-Syazwani et al. (2018) also identified fermentation quotient, a parameter in showing value of sourdough, using concentrations of lactic and acetic acids. The organic acid production rates are higher when storage temperature of sourdough during fermentation increases (Venturi et al., 2013).

Bartkiene et al. (2020) studied microbial populations in sourdough and some of their antimicrobial and antifungal properties, which are important for extension of shelf life, whereas Çakır et al. (2020) tested tolerances of LAB towards conditions with high acidity and salt concentration to ensure LAB in sourdough are compatible as probiotics. Some researchers were also interested in dough leavening during sourdough fermentation. They measured leavening height, carbon dioxide production

and carbon dioxide retention of gluten-free sourdough so that the time allowed for leavening before collapsing due to weak dough structure can be applied in the industry (Cappa et al., 2016). By looking into characteristics of various types of sourdough starters, comparisons and analyses can be worked out for better control of sourdough fermentation, resulting in sourdough bread with excellent quality.

1.2 PROBLEM STATEMENT

Sourdough starter, which is very beneficial for the bakery industry, is still not very common in Malaysia but has been popular in the other corners of the world, especially the European countries. One of its natural properties includes enhancing quality of bakery products by dough leavening, extension of product shelf life and being able to replace chemical additives in bakery products. A commonly added chemical additive in bakery products is azodicarbonamide (ADA) that strengthens dough structure but brings potential health risks when semicarbazide and ethyl carbamate are present as by-products (Karunaratne & Pamunuwa, 2017). Gioia et al. (2017) also discussed the possible adverse effects of intaking too much calcium propionate, which is one of the bread preservatives, in children's health. The health of consumers is at risk if manufacturers add too much chemical additives which are meant for enhancing bread quality.

With relatively low acceptability of local consumers towards bread with sour, tangy taste, acidity of sourdough starter as well as sourdough bread should be controlled cautiously in view of increasing sourdough bread acceptability in local market. Hanis-Syazwani et al. (2018) compared bread from wheat flour (control)

with wheat bran and rice bran sourdough bread. The scores for flavour and taste were slightly lower for sourdough breads as compared to bread from wheat flour.

Rye flour is regularly seen as the main ingredient for sourdough starter as it makes the most stable starter while sorghum flour has a great potential in starter making for gluten-free breads due to market demand for such. Main sourdough ingredient such as the rye and sorghum flour are not primitive in our region and most researches reported on sourdoughs are also not climatised to the weather in our region. Thus, in order to initiate the involvement of sourdough starter in our local food manufacturing industry, a study investigating properties of sourdough starter from wheat, sorghum and rye flours locally is essential for comparison and further understanding.

1.3 OBJECTIVES

This research was conducted to study the effect of different types of flour on the changes of sourdough starter properties during fermentation in understanding its behavior for quality control for its final product. To achieve this, the following specific objectives were made:

(i) to measure and predict physicochemical properties of sourdough starters during fermentation,

(ii) to identify beneficial bacteria in sourdough starter, and

(iii) to compare sourdough starter from different types of flour.

1.4 SCOPE OF WORK

This study focused on the comparison for sourdough starter fermentation by using different types of flour which are wheat flour, sorghum flour and rye flour. The mixture of flour and water on day 0 was taken as the control and its physicochemical properties were determined. During the fermentation period of 10 days at room temperature, samples were extracted daily from each sourdough starter type for physicochemical analysis which included measurement of pH, total titratable acidity (TTA), concentrations of water-soluble free sugars and leavening height. All the starters were also refreshed by discarding a small portion and then adding with their respective flour and water daily after the analysis as per the process of making sourdough starter. Microbiological test was conducted at the end of the fermentation. The changes of physicochemical properties for all types of starter with fermentation period were statistically modeled to show exact relationship between each physicochemical property and fermentation time.

1.5 THESIS STRUCTURE

This research is introduced by an overview on sourdough starter addressing its related issues and objectives. The following chapter reviews the literatures related to this research which include substantive findings of theoretical and methodological contributions to this research. Chapter 3 presents materials and methods used in conducting this research. The techniques and analyses used are compiled and described. Then, results determined from this research are displayed and discussed in Chapter 4. The thesis ends with conclusions on research findings and recommendations.

CHAPTER 2

LITERATURE REVIEW

2.1 SOURDOUGH

The existence of sourdough has been recorded since several hundreds years ago in Egypt, as early as 3000BC (Wood, 1996; Hui & Sherkat, 2005). Sourdough was initially the only technique in baking before generalisation of yeast usage in bakery industry. Nowadays, sourdough can be found in the manufacture of various types of bakeries, for instance breads, cakes and crackers (Aplevicz et al., 2013; De Vuyst & Gänzle, 2005). Many artisan bakers are also persistent in using sourdough for their bakery products. Some famous examples are San Francisco sourdough bread, many traditional Italian sourdough breads, and German three-stage rye sourdough bread (De Vuyst et al., 2017). Hui & Sherkat (2005) suggested that all range of sourdough products containing 'living' lactic acid bacteria (LAB) should also be considered as sourdough and not just breads alone. Made from flour and water, the sourdough has LAB which induce lactic acid fermentation within.

One of the reasons to involve sourdough in bakery products is better dough properties. By addition of sourdough, the dough has better gas retention capacity and possible enhancement in the gluten network structure during proofing (Tamani et al., 2012). Therefore, the product, especially bread, may have increased loaf size and

softer texture. Furthermore, the flavour of sourdough bread is richer and more aromatic than usual bread without sourdough. The taste and nutritional values are strengthened too. Sourdough is claimed to be helpful in extending shelf life due to the presence of organic acids and carbon dioxide released by lactic acid bacteria (Chavan & Chavan, 2011).

A piece of a high-quality sourdough refers to the food product which is both consistent and microbially stable. Besides, the natural presence of sourdough yeast is also possible in sourdough. After achieving the stability in the microbiota within the sourdough, the sourdough can be said to have a good fermentation capacity, which may be affected by the activity of microbial flora (lactic acid bacteria and yeast), types of flour (wheat flour, rye flour, etc.) and some process parameters like temperature, fermentation time and quantity of starter (Hui & Sherkat, 2005). In short, sourdough is majorly influenced by the sourdough starter, which supports the microbiota and gives main characteristics of a typical sourdough.

2.2 SOURDOUGH STARTER

Sourdough starter refers to a dough or batter, which has a detectable acidity as a product of fermentation of wild yeasts and bacteria, used to leaven other dough (Gisslen, 2012). By referring to Figure 2.1, sourdough can be categorised according to how it is being started, whether through spontaneous fermentation, adding a piece of mature sourdough (mother sponge) or adding a known starter culture. Type I sourdough, which is prepared at room temperature (ranged from 25-30°C), is continuously passed on through backslopping using newly prepared batch of flour and water; whereas type II is obtained from the industry under higher fermentation temperature with known strain of starter culture. As for type III, the sourdough starter is initiated by some adapted starter strains in dried form and followed by backslopping (Chavan & Chavan, 2011). Backslopping is a common fermentation technique by utilizing some of previous sourdough starter through continuous inoculation and mix them with fresh flour and water to start the next fermentation.

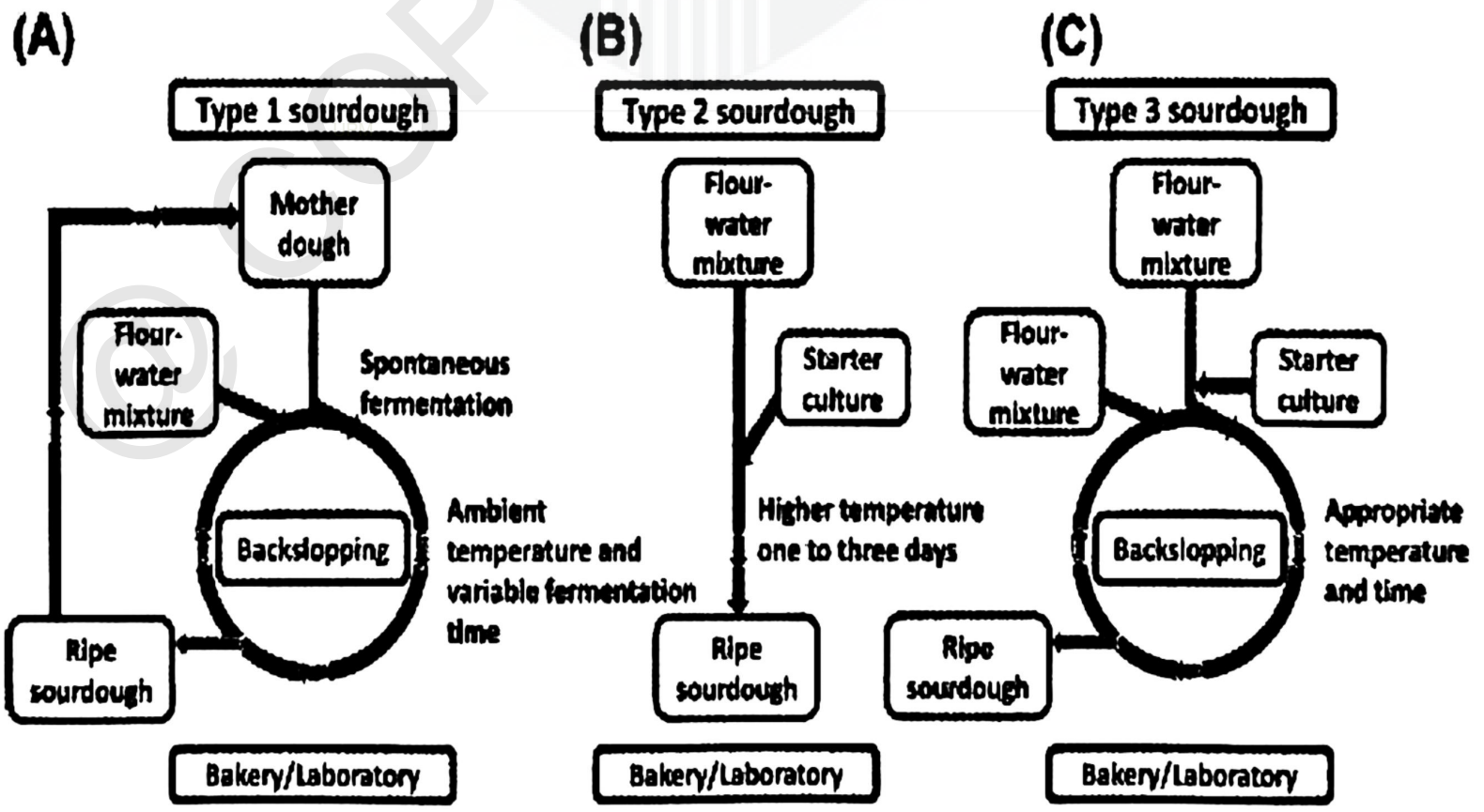

Figure 2.1: Types of sourdough from different sourdough starter preparation methods (De Vuyst et al., 2017)

Type I is the most traditional type of sourdough. The formation of type I sourdough has been extensively studied in laboratories, substantially backslopped wheat and rye sourdough fermentation processes (De Vuyst et al., 2017). Thus, this type of sourdough is also frequently started for research purposes. In the process of starting a type I sourdough starter, only flour and water are needed. After the mixture of flour and water is left to stand for a long enough period, it starts fermenting eventually by the wild yeast or bacteria originated from the ambient air or the flour. The initial fermentation time usually takes at least two to three days. The microorganisms creating sourdough starter vary from place to place due to difference in temperature, nutrition, humidity and other conditions (Gisslen, 2012).

In order to ensure the constant quality of sourdough as a commercial product, sourdough starter (Böcker Reinzuchtsauerteig, referring to Böcker pure breed sourdough) appeared to be commercially ready in the market since 1910 by referring to the methods in production of yeasts for breweries (Hefe-Reinzucht, which means yeast pure culture in English). Following that, types II and III sourdoughs were then introduced to the industry and society. The development of starter cultures boosts the quality and food safety in sourdough breads and yet giving no effect on the process time (Brandt, 2007).

2.3 SOURDOUGH INGREDIENTS

Due to different methods in preparation of sourdough starter, properties of sourdough can also be greatly influenced by the only non-sterile ingredient, *i.e.* the flour. The flour chosen for preparing the sourdough starter is the substrate for the microbial fermentation. The flour components have great impacts on various properties of sourdough, including flavour, texture, shelf life and nutritional quality during the stages of preparing starter and dough as in Table 2.2 under Section 2.4.3 (Gänzle, 2014). Therefore, starters prepared from different ingredients, for example, wheat flour, sorghum flour and rye flour used in this study, exhibit different characteristics too since they are consisted of different composition of flour components, such as starch, gluten, pentosans and so on. Whole grains or fruits are common ingredients used for preparing sourdough starter since wild LAB and yeasts can be naturally found on their surfaces. Gisslen (2012) reported that the usage of whole grain flour is more common as this is one of the most reliable and best methods in creating a sourdough starter.

2.3.1 Wheat Flour

Figure 2.2: Wheat (*Triticum aestivum*) grain (Lyddon, 2018)

Wheat (*Triticum aestivum*), as shown in Figure 2.2, is used widely as carbohydrate source in many countries such as China, European Union, India, etc. In the marketing year of 2018/2019, China had already consumed 125 million metric tons of wheat (Shahbandeh, 2020). Referring to statistics from United States Department of Agriculture (Organisation for Economic Co-operation and Development, 2018), Malaysia contributes to an average annual wheat consumption of 1.425 million metric tons, in which the consumption as food by itself is already 69.54%. Wheat is mostly milled into wheat flour, which is the ingredient for various staple food like bread and noodles. Wheat flour can be differentiated into many different types due to different level of milling. For example, whole wheat flour is milled of the whole grain, whereas white flour only consists the endosperm (Marcus, 2013). In terms of extraction rate, which refers to the percentage of original grain left in the milled flour, De Angelis et al. (2019) deduced that the whole wheat flour has a

value of 100% while white flour refers to the range of flour with lower extraction rates. Table 2.1 summarises the types of white flour with the respective extraction rates under the system in Italy. The degree of refinement and nutrient loss increases as the type number decreases (Whitley, 2009). The flour also appears whiter when type number decreases.

Table 2.1: Types of white flour with respective extraction rates in Italy (Whitley, 2009)

Type	Extraction rate (%)
00	50
0	72
1	80
2	85

Wheat flour can be consisted of 6 basic components in its dry form, which are 70-75% of starch, 10-12% of total protein, including 70-80% storage proteins (gluten) and some water-soluble proteins, 2-3% non-starch polysaccharides (pentosans), 2% lipids and inorganic compounds (ash) (De Vuyst et al., 2017). 1-2% of fermentable carbohydrates is usually found in wheat flour. The presence of glucose, fructose and maltose is detected with the usage of wheat flour in sourdough fermentation. Besides, lactic acid, acetic acid and ethanol are also produced in the starter as by-products (Lefebvre et al., 2002). In wheat flour, the 2 basic types of protein that wheat flour contains are gliadin and glutenin, which are main components of gluten. The presence of gluten plays an important role in the viscoelastic properties of dough since an elastic and cohesive network can be built up by the wetted gluten. Besides that, there are also some phenolic acids found in wheat grains, such as hydroxycinnamic acids (C6 - C3 compounds) and hydroxybenzoic acids (C6 - C1 compounds), which have antimicrobial properties, protecting the grains from being attacked by pathogens (Ripari et al., 2019).

2.3.2 Sorghum Flour

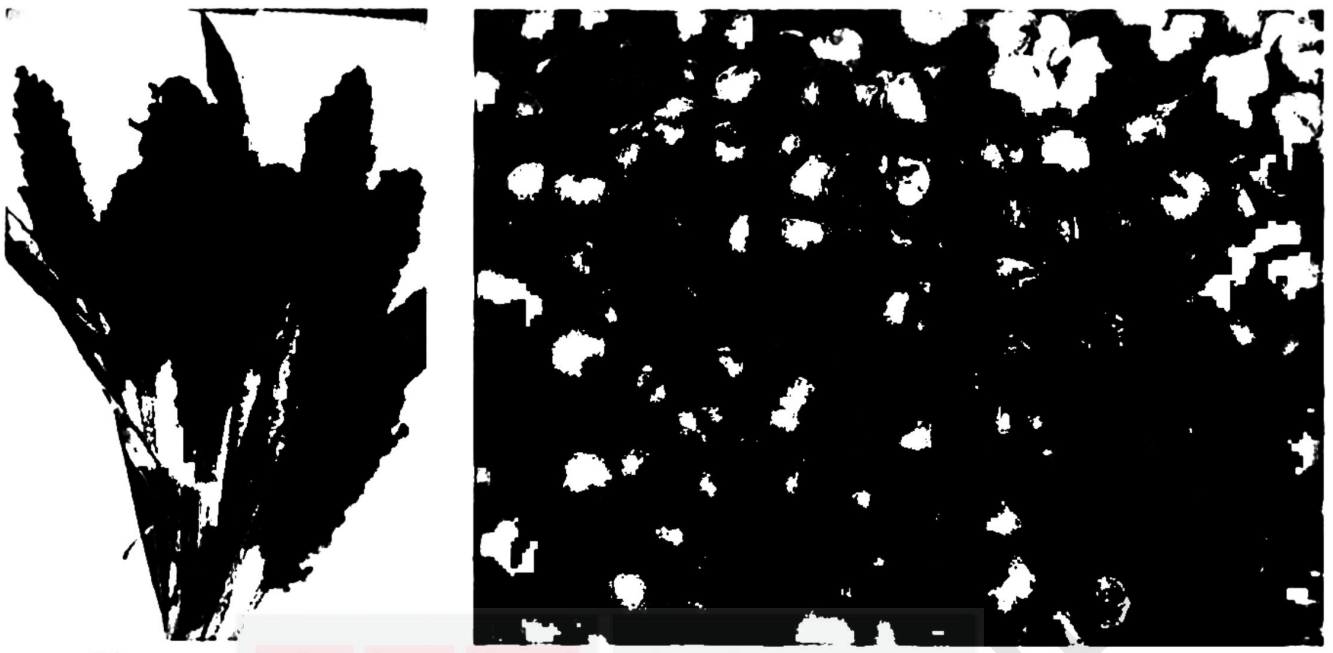

Figure 2.3: Sorghum (*Sorghum bicolor*) grain (Heuzé et al., 2015)

Originating in Africa and India, sorghum (*Sorghum bicolor*) is one of the oldest known as well as the fifth most important grains in the world (Ogunsakin et al., 2015). Referring to the statistics from United States Department of Agriculture (USDA), Nigeria with the consumption of 6.8 million metric tons, United States and Mexico are ranked as the top three among the countries which consumes sorghum in the year of 2019. Sorghum, as shown in Figure 2.3, is usually used in cooking porridge, thickening stews as well as production of alcoholic drinks and syrup. The usage of sorghum flour is common too, for instance, in the making of Indian flatbread and jowar roti. Sorghum flour has light or beige colour with mild sweet flavour (Hartley, 2019).

Nordin (1959) claimed that sorghum grains contain significant quantities of free sugars (1-1.4% of ketose sugars and 0.2-0.5% of reducing sugars). Moreover, the amount of pentosans present in sorghum flour is ranged between 2.51% and 5.57%, which is higher than in wheat flour (Karim & Rooney, 1972). However, the usage of sorghum flour in baking breads is difficult due to some limitations. For example, some varieties of sorghum can be easily contaminated with geographically

widespread mycotoxigenic fungal species, which can also be sourced from other grains. The fungal genera *Aspergillus* and *Fusarium* are most commonly found to produce mycotoxins that can contaminate sorghum. The breads baked from sorghum flour are claimed to be of low quality and have dissatisfying mouthfeel and flavour (Moroni et al., 2009). Dicko et al. (2015) whereas related resistance of sorghum with concentrations of phenolic compounds, particularly proanthocyanidins (PAs) and 3-deoxyanthocyanidins (3-DAs) in which their concentrations are good indicators of resistance towards fungi and mold.

Flour milled from sorghum is usually chosen for making of gluten-free sourdough starter despite the lack in natural sponginess as well as strong smell and taste during fermentation (Kane, 2013). Presence of gluten which is necessary in bread baking for the elasticity, strength and stability can be omitted by incorporation of sourdough starter into sorghum flour. Olojede et al. (2019) reported the activities of cultures (*Pediococcus pentosaceus, Weissella confusa, P. pentosaceus and Saccharomyces cerevisiae*) in the sourdough starter are believed to improve the rheological, textural and nutritional characteristics of the bread made from sorghum flour. The incorporation also enhances the swelling properties of polysaccharides through acidification, provides structure through exopolysaccharides produced in situ by the fermenting lactic acid bacteria, improves the shelf life of bread by delivering antifungal compounds, or improves flavour by releasing organic acids, amino acids, or other metabolites during fermentation (Arendt & Renzetti, 2009). Besides being gluten-free, it possesses rich fiber as well as low starch digestibility, hence it is suitable for consumers with health issues such as celiac disease (severe gluten intolerance), diabetes, obese and so on. This widens the marketability of sorghum sourdough bread as gluten-free bread.

2.3.3 Rye Flour

Figure 2.4: Rye (*Secale cereale*) grain (Staughton, 2020)

Rye (*Secale cereale*) flour is ordinarily used for bread making in countries with northern climate, for example, Germany, Russia, and countries of Central Europe, as rye is tolerant of the extreme conditions, both frost and drought. The appearance of rye grains are displayed in Figure 2.4. After wheat flour, rye is the second mostly used flour by bakers in the world. Rye flours are generally classified based on their colour, white, medium or dark. They are different due to the different milling methods. The milling from the center of kernel gives white rye flour, the patent grade which consists of the least percentage of bran. Then, the medium rye flour is known as the straight grade with a darker greyish appearance. Medium rye flour contains the whole starchy endosperm, whereas most the bran and germ are removed after milling. The clear flour grade corresponds to the dark rye flour. It is the fraction of flour which removed from the process of producing patent flour. The colour is darker and the enzymatic activity is higher due to the closer origin from outer bran layer (Kovacic, 2011). Although rye flour is not commonly utilised in Malaysia yet, this type of flour attracts the attention of many bakers, as the mixing of whole-grain rye flour into a dough with water is known as the most reliable methods in creating a sourdough starter (Gisslen, 2012).

As compared to wheat, rye has the almost similar total proteins fraction. However, rye flour has lower forming ability of gluten, hence is unable to form an elastic dough structure like wheat flour. This characteristic of rye flour is also because of its higher percentage of pentosans, which inhibits hydration and stickiness of rye dough as well as lengthen the shelf life of rye products. Phenolic compounds in rye grains help in raising tolerance of rye grains towards pathogenic attack, thus further extending shelf life of rye products. Some examples of antimicrobial phenolic compounds found in rye grains are benzoxazinoids (Andersson et al., 2014), hydroxycinnamic acids and hydroxybenzoic acids (Ripari et al., 2019). Rye flour also contains more amylase than wheat flour, which degrades starch, thus makes rye dough become extremely sticky and difficult to work once this biochemical reaction is not controlled well (Kovacic, 2011). To tackle this problem, the involvement of sourdough starter is therefore necessary for rye bread production as the acidifying process caused by the lactic acid bacteria (LAB) leads to the inhibition of amylase activities. Excessive starch degradation during baking of rye bread can then be stopped due to the low pH condition.

In order to bake rye bread with high quality, the demand for dough acidifiers, which were comparatively more convenient than sourdough starter, grew rapidly with the increasing use of baker's yeast. In the 1920s, the first dough acidifier, a mixture of pregelatinised flour and lactic acid came to the market, especially in Germany and Austria. As the acceptance of rye bread was affected due to the unsatisfying flavour after adding dough acidifiers, the development of dried sourdoughs as convenient bakery ingredient was also initiated at that time and resulted finally in the early 1970s in the development of naturally fermented dried sourdoughs with total titratable acidity (TTA) of more than 200 (Brandt, 2007).

2.4 PHYSICOCHEMICAL PROPERTIES

As sourdough starters made from different types of flour are also distinctly different, the comparison among them can be carried out by studying their physicochemical properties. Some common physicochemical properties which are closely related to sourdough starter are pH, total titratable acidity (TTA), concentration of organic acids, water-soluble free sugars and ethanol. Significant changes in pH and TTA of sourdough starter are usually reported since fermentation processes usually produce organic acids as by-products. Hanis-Syazwani et al. (2018), Lim et al. (2017), Tamani et al. (2012) and Banu et al. (2011) are among the researchers who proposed the drop in pH and rise in TTA after fermentation of sourdough starter. Concentrations of water soluble free sugars (sucrose, maltose, glucose and fructose) are different from sourdough starter which are inhabited by different types of sourdough microbiota (Hanis-Syazwani et al., 2018; Lim et al., 2017) and from sourdough starter which experienced different length of fermentation period (Lefebvre et al., 2002).

2.4.1 pH

pH is frequently used to characterise quality of sourdough starter. pH is defined as the logarithm of the reciprocal of the hydrogen ion concentration. The definition of pH is also the negative logarithm of molar concentration of hydrogen ions. In order to define and express acidity, pH and total titratable acidity (TTA) are both frequently used as they are interrelated. The same amount of acid in the samples can result in different pH. This is because food acids usually dissociate partially but strong acids are fully dissociated, hence results in different concentration of hydrogen ions in samples.

Chin (2020) claimed the range of pH in most sourdough starters is between 3.5 and 5 whereas Komlenić et al. (2012) reported the pH of sourdough starters from wheat flour, rye flour or mixture of them are approximately 4.0. For sourdough starters made from wheat flour, Aplevicz et al. (2013) found pH of them at 3.44. Study of Banu et al. (2011) for rye sourdough starters deduced the final pH ranged from 3.93 to 3.97 while 3.5 was pH of sorghum sourdough starters measured by Karrar et al. (2019). The low pH of sourdough starter is mainly due to the organic acids, such as lactic acid and acetic acid, released from activities of LAB. The pH of sourdough starter affects the mixing of dough, as shorter mixing time is needed for dough with lower pH (Chavan & Chavan, 2011). The shelf life of final product can be extended as well with lower pH because some pathogenic bacteria and mould cannot grow in acidic conditions (Sadeghi, 2008).

2.4.2 Total Titratable Acidity (TTA)

TTA is a major quality criterion of sourdoughs and is defined by the consumption of sodium hydroxide (NaOH) during the titration. Sadler and Murphy (2010) explained that the endpoint of titration is commonly determined by achieving a targeted pH or showing change in colour of pH-sensitive dye, like phenolphthalein (changes from colourless to pink). It measures total acids produced by yeast and lactic acid bacteria during fermentation. Some of the commonly found acids in sourdough starter are lactic and acetic acids.

Generally, TTA is expressed in millilitre of 1.0N sodium hydroxide used for titration involving 10 grams of sourdough starter. Hanis-Syazwani et al. (2018) found that TTA values of rice bran sourdough starter fermented with selected LAB strains of *L. brevis*, *L. plantarum* and *L. sanfranciscencis* ranged from 20.6 to 21.83 ml/10g sourdough. The range of TTA was reported to be in between 12.3 and 13.4 ml 10g sourdough in another research of wheat sourdough starter by using different strains of *Lactobacillus delbrueckii subsp. bulgaricus* (Tamani et al., 2012). If flour with lower extraction rate is used, the sourdough starter usually possesses TTA values from 8 to 11. TTA values are higher, *i.e.* between 16 and 22, if wholemeal flour is used in fermentation of sourdough starter (Hui & Sherkat, 2005). After 1 day of fermentation, Banu et al. (2011) depicted final TTA of whole rye sourdough starter at 14.52 ml whereas dark rye sourdough starter at 12.27 ml.

By measuring TTA during a sourdough fermentation process, the fermentation rate and period for achieving mature fermentation are determined. Analysis using TTA is sometimes preferable as the value is not affected by the presence of buffering in samples unlike pH. Nonetheless, TTA only shows basic estimation of the total

acid amount in food. It is more suitable in predicting the level of tartness of food which can be easily affected by presence of sugars (Sadler & Murphy, 2010). Hence, the extent of fermentation and increase in acidity in sourdough starter can be controlled precisely by monitoring both pH and TTA. Typically, higher TTA results in stronger sour odour and flavour. For research with known predominant acid, for instance, lactic acid, TTA can be calculated using the formula as below in the unit of % lactic acid:

$$\%acid(wt/wt) = \frac{(N)(V)(Eq_{wt})}{W(1000)} X100,$$

where N = normality of titrant (mol/ml)
V = volume of titrant (ml)
Eq_{wt} = molar weight of predominant acid (mg/mol)
W = mass of sample (g)
1000 = factor relating mg to gram (mg/g)

2.4.3 Water-soluble Free Sugars

In different types of flour, the amount of water-soluble free sugars such as maltose and glucose also vary. Soccol et al. (2013) reported that the concentration of water-soluble free sugar is generally low in both rye and wheat flours, ranged between 1.55% and 1.85%. In wheat sourdough, maltose is usually more abundant than glucose, whereas contrary glucose concentration is higher in sorghum sourdough (Sekwati-Monang et al., 2012).

Water-soluble free sugars act as substrate for the fermenting microorganisms. This means that, during fermentation, LAB consumes these fermentable sugars that are available in sourdough starter for production of carbon dioxide, ethanol and organic acids. Despite this, oligosaccharides and polysaccharides are not being

metabolised in sourdough starter (Batt & Tortorello, 2014). The metabolism of sugars is not necessarily same for fermentation under all conditions because the final sugar concentrations can be contrary from one sourdough starter to another when the type of fermentation is different.

The type of fermentation is classified according to the end products of sugar metabolism. Homofermentation occurs when conversion of sugars in starter produces more than 90% of lactic acid while only little acetic acid is found. End products of heterofermentation, another type of fermentation, comprise of lactic acid (around 50%) and other organic substances, for instance, ethanol, acetic acid, carbon dioxide and acetaldehyde (Chavan & Chavan, 2011; Donnelly, 2014; Hui & Sherkat, 2005). Sugar metabolism of homofermentative LAB is less studied as this type of LAB is usually outcompeted by heterofermentative LAB in the sourdough starter system (Van der Meulen et al., 2007). Gobbetti and Gänzle (2012) proposed some reasons of heterofermentative LAB as the dominating ones in sourdough system are carbohydrate metabolism with better adaptation, stronger amino acid assimilation as well as being more resistant to environmental stresses.

Glucose, one of the water-soluble free sugars, is a simple sugar with a molecular formula of $C_6H_{12}O_6$. Due to enzymatic activity on flour, glucose is formed through hydrolysis of starch. At the same time, glucose is also converted by metabolism of heterofermentative LAB via phosphoketolase pathway to lactate, ethanol or acetate and carbon dioxide gas (Chavan & Chavan, 2011). Thus, the concentration of glucose usually does not deviate much from the beginning of fermentation if the rate of consumption for fermentation and rate of generation from starch hydrolysis is almost the same (Leroy et al., 2006). The enzymatic activity on rye flour is faster

than wheat flour, hence providing more available glucose for fermentation of sourdough starter (Soccol et al., 2013).

Two units of joined glucose exist as maltose ($C_{12}H_{22}O_{11}$). The concentration of maltose in rye flour is high among the fermentable sugars (Hui & Sherkat, 2005). Lefebvre et al. (2002) reported that maltose may be increased along fermentation period due to hydrolytic activity of amylase on starch damaged during milling. Besides, the amount of maltose is also affected by sourdough microbiota. For instance, *L. sanfrancisco* reacts with maltose by hydrolysis, then one of the glucose molecules can be excreted and free for fermentation of starter (Hui & Sherkat, 2005).

Other than that, the chemical formula of maltose is similar as sucrose, *i.e.* another type of soluble sugar. Sucrose, present naturally in low quantity in flour, is hydrolysed rapidly due to sourdough yeast invertase activity so that some of the released glucose and fructose is available for LAB activities (Chavan & Chavan, 2011; Leroy et al., 2006). Fructose is also possible to appear in sourdough starter. It joins with glucose to form sucrose. If fructose is present, heterofermentative LAB utilise it as an electron acceptor by reducing it to mannitol with concomitant oxidation of NADH to NAD+ (Batt & Tortorello, 2014). Ray and Montet (2017) claimed that this mechanism is essential as the conversion of acetyl CoA, a crucial cofactor, to acetyldehyde and consecutively ethanol is not necessary anymore. The starter hence does not release the ethanol odour which may be undesirable in sourdough products. As summarised in Table 2.2, the cofactor can be regenerated and utilized in Kreb's Cycle, which supplies energy to LAB. The other functions of carbohydrate metabolism can be studied from the same table too.

Table 2.2: Roles of carbohydrate metabolism during sourdough fermentation in microbial physiology and contribution to bread quality

Role in microbial physiology	Contribution to bread quality
Energy metabolic (maltose[a], sucrose[a], glucose[b])	Texture (starch)
Cofactor regeneration (fructose)	Water binding, staling (starch, pentosans, EPS)
Protection against environmental insults (oligosaccarides, exopolysaccharides)	Taste and shelf life (organic acids)
Biofilm formation (exopolysaccharides)	Generation of reducing sugars for flavour generation during baking
	Dietary fibre and prebiotic oligosaccharides

[a] requires further hydrolysis into simpler sugars
[b] readily available source of energy
Note: Adapted from Gänzle, (2014) and Jurtshuk (1996)

The changes of sugar content with fermentation time can be determined using high performance liquid chromatography (HPLC) which consists of components shown in Figure 2.5, including solvent, pump, sample injector, column, detector, waste removal site as well as computer for data acquisition (Aryal, 2018). Different mechanisms can be applied for the separation of the fermentable sugars in the starter according to the structural diversities and isomeric multiplicity. As for sourdough starter, it is frequent for studies to incorporate concept of hydrophilic interaction in HPLC by using an amino-bonded silica gel column with acetonitrile-water as the mobile phase. This mechanism is useful in separating monosaccharides and disaccharides. However, the amino groups on column loss easily when hydrolysis occurs. It also readily reacts with aldehydes and ketones, which are commonly found in food samples. Polyamine-bonded polymer gel columns, being more stable and better in performance, are then introduced as the amino-bonded silica gel column had a short lifetime (Soga, 2002).

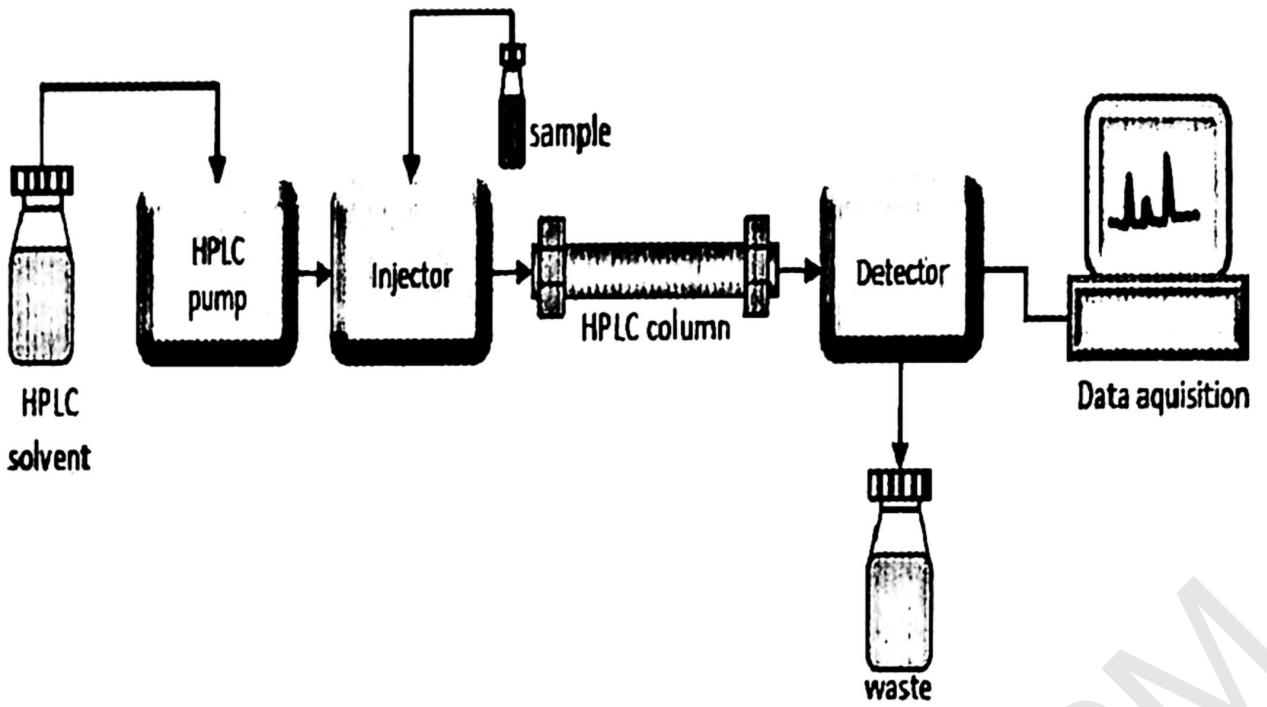

Figure 2.5: Main components of HPLC system (Aryal, 2018)

For measurement of sugar concentration using HPLC, the sample is injected using a syringe into the system to join the flow of solvent (mobile phase), which is controlled by pump, towards separation column (stationary phase). Following that, targeted analytes, such as water-soluble free sugars in this research, move across HPLC column at different rates due to their respective degree of interactions with the column (Aryal, 2018). By taking refractive index of solution as function of component concentration in solution, deviation of directed light beam in solution at the detector hence can be one of the quantitative methods in HPLC. Lastly, the quantity of analytes can be acquired using HPLC software installed in computer when the peaks of graph are identified at specific retention time (Moldoveanu & David, 2012).

2.5 LEAVENING HEIGHT

Leavening height is one of the important parameters of bakery products. If the leavening reaches certain amount of height, volume of bakery products can be raised and the texture can be enhanced too. Hui et al. (2008) suggested that there are a few types of agents applied in bakery industry for the leavening purpose including yeast, natural agents (sourdough starter) and chemical agents (baking soda, cream of tartar, baking powder, etc.). The usage of sourdough starter in leavening is receiving more attention since it is natural and capable in providing good gas holding capacity as well as desirable structure in bakery products. The leavening is achieved by expansion due to formation of gluten network structure which entraps carbon dioxide gas released as by-products from the starter (Preedy & Watson, 2019). Leavening height is generally determined by measuring the reading of starter height along the fermentation, more specifically, before and after each refreshing.

Edema and Sanni (2008) discussed leavening height, by placing a short meter ruler along the side of the bottle containing fermenting starter, while researching the functional properties of sourdough starter in maize bread. Ogunsakin et al. (2015) also measured the leavening height for sorghum starter after 0 hour, 8 hours, 24 hours and 48 hours of fermentation by using the similar method. Due to poor leavening ability of gluten-free flour, sourdough starter was therefore being investigated in its ability in affecting dough maximum height (H_m) and dough height at the end of the test (H_f) by using Chopin rheofermentometer (Cappa et al., 2016).

2.6 MICROBIOLOGICAL PROPERTIES

Sourdough starter exists as a satisfactory habitat for wide variety of microorganisms, particularly lactic acid bacteria, because of the conditions during fermentation of starter, such as fermentable sugar in flour, water content, temperature and pH that support the growth and reproduction of LAB. At the same time, the unique characteristics of sourdough, such as leavening capability and sour taste, are also due to the microbial activities. Recent researches on microbiological properties of sourdough starter discuss the interactions between different microorganisms in sourdough starter, identification of new sourdough species, inhibitory substances from sourdough LAB as well as the induced specific enzymatic activities (Hui & Sherkat, 2005). One of the studies reported the increase of microbial content in each sourdough starter containing *Lactobacillus plantarum* and *Leuconostoc* respectively after 20 hours of fermentation with the decrease in starter pH and increase in TTA (Robert et al., 2006). Sekwati-Monang et al. (2012) investigated the competitiveness between LAB including *L. sanfranciscensis*, *L. parabuchneri* and *L. casei* and realised that the number of *L. sanfranciscensis* increased progressively in wheat sourdough starters but this LAB did not grow well in sorghum starters. From the studies of Ercolini et al. (2013), after fermentation of 1, 2, 5 and 10 days, the spontaneous wheat and rye starters were all dominated by *Firmicutes*, which LAB belongs to, *Proteobacteria* and *Cyanobacteria*.

Researchers have applied various approaches in studying microbiological properties of sourdough starter. Most focused on total viable count, a quantitative estimation about concentration of microorganisms. For example, Aplevicz et al. (2013) deduced that lactic acid bacteria showed the highest microbial counts over

time in the sourdough starter including *Lactobacillus paracasei* and *Saccharomyces cerevisiae*. LAB counts achieved the value of approximately 8.5 log CFU/g, whereas there is about 7.1 log CFU/g of yeast in the culture. The same trend was observed when using sorghum starter (Ogunsakin et al., 2015). In another research by Edema and Sanni (2008), the higher LAB counts initially increased to 6.45 log CFU/g after 48 hours fermentation while yeast counts recorded a rise-up to 6.64 log CFU/g over the same period of fermentation. De Angelis et al. (2019) used Genomix4Life to conduct 16S metagenetics using the Illumina MiSeq platform which deduced the domination of *Lactobacillaceae* and *Leuconostocaceae* in wheat sourdough starters after 10 days of fermentation. LAB (*L. lactis*) was also found to be the bacteria with the highest relative abundance of 37.1% in traditional Mexican sourdough by utilizing GenBank® (Escalante et al., 2001). In Lhomme et al.'s (2015) study, 63% of total number of strains identified was reported to be *L. sanfranciscensis* when the software of GSMapper was used for determining the microbiota in some French traditional sourdoughs.

2.6.1 Lactic Acid Bacteria (LAB) Identification

LAB, being gram-positive are non-spore forming rods or cocci that produce lactic acid as one of the fermentation products. LAB are also aerotolerant anaerobes, possessing anaerobic metabolism without being sensitive to presence of oxygen (Florou-Paneri et al., 2013). Bacteria which are classified under LAB are rapidly increasing. Holzapfel and Wood (2014) suggested that LAB should include six families of *Aerococcaceae* (7 genera), *Carnobacteriaceae* (16 genera),

Enterococcaceae (7 genera), *Lactobacillaceae* (3 genera), *Leuconostoccaceae* (4 genera) and *Streptococcaceae* (3 genera).

Table 2.3: Group of lactobacillus isolated from sourdoughs (Hui & Sherkat, 2005)

Characteristics		Obligately homofermentative	Facultatively heterofermentative	Obligately heterofermentative
Growth at	15°C	-[a]	+[b]	+/-
	45°C	+	-	+/-
Pentose fermentation		-	+	+
CO_2 from	glucose	-	-	+
	gluconate	-	+	+
FDP aldolase present		+	+	-
Phosphoketolase present		-	+	+
Lactobacillus		*L. acidophilus* *L. amylovorus* *L. delbrueckii spp. bulgaricus* *L. delbrueckii spp. delbrueckii* *L. farciminis* *L. helviticus* *L. leichmanni* *L. mindensis*	*L. alimentarius* *L. casei* *L. curvatus* *L. paralimentarius* *L. plantarum* *L. rhamnosus*	*L. brevis* *L. buchneri* *L. fermentum* *L. fructivorans* *L. frumenti* *L. panis* *L. pontis* *L. reuteri* *L. sanfranciscensis* *L. viridescens*

[a] The group of LAB does not have the specific characteristic.
[b] The group of LAB possesses the specific characteristic.

LAB can be divided according to their carbohydrate fermentation patterns, namely obligately homofermentative LAB, obligately heterofermentative LAB and facultatively heterofermentative LAB. They breakdown carbohydrates under different pathways, hence resulting in different end-products. The more detailed explanations can be found under section 2.4.3 which discussed about the water-soluble free sugars. In short, only obligately heterofermentative LAB release carbon dioxide as one of the by-product, and not by facultatively heterofermentative LAB which produce equimolar of lactic acid and acetic acid if gluconate is not

present (Chavan & Chavan, 2011). Table 2.3 summarizes this type of classification and some common LAB found in sourdough starters.

The dominance of *L. sanfranciscensis* was observed in traditional sourdoughs, both from wheat and rye (Lhomme et al, 2015; Ganchev et al., 2014; Randazzo et al., 2005). The growth of this LAB is conducive under pH between 3.9 and 6.7 (Gobbetti & Gänzle, 2012). It is an obligately heterofermentative LAB, thus contributes to the sour, tangy taste as well as the excellent leavening ability of sourdough products. *L. sanfranciscensis* preferentially metabolises maltose over glucose to generate large amount of lactic acid and acetic acid (De Vuyst & Neysens, 2005). This reaction also indirectly rises the concentration of glucose in the sourdough after the hydrolysis of maltose by *L. sanfranciscensis.*

Apart from that, *L. fermentum* is frequently found in Type I sourdough as an obligately heterofermentative LAB (Randazzo et al., 2005). The presence of *L. fermentum* in Type II sourdough that involves longer fermentation period and higher temperature is prevalent too since it has better acid tolerance (Gobbetti & Gänzle, 2012). It is always discussed together with *L. brevis*, one of the oldest heterofermentative LAB, as both of them fall under the classification of probiotics which are used to enhance gut health through improvement of the microecology of the gastrointestinal tract. De Angelis and Gobbetti (2011) proposed that *L. fermentum* and *L. brevis* can also grow in cheese under conducive temperature and salt concentration.

Being susceptible to higher temperature of more than 45°C and higher concentration of undissociated acetic and lactic acids, *L. pontis* can still grow due to excellent adaptability under unfavourable fermentation conditions (Hui & Sherkat,

2005). Thus, it always predominant over *L. sanfranciscensis* in Type II sourdough (Kulp & Lorenz, 2003). Different from acidity, the minimum pH for survival of *L. pontis* is not that low, around the value of 3.5 (Gobbetti & Gänzle, 2012).

The inoculation of *L. plantarum*, one of the facultatively heterofermentative LAB, in bread is beneficial due to its antifungal property. The usage of calcium propionate, for the purpose of extending shelf life, can be reduced by 30% once *L. plantarum* is involved in the sourdough starter of bread making (Ryan et al., 2008). From studies of Dal Bello et al. (2007), growth of fungi, such as Aspergillus, Fusarium and Penicillium spores, were prevented through the incorporation of this LAB through in vitro and in wheat bread. Furthermore, *Lactobacillus plantarum* preferably metabolises maltose and glucose over fructose and sucrose in sourdough starters (Kulp & Lorenz, 2003).

Other than that, species like *Lactobacillus kimchii* and *Lactobacillus alimentarius* are also commonly found as they readily utilise all soluble sugars, maltose, glucose, sucrose and fructose, for energy metabolism (Randazzo et al., 2005). Ganchev et al. (2014) found that *Lactobacillus spicheri*, *Lactobacillus paralimentarius* and *Lactobacillus kimchii* as some dominant LAB in spontaneously fermented rye bread.

2.7 SUMMARY

Sourdough is beneficial from many aspects, such as, increasing loaf size, softening texture, enriching flavour and nutrients, as well as extending shelf life. By daily addition of flour and water, lactic acid fermentation occurs due to growth of LAB, thus converting dough into a stable and acidic microbial system, known as sourdough starter, which is used for dough leavening. The flour involved induces great impacts on properties of sourdough starter due to different flour composition. Usage of wheat flour for sourdough starter is common since it is widely consumed, whereas gluten-free sorghum flour has stronger structural properties with sourdough. In the meanwhile, rye flour requires the acidic condition to prevent excessive starch degradation. To further understand behaviour of sourdough starters, properties including concentration of water-soluble free sugars, pH and TTA because of organic acids accumulation, leavening height due to carbon dioxide release, together with microbiological properties, are usually investigated by researchers.

CHAPTER 3

METHODOLOGY

3.1 OVERVIEW OF RESEARCH FRAMEWORK

This chapter explains the outline of methods used in this research. The important materials and equipment are described. Figure 3.1 shows the summary of the research in a flow diagram. 50 grams from each type of flour, wheat, sorghum and rye flours, was mixed with 50 grams of distilled water respectively. Each sample was also replicated, thus there were a total of 6 samples. All the mixture of flour and water, known as sourdough starters, were left at room temperature of 30.5 $\pm$ 2.5°C for 10 days for fermentation. During the fermentation process, analysis on sourdough starter was done daily for each sample before refreshing with flour and water. The analysis included measuring leavening height of each sourdough starter and by extracting 1 gram for pH test, 10 grams for total titratable acidity (TTA) measurement and 10 grams for high performance liquid chromatography (HPLC) analysis to identify amount of soluble sugar followed by refreshing each sourdough starter through discarding 50 grams for the first refresh and 100 g for subsequent refresh thereafter adding 50 grams of fresh flour and 50 grams of distilled water. At the end of fermentation of 10 days, additional analysis of taking 10 grams of sourdough starter for microbiological test.

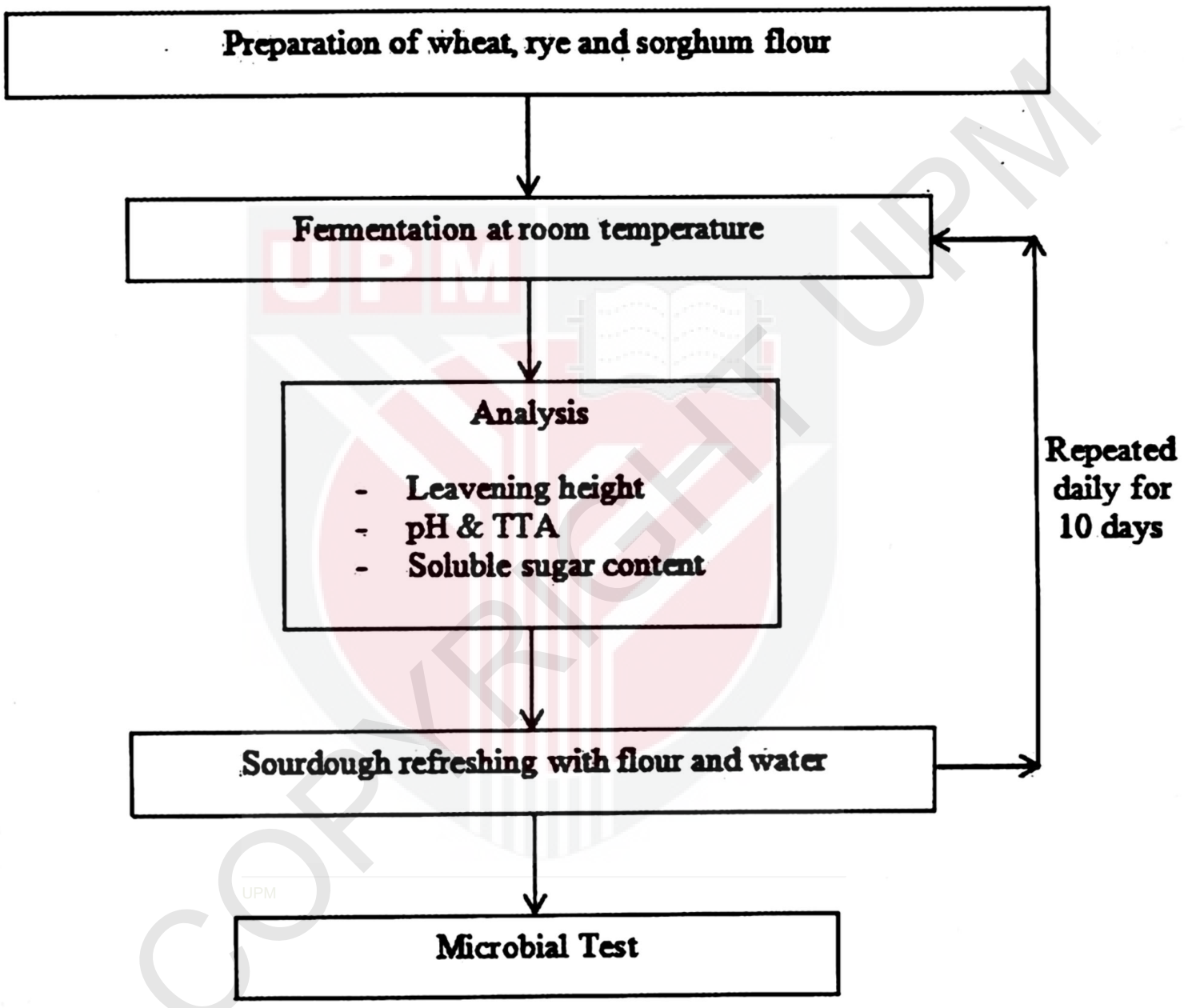

Figure 3.1: Summary of research framework

3.2 PREPARATION OF SOUDOUGH STARTER

Sourdough starters were made following Type I (Section 2.2). Figure 3.2 shows the flours used, *i.e.* wheat flour originated from Turkey and certified organic by United States Department of Agriculture (USDA); sorghum flour originated from inner Mongolia and certified organic by Kiwa BCS OEKO-GARANTIE, a registered German certification body as well as rye flour originated from Finland which certified organic by Evira (Finnish Food Security Authority). All types of flour mentioned were purchased from EZ Clean Eating Enterprise (Selangor).

(a) (b) (c)

Figure 3.2: Types of flour used for fermentation of sourdough starters included (a) wheat flour, (b) sorghum flour and (c) rye flour

The methods described by Lonner et al. (1986) and Akinola and Osundahunsi (2017) were adopted to prepare the sourdough starters. Each of them was mixed with distilled water at ratio of 1:1 (weight per weight), which is equivalent to 50g flour and 50g water. Then, each mixture was allowed to ferment naturally at room temperature for ten days in transparent 250 ml beakers. For refreshing of sourdough sample, 50 grams for the first refresh and 100 g for subsequent refresh was removed

and subsequently equal amount of flour (50g) and water (50g) were added to the fermenting mixture daily. The discard of starter was done to ensure the fermentation process was manageable so that amount of flour and water required everyday will be constant instead of rising exponentially day by day. The entire experiment of sourdough fermentation for 10 days was repeated once.

3.3 PHYSICOCHEMICAL ANALYSIS

To identify the physicochemical properties of sourdough starters from wheat, sorghum and rye sourdough starters, a series of analysis was conducted for each of the property. Sampling was done from each sourdough starter fermented according to the method explained under section 3.2. Each sample was tested for its pH, total titratable acidity (TTA) and concentration of water-soluble free sugars. Measurements were done in triplicates.

3.3.1 pH

1 gram of each sample was suspended in 9ml of sterile distilled water and homogenised. At room temperature, the pH meter (Mi 805, Miwaukee, U.S.A.) was calibrated with buffer solutions of pH 4 and 7 respectively. Then, the sensor tip was rinsed with distilled water before being dipped into the homogenate for pH measurement (Ogunsakin et al., 2015; Tansey, 1973).

3.3.2 Total Titratable Acidity

10 grams of each sample was suspended in 90ml of sterile distilled water and homogenised. The total titratable acidity (TTA) was measured by titrating each homogenised sample against 1.0N NaOH using 2-3 drops of phenolphthalein indicator until endpoint is reached. TTA value can be defined as the amount of 1.0N NaOH used to neutralize 10g of sample (Hanis-Syazwani et al., 2018; Lefebvre, 2002).

3.3.3 HPLC Analysis for Soluble Sugar

For each sample, 10 grams of starter was blended with 90 ml of distilled water and the suspension was centrifuged (Universal 320, Hettich, Germany) at 4,000 ×g for 15 min. The supernatant was boiled for 5 min and centrifuged again. The resulting clear broth was filtered through a 0.2 μm syringe filter (Lim et al., 2017). The filtered solution was then used for filling a vial which will then be sent for chromatography test.

The separation method using chromatography was adapted from Hanis-Syazwani et al. (2018) as well as Martínez-Anaya et al. (1993). A total of 6 vials were placed into the vial-holding tray so that the fractions in vials can be collected by the high-performance-liquid-chromatography (HPLC) system (LC-10AT, Shimadzu, Japan). Detection was performed with a refractive index detector. Chromatographic separation was done using SHIM-PACK carbohydrate analysis column (4.6mm x 150mm). The mobile phase was 80% acetonitrile and 20% water. The temperature of the column, flow rate, and injection volume were 30 °C, 1.0 mL/min and 20 microliter respectively. The graphs generated were collected for further analysis of soluble sugar content in samples.

3.4 LEAVENING HEIGHT

As shown in Figure 3.3, using a ruler, the height for leavening of sample in each scaled beaker was recorded as final height for day x, h_{fx}, and after refreshing included discarding, as initial height of day x+1, h_{ix+1}, every day. The difference between the initial and the final readings were taken as the level of leavening (Edema & Sanni, 2008).

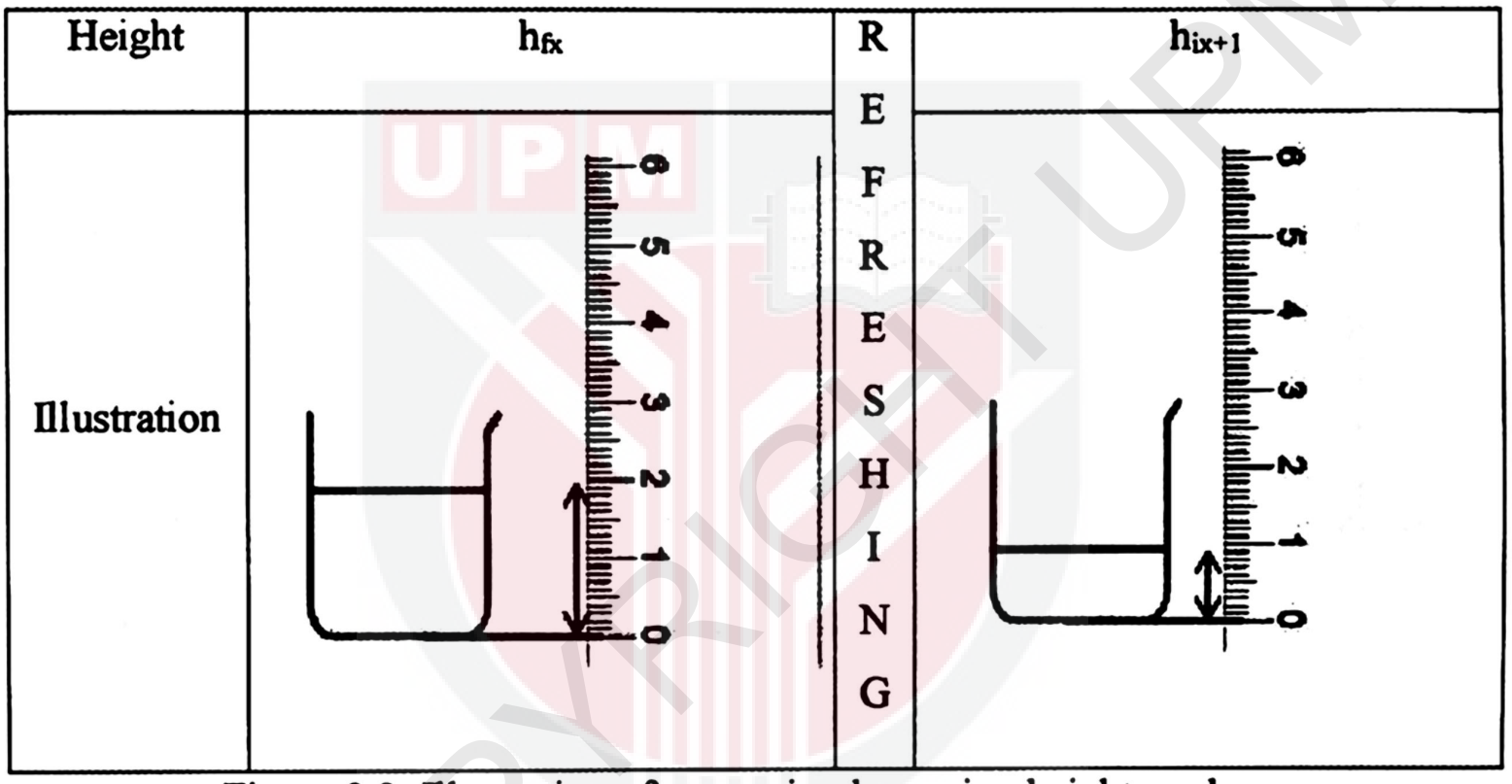

Figure 3.3: Illustration of measuring leavening height on day x

3.5 MICROBIOLOGICAL ANALYSIS

Microbial profile of each wheat, sorghum and rye sourdough starters were made following steps for DNA extraction, amplification and amplicon sequencing and lastly bioinformatics data analysis.

The method of DNA extraction was adapted from Tung et al (2016). Two samples, weighed 10 grams individually, were extracted from each flour type and then homogenized using Taco™ Prep Bead Beater (GeneReach Biotechnology Corp). Steel beads and silica beads were both used in this homogenization process with alkaline lysis buffer (250 mM NaOH, 10 mM EDTA, 1% SDS). Then, each suspension was incubated for 1 minute at 90°C before being neutralized using 1M Tris-HCl (pH 8). Right after that, centrifugation was done at 10,000 rpm for 1 minute. Next, 10 µL supernatant was mixed with OmegaBiotek NGS totalpure beads in new tubes respectively. The beads were washed with 75% (v/v) ethanol and dried at room temperature. They are then dissolved in 25 µL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) so that their DNA can be eluted.

PCR amplification was amplified using Q5 Hot Start High-Fidelity PCR master mix (NEB, Ipwich, USA) with barcoded primer pair (515F-806R) (Walters et al., 2016). The amplicons were then clustered according to their yield and quantified using Denovix high-sensitivity fluorescence quantification kit (Denovix, Delaware, USA). For bioinformatics analysis, Linux-based bioinformatic tools was used (Callahan et al., 2016).

3.6 STATISTICAL MODELLING

Properties of sourdough starters, *i.e.* pH, TTA, concentration of fermentable sugars and leavening height were predicted with fermentation time via statistical modelling. Using polynomial regression, dependent variable, *y*, representing a sourdough property, was regressed against a single independent variable, *x*, fermentation time using a polynomial regression as below:

$$y_i = \beta_0 + \beta_1 x_i + \beta_2 x_i^{\,2} + \beta_3 x_i^{\,3} + \ldots + \beta_k x_i^{\,k} + e_i, \text{ for } i = 1,2,\ldots,n,$$

where y_i = dependent variable
k = degree of polynomial
β_0 = y-intercept
β_j = slope with respect to independent variable (j = 0,1,2,…,k)
e_i = random error

As models are impossible to fit the real values perfectly due to systematic errors (Arminger et al., 2013), the best fitted statistical models are always chosen by referring to sum of squared estimate of errors (SSE), coefficient of determination (r^2) and various other properties (Abedfar & Sadeghi, 2019). SSE is used for measuring how well the mathematical model fits to the experimental data. Among the formed models for each property, they are always compared and only the model with minimised SSE is selected (Ostertagová, 2012). Value of SSE can be calculated by using the following formula:

$$SSE = \sum_{i=1}^{n} (y_i - \hat{y}_i)^2$$

where y_i = experimental value
$\hat{y}_i$ = fitted value for dependent variable

Coefficient of determination, often known as r^2, is more frequently applied for statistical modelling than SSE. The coefficient is defined as degree of variation for dependent variable (y) explained by independent variable (x), with the values ranged from 0 to 1 (Ostertagová, 2012). The higher the value of r^2, the better the model fits the experimental data. The formula of r^2 is as below:

$$r^2 = 1 - \frac{SSE}{SST} = 1 - \frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{\sum_{i-1}^{n}(y_i - \bar{y})^2}$$

where SST = total sum of squares
$\bar{y}$ = arithmetic mean of dependent variable

Following the above formulae, manual calculation can be complicated and time-consuming for each type of sourdough starter as well as each of related property. For fast and accurate generation of statistical models, Microsoft Excel is commonly used (Motulsky & Christopoulos, 2004).

3.7 SUMMARY

From each of wheat, sorghum and rye flours, 50 grams was taken out and mixed with 50 grams of distilled water respectively. The samples were replicated hence 6 samples were prepared in total. The mixture, known as sourdough starters, were fermented for 10 days at room temperature of 30.5±2.5 °C. On a daily basis, analysis was done for each sample before refreshing through discarding 50 grams for first refresh and 100 grams for subsequent refresh thereafter adding 50 grams of fresh flour and 50 grams of distilled water. The analysis carried out for each sample everyday comprised measuring leavening height along with extracting 1 gram for pH test, 10 grams for TTA measurement and 10 grams for HPLC analysis to identify amount of soluble sugars, including fructose, glucose, sucrose and maltose. For microbiological analysis, 10 grams from each sample was extracted to conduct laboratory test after fermentation was completed on day 10. The whole experiment involving sourdough fermentation for 10 days was repeated once. The values of properties for each type of sourdough starter were predicted when changes in properties with fermentation time were identified and applied for statistical modelling.

CHAPTER 4

RESULTS & DISCUSSIONS

4.1 PHYSICOCHEMICAL PROPERTIES OF SOURDOUGH STARTERS DURING FERMENTATION

The dough from each of wheat, sorghum and rye flour was fermented under consistent conditions of ambient temperature at 30.5 $\pm$ 2.5°C by daily refreshment using fresh flour and distilled water. As time goes, physicochemical properties of sourdough starters were measured and recorded. The carbohydrates in flour were hydrolysed into water-soluble free sugars as substrates for sourdough fermentation. In the meanwhile, organic acids including lactic acid and acetic acid were released as by-products, which then affected pH and TTA of all sourdough starters.

4.1.1 pH

Figure 4.1 shows the changes of pH of each sourdough starter during fermentation. The fresh mix of wheat flour and water into dough gave the lowest pH at around 4.20 on day 0, whereas higher pH of 5.79 and 6.01 were recorded for sorghum and rye samples respectively. Generally, pH of all sourdough starters decreased after fermentation period of 10 days similar to its initial trend with wheat

flour having the lowest pH (3.23) followed by sorghum (3.36) and rye (3.86). The pH plunge in sourdough starter is because of the organic acids production from LAB activities, particularly metabolism of fermentable sugars (Komlenić et al., 2012). The longer the fermentation time, a greater amount of organic acids was produced, resulting in more dissociated hydrogen ions and lower pH in the sourdough starters.

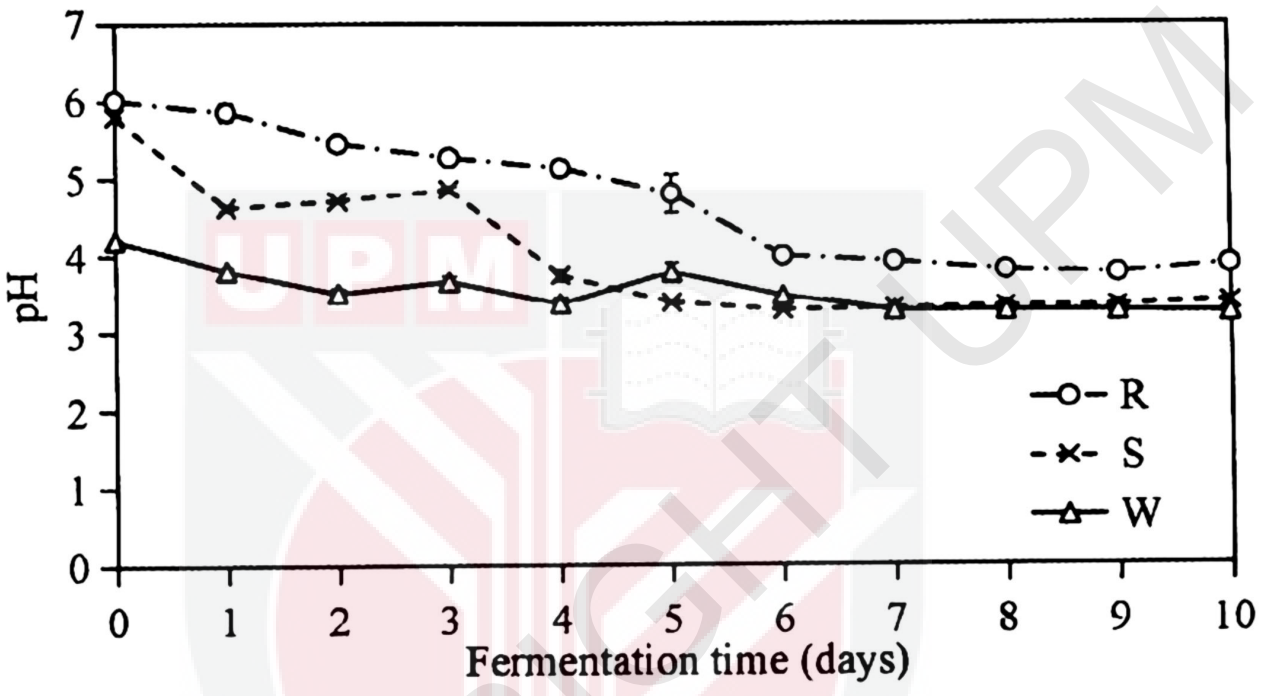

Figure 4.1: Graph of pH for wheat (W), sorghum (S) and rye (R) flour sourdough starters against fermentation time

In overall, sorghum sourdough starter achieved the greatest drop of pH by 2.43 or equivalent to 41.94%, followed by rye sourdough starter (35.77%) and wheat sourdough starter (27.14%). Mature sorghum sourdough starter reached pH of 3.28 on day 6 with minimum fluctuation after that while 3.75 was the lowest pH achieved by rye sourdough starter during the fermentation period. The lowest pH for wheat sourdough starter was 3.23, which was also the lowest pH among different types of sourdough starters, on day 10 of fermentation. This can be explained by the highest moisture of wheat sourdough starter due to the least abundance of pentosans in wheat flour (De Vuyst et al., 2017). The dissociation of organic acids utilises liquid as medium to release free hydrogen ions, therefore the higher the moisture of

sourdough starter, the greater amount of free hydrogen ions can be released, and the lower the pH can be achieved (Ognean, 2015).

In this research, pH of wheat sourdough starter had reached the typical value at around 4.0 after one day for wheat and rye sourdough starters, whereas rye sourdough starter took 6 days to achieve this (Komlenić et al., 2012). At this pH, the sourdough starters are said to be mature as organic acids tend to exist in their undissociated forms, hence they will be transported through the cell membrane and metabolism reactions will be inhibited by their presence (Ognean, 2015). After two days of fermentation, the pH of sorghum sourdough starter with the value of 4.71 was slightly higher than the pH proposed by Ogunsakin et al. (2015) at 4.31 since the sorghum used were from different origin and species. Banu et al. (2011) also deduced the final pH of rye sourdough starter ranged between 3.93 and 3.97, which was approximately similar to pH measured on day 6 (3.98) for this research. As for wheat sourdough starter, pH of 3.44 was claimed by Aplevicz et al. (2013) that was slightly higher than value from this research. This may be due to different carbohydrate metabolism rate of inoculated *Lactobacillus paracasei,* which was not being found in this research. Moreover, under optimum temperature of 37 °C, 3.54 was pH of sorghum sourdough starters measured by Karrar et al. (2019), which was achieved after day 4 of fermentation in this research at lower temperature of 30 °C.

As sourdough starters are added as one of the baking ingredients during bread making, the pH of final bread products will be higher and it also varies from one bread formulation to another when the percentage of sourdough starters are varied. Sanz-Penella et al. (2012) studied customer acceptance towards whole wheat sourdough bread and most of them accepted sourdough bread with 5-15% of

sourdough starter. Final pH of bread, ranging from 4.75 to 5.00, are accepted by most of the panelists, thus can be referred as guidance for necessity of further addition of sourdough starter in bread (Yan et al., 2020).

For easy and rapid tracking of pH changes with fermentation time, statistical modelling was made for each type of sourdough starter using curve fitting based on the pH values measured in this research (Figure 4.2). The accuracy of the models were increased by following the curves with lowest possible root mean square error and highest possible coefficient of determination (r^2). By doing so, pH of each sourdough starter was modeled to be the function of fermentation time with the general formula of $y = ax^2 + bx + c$, whereby y refers to pH, x refers to fermentation time while a, b and c are the constants summarised in Table 4.1.

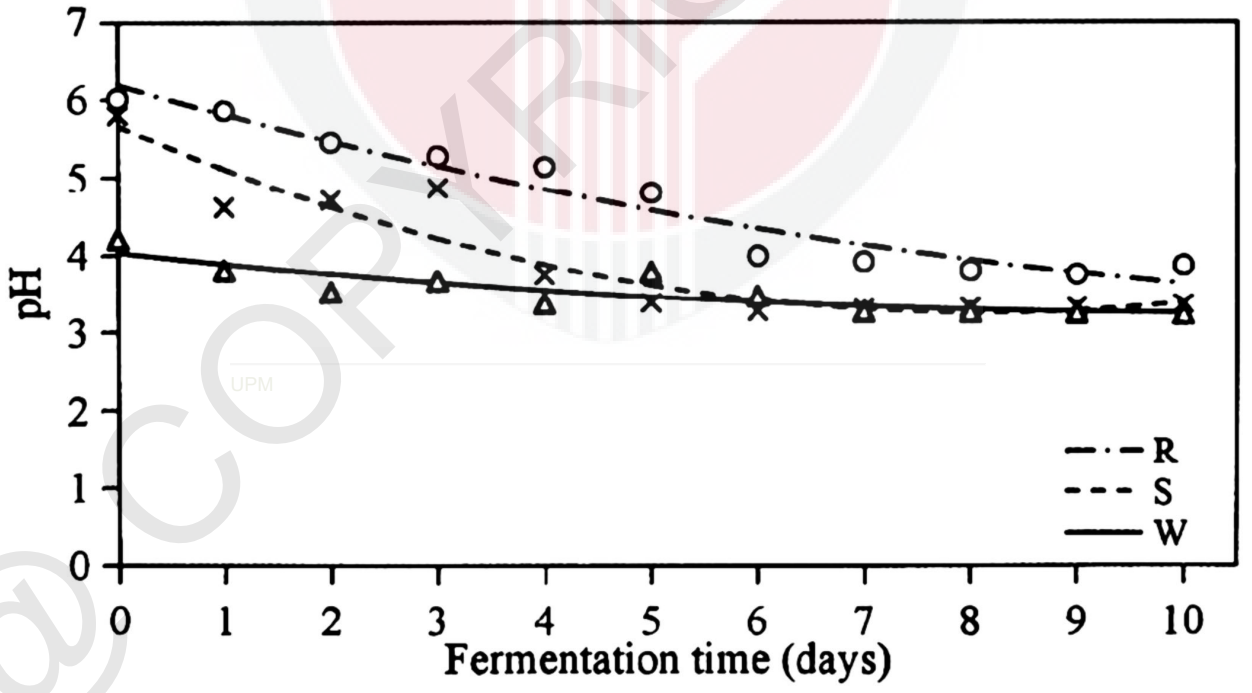

Figure 4.2: Statistical modeled curves of wheat (W), sorghum (S) and rye (R) sourdough starters for pH changes with fermentation time

Table 4.1: Statistical models of different sourdough starters for pH (y) changes with fermentation time (x) with general formula, $y = ax^2 + bx + c$

Type	Day	a	b	c	r^2
Wheat	0-10	0.0071	-0.1481	4.0209	0.7032
Sorghum	0-10	0.0362	-0.5897	5.653	0.8979
Rye	0-10	0.0131	-0.3859	6.1776	0.9449

4.1.2 Total Titratable Acidity (TTA)

During spontaneous fermentation using wheat, sorghum and rye flour, which lasted for 10 days, lactic acid and acetic acid were produced and total titratable acidity (TTA) for every type of sourdough starter increased (Figure 4.3). TTA was expressed as the volume of 1.0N sodium hydroxide (NaOH) required to neutralise 10g of sourdough starter. TTA values for wheat sourdough starter before and after fermentation were 4.8 ml and 14.8 ml separately, which both also were the highest as compared to sorghum and rye. In order to titrate 10 grams of rye sourdough starter, 3.1 ml of 1.0N NaOH was utilised on day 0 while on day 10, 13.9 ml was consumed. As for sorghum sourdough starter, it possessed a slightly higher TTA of 14.6 ml on day 10. By referring to study from Tamani et al. (2012), wheat sourdough starter had achieved similar maturity with TTA at 13.5 ml of 1.0N NaOH on day 2 of fermentation. 14.52 ml of 1.0N NaOH was TTA proposed for whole rye sourdough starter by Banu et al. (2011) and this value of TTA was reached during fermentation day 8.

In general, the longer the fermentation time, the higher the TTA in sourdough starters. This is in agreement with other studies that showed the rise in TTA after certain period of sourdough starter fermentation (Lim et al., 2017; Ogunsakin et al., 2015; Aplevicz et al., 2013; Robert et al., 2006). Sorghum sourdough starter showed the largest rise of TTA by 13.3 ml of 1.0N NaOH (324%) on day 7 of fermentation. This corresponds with the results whereby sorghum sourdough starter achieving the greatest drop in pH during fermentation. Besides, 12 ml of 1.0N NaOH was the greatest increase of TTA achieved by rye sourdough starter whereas for wheat sourdough starter, the value was 11 ml.

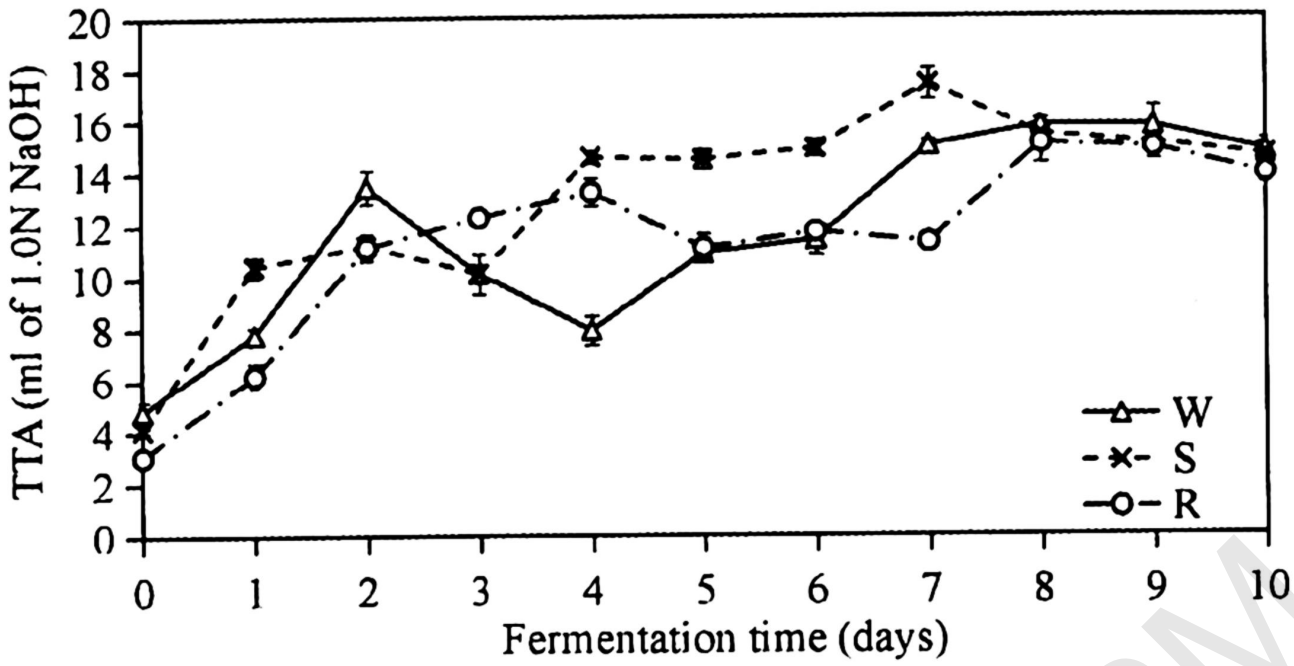

Figure 4.3: Graph of total titratable acidity (TTA) for wheat (W), sorghum (S) and rye (R) flour sourdough starters against fermentation time

To prevent consumers from having an unpleasant tangy taste in sourdough bread, pH and TTA of both sourdough starters and sourdough bread products are all monitored and controlled. TTA of sourdough bread is usually lower than TTA of sourdough starter as the mixture of other baking ingredients dilutes the acidity. Katina (2005) suggested a maximum TTA at 6.0 for sourdough bread. Hence, sourdough starters with higher TTA should be added at lesser percentage in mixture of dough so that sourdough bread TTA does not exceed 6.0.

TTA was chosen as one of the parameters in investigating properties of sourdough starter because organic acids, produced from fermentation, are usually weak acids. Buffering, the resistance of solution in pH changes, thus commonly happens and affects accuracy of pH. Using titration method, all free hydrogen ions can be detected since initial free hydrogen ions in solution can be removed before further dissociation of organic acids (Sadler & Murphy, 2010).

For easy and rapid tracking of TTA changes with fermentation time, statistical modelling was made in Figure 4.4 for each type of sourdough starter using curve fitting according to the TTA values obtained from this research. Therefore, TTA of

each sourdough starter was modeled to be the function of fermentation time with the general formula of $y = ax^3 + bx^2 + cx + d$, whereby y refers to TTA, x refers to fermentation time while a, b, c and d are the constants summarised in Table 4.2. To boost the accuracy of the models, models were done by following the curves with lowest possible root mean square error and highest possible coefficient of determination (r^2).

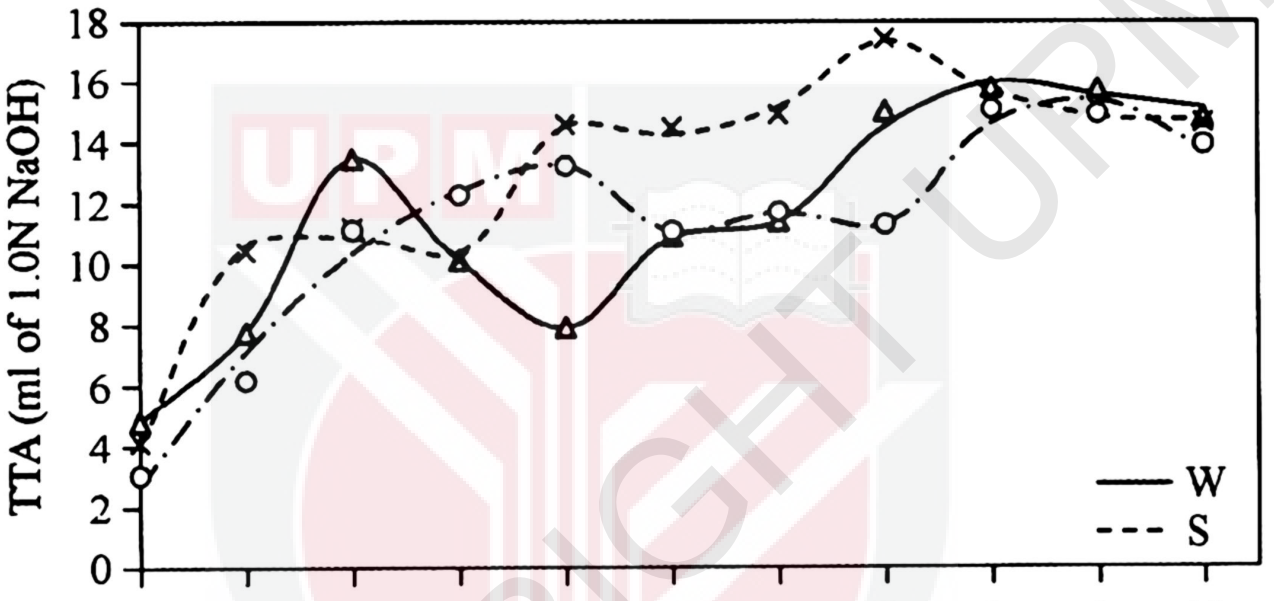

Figure 4.4: Statistical modeled curves of wheat (Wm), sorghum (Sm) and rye (Rm) sourdough starters for TTA changes with fermentation time

Table 4.2: Statistical models of different sourdough starters for TTA (y) changes with fermentation time (x) with general formula, $y = ax^3 + bx^2 + cx + d$

Flour	Day	a	b	c	d	r^2
Wheat	0-3	-1.9667	7.275	-2.3583	4.8	1
	3-6	-1.2708	17.837	-80.042	124	1
	6-10	-	-0.7071	12.069	-35.338	0.9675
Sorghum	0-4	0.9188	-5.9107	11.562	4.0461	0.9965
	4-7	-	0.65	-6.26	29.293	0.9792
	7-10	-	0.375	-7.24	49.615	0.9637
Rye	0-4	-	-0.5821	4.9736	2.7157	0.9787
	4-7	-0.6333	10.887	-61.504	125.57	1
	7-10	-	-1.1938	21.066	-77.516	0.9484

4.1.3 Water-soluble Free Sugars

Water-soluble free sugars, which refer to fructose, glucose, sucrose and maltose in this thesis, support the fermentation of sourdough starter as LAB consume them to generate energy in the form of ATP for growth. At the same time, organic acids, ethanol and carbon dioxide are usually released as by-products, thus lead to drop in pH, rise in TTA and rise in leavening height (Hui & Sherkat, 2005). Maltose and sucrose are both disaccharides which have to be hydrolysed into monosaccharides so that they can be utilised directly as fermentation substrates in the form of glucose or fructose (Chavan & Chavan, 2011). In wheat, sorghum and rye sourdough starters, the amount of each sugar mentioned was measured daily during the fermentation period. The results were plotted in graphs as Figure 4.5 (wheat sourdough starter), Figure 4.6 (sorghum sourdough starter) and Figure 4.7 (rye sourdough starter). Generally, concentrations of maltose and sucrose were lower, with values less than 0.5g/100g sample, as compared to concentrations of fructose and glucose in all types of starters.

Figure 4.5 shows that concentrations of all sugars peaked on day 2 of fermentation. There were 2.167g of fructose, 2.412g of glucose, 1.161g of sucrose and 0.136g of maltose in 100g of wheat sourdough sample. After achieving its highest concentration, sucrose almost disappeared for the following days. This is resulted from rapid hydrolysis by sourdough yeast invertase into glucose and fructose as readily available substrates for sourdough fermentation (Chavan & Chavan, 2011; Leroy et al., 2006). As for maltose and glucose, their abundances increased significantly on day 6 to 0.181g/100g sample and 0.580g/100g sample respectively, probably caused by lower metabolism rate during transition of

microbiota succession (Van der Meulen et al., 2007), thus the sugars accumulated after being hydrolysed by amylase activity upon wheat flour. Fructose concentration also rose notably on day 7 up to 0.5344g/100g sample due to the transition as fructose played the role as an electron acceptor during heterofermentative sugar metabolism (Batt & Tortorello, 2014).

Figure 4.7 shows an almost similar trend of changes in sugar concentration in rye sourdough starter as wheat sourdough starter. On day 1, the concentrations of fructose and glucose in rye sourdough starter were the highest at 2.122g/100g sample and 2.342g/100g sample individually. The next peak was observed on day 6 for concentrations of glucose (1.688g/100g sample) and fructose (0.515g/100g sample). For sorghum sourdough starter, 0.596g of fructose was detected as the greatest amount of sugar in 100g of sample. Then, the concentration of glucose achieved its maximum concentration of 0.936g/100g sample on day 5. On the same day, the concentration of fructose also rose to 0.661g/100g sample. In both sourdough starters, sucrose was almost depleted after reaching its maximum value, similar to the metabolism of sucrose in wheat sourdough starter. For maltose, the concentration was fluctuating around its initial one on day 0, which were both low in sorghum and rye sourdough starters.

Between maltose and glucose, the concentration of maltose was higher in wheat sample during most of the days, whereas glucose was found to be mostly higher in sorghum sourdough starter. Sekwati-Monang et al. (2012) made a similar conclusion in their studies too. The low concentration of maltose in sorghum sourdough starter was because β-amylase activity in ungerminated sorghum is low for starch hydrolysis (Taylor et al., 2006). On the other hand, glucose concentration of rye

sourdough starter was higher than wheat sourdough starter once the fermentation process reached maturity which was around day 5. The more rapid enzymatic activity in rye flour contributed to this (Soccol et al., 2013).

The concentrations of all water-soluble free sugars were low at the end of fermentation due to their metabolism into sourdough byproducts, especially the organic acids. This indicates that sourdough helps bringing health benefits when being involved in breadmaking. Maioli et al. (2018) claimed that surge of plasma glucose and insulin were both reduced after intaking sourdough bread as compared to regular bread. Therefore, sourdough bread is suitable for consumption by patients with impaired glucose tolerance (IGT), which is a condition usually referred as 'prediabetes'. Besides, the acidic condition of sourdough starter reduces digestion rate of starch in flour, thus slowing down rise of blood glucose and reducing its glycemic index. Thus, body weight and body fats may be decreased due to slower digestion rate and gastric emptying rate after intaking sourdough bread (Capurso & Capurso, 2020).

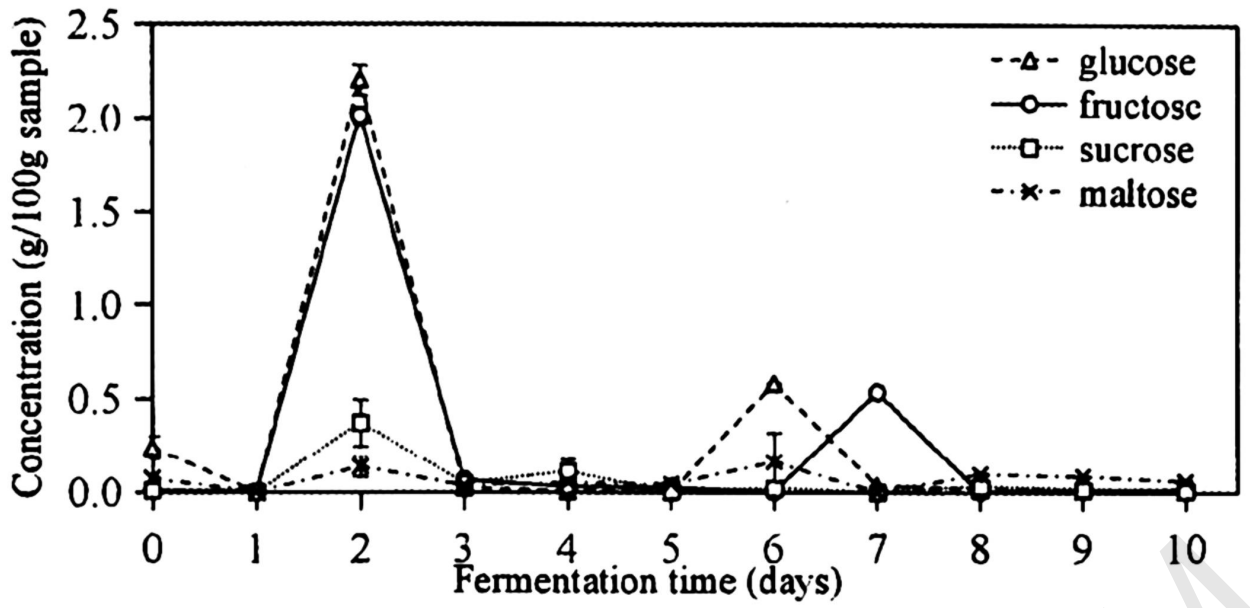

Figure 4.5: Changes of sugar concentrations including fructose, glucose, sucrose and maltose in wheat sourdough starter during 10-day-long fermentation

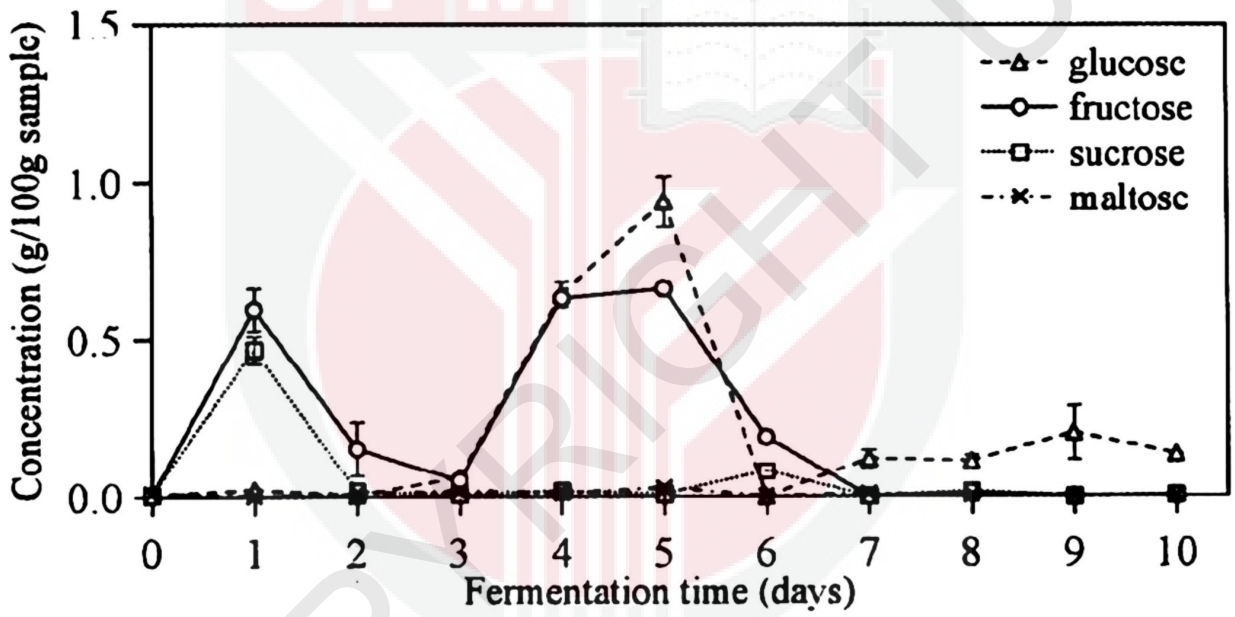

Figure 4.6: Changes of sugar concentrations including fructose, glucose, sucrose and maltose in sorghum sourdough starter during 10-day-long fermentation

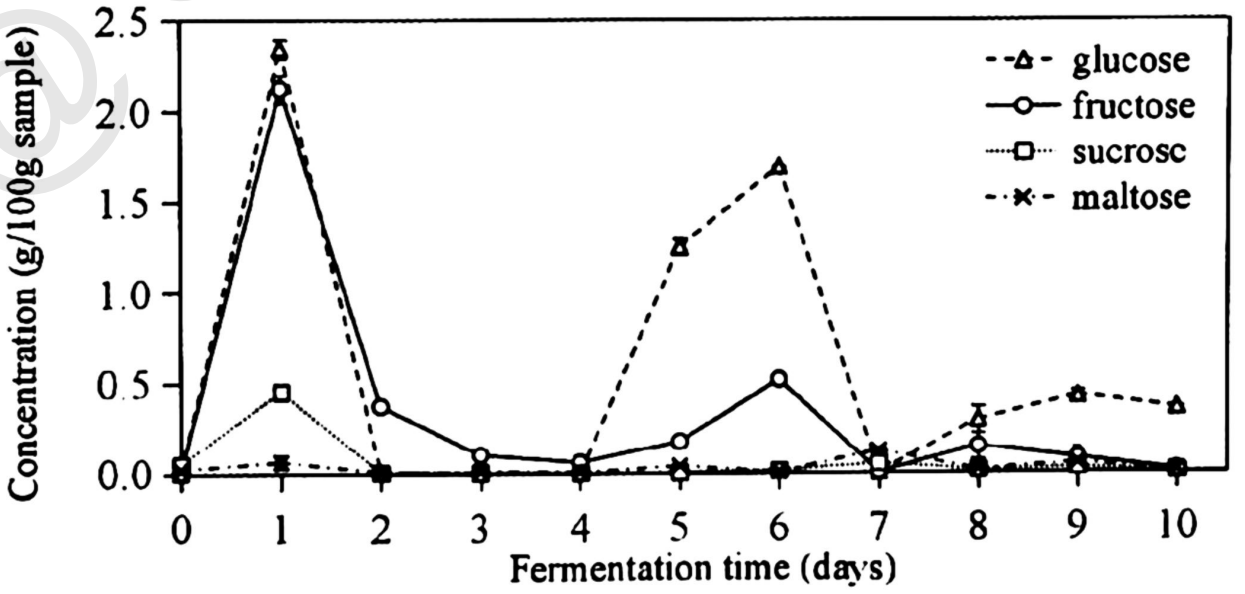

Figure 4.7: Changes of sugar concentrations including fructose, glucose, sucrose and maltose in rye sourdough starter during 10-day-long fermentation

For sourdough starters to reach maturity, they usually experience a three-phase succession of microbiota. Particularly for wheat sourdough starter, the first phase is roughly from days 0-2, dominated by microorganisms from water and cereal grains, followed by second phase (days 2-5) as well as third phase comprising the following days. Acidification starts to occur during phase 2 hence sourdough-specific LAB, such as *Lactobacillus*, *Pediococcus*, and *Weissella*, survive in the acidic environment whereas phase 3 will be dominated by more well-adapted sourdough strains such as *L. fermentum* and *L. plantarum* (Van der Meulen et al., 2007). Hence, the properties of sourdough starters were correlated to the succession process.

For easy and rapid estimation of changes in concentration of each fermentable sugar with fermentation time, statistical modelling was made for wheat, sorghum and rye sourdough starters using curve fitting based on the concentrations measured in this research (Figures 4.8, 4.9 and 4.10). The accuracy of the models were increased by following the curves with lowest possible SSE and highest possible coefficient of determination (r^2). By doing so, fermentable sugar concentration of each sourdough starter was modeled to be the function of fermentation time with general formula of $y = ax^3 + bx^2 + cx + d$, whereby y is concentration of fermentable sugar, x refers to fermentation time while a, b, c and d are constants summarised in Table 4.3.

Table 4.3: Statistical models of different sourdough starters for fermentable sugar (y) changes with fermentation time (x) with general formula, $y = ax^3 + bx^2 + cx + d$

Flour	Sugars	Day	a	b	c	d	r^2
Wheat	Fructose	0-3	0.8913	-4.6087	5.8368	0.0033	1.0000
		3-7	-0.0844	1.2153	-5.5069	7.9794	0.8855
		7-10	-	-0.0519	0.8790	-3.5990	0.8330
	Glucose	0-3	1.1672	-5.8393	7.0124	0.0022	1.0000
		3-6	-0.3427	4.7361	-20.4720	28.0430	1.0000
		6-10	-0.1816	4.5792	-38.0030	104.0600	0.9636
	Sucrose	0-3	0.2173	-1.0796	1.2670	0.0494	1.0000
		3-7	-	0.0059	-0.0487	0.0968	0.9726
		7-10	-0.0136	0.3498	-2.9915	8.5001	1.0000
	Maltose	0-3	0.0264	-0.1283	0.1434	0.0209	1.0000
		3-7	0.0095	-0.1298	0.5745	-0.8065	0.8452
		7-10	-0.0364	0.9442	-8.1206	23.1760	1.0000
Sorghum	Fructose	0-3	-0.9944	3.9896	-2.9980	0.0043	1.0000
		3-7	0.0435	-0.5713	2.3948	-3.1584	0.9732
		7-10	-0.0900	2.4291	-21.7680	64.7390	1.0000
	Glucose	0-3	-1.1385	4.6367	-3.7358	0.2376	1.0000
		3-6	0.0942	-1.1222	4.3542	-5.4842	1.0000
		6-10	-0.0434	1.1194	-9.5538	26.9650	0.9863
	Sucrose	0-3	-0.1784	0.7250	-0.556	0.0096	1.0000
		3-7	0.0112	-0.1716	0.8160	-1.1524	0.6539
		7-10	0.0087	-0.2309	2.0206	-5.8179	1.0000
	Maltose	0-3	-0.0741	0.3269	-0.3265	0.0739	1.0000
		3-7	-0.0213	0.3027	-1.3559	1.9618	0.7051
		7-10	0.0152	-0.4191	3.8053	-11.3310	1.0000
Rye	Fructose	0-3	0.2295	-1.2064	1.5703	0.0023	1.0000
		3-7	0.0694	-1.1872	6.3742	-10.272	0.9807
		7-10	0.0039	-0.1023	0.8772	-2.4782	1.0000
	Glucose	0-3	0.0175	-0.0686	0.067	0.0058	1.0000
		3-6	-0.1571	1.7413	-5.7951	6.0159	1.0000
		6-10	-	-0.0199	0.3540	-1.4025	0.8395
	Sucrose	0-3	0.2257	-1.1343	1.3719	0.0053	1.0000
		3-6	0.0173	-0.2174	0.8943	-1.1853	1.0000
		6-10	-0.0064	0.1629	-1.3752	3.8445	0.8984
	Maltose	0-3	-0.0030	0.0184	-0.0260	0.0113	0.9839
		3-7	0.0127	-0.2116	1.1470	-2.0037	1.0000
		7-10	-	0.0013	-0.0249	0.1193	0.9971

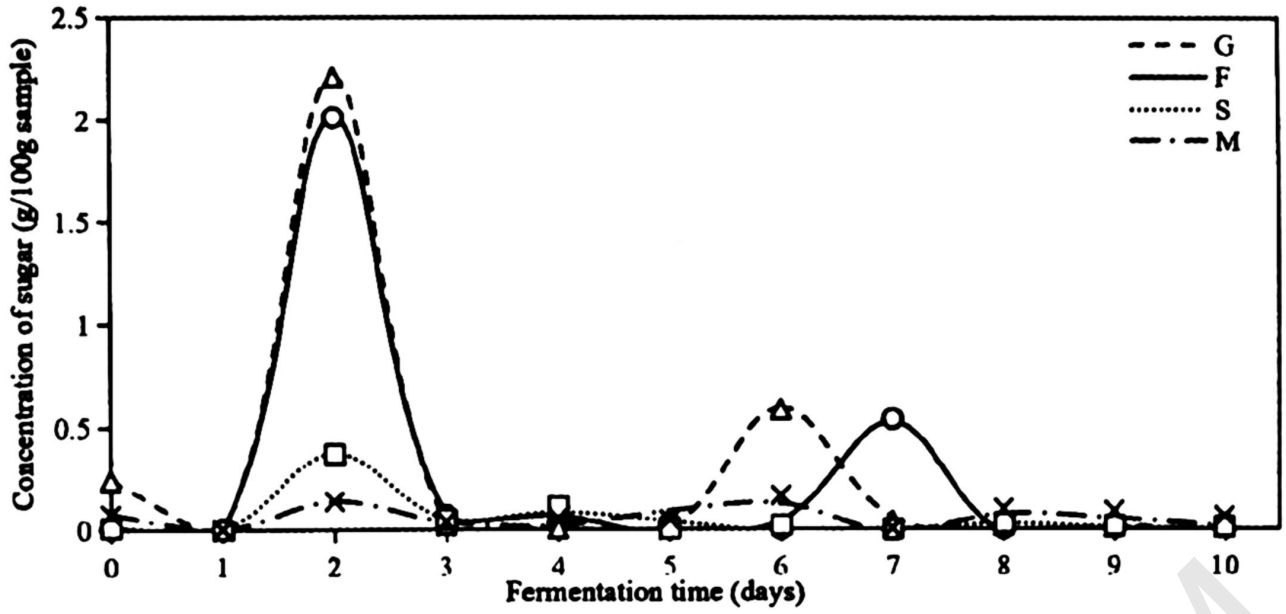

Figure 4.8: Statistical modeled curves of fructose (F), glucose (G), sucrose (S) and maltose (M) changes in wheat sourdough sample with fermentation time

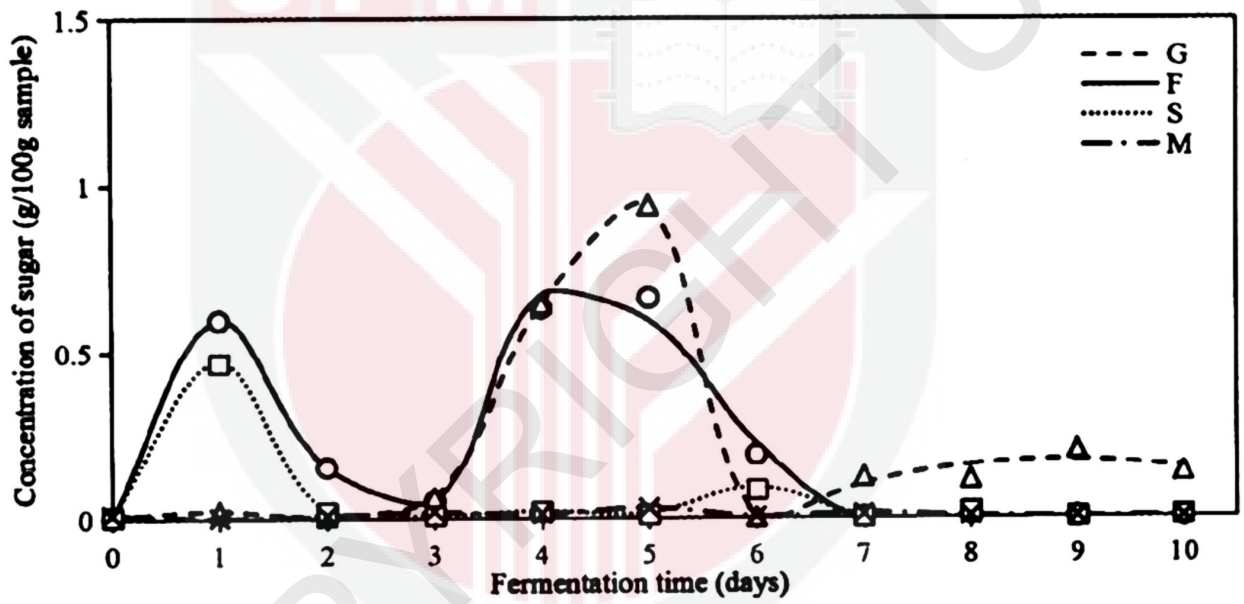

Figure 4.9: Statistical modeled curves of fructose (F), glucose (G), sucrose (S) and maltose (M) changes in sorghum sourdough sample with fermentation time

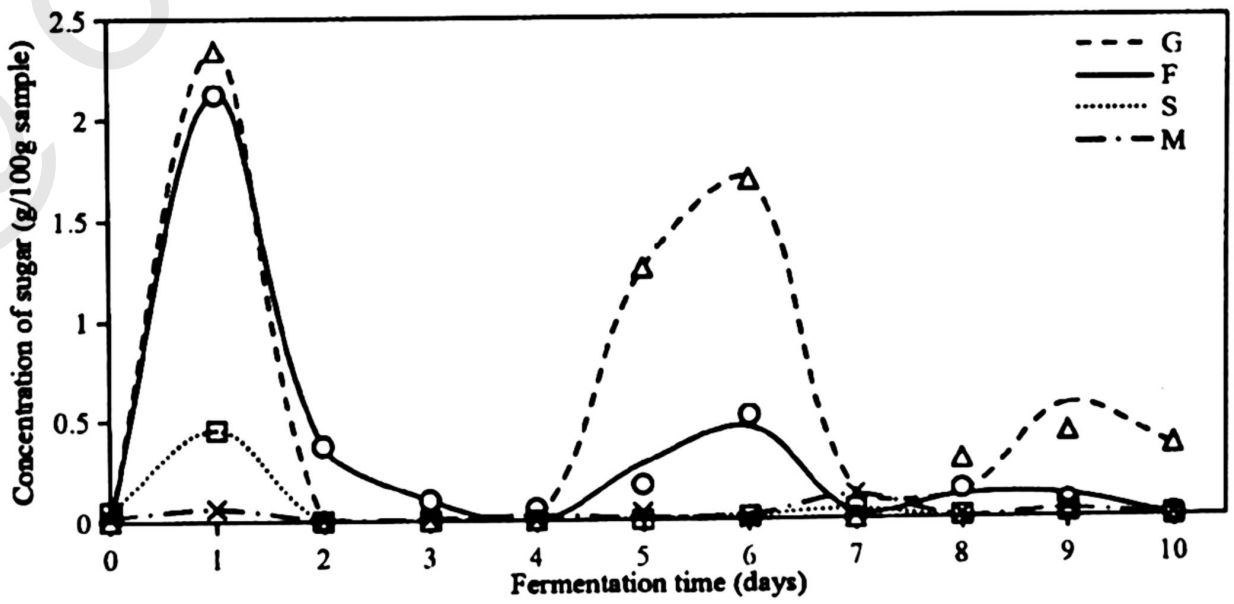

Figure 4.10: Statistical modeled curves of fructose (F), glucose (G), sucrose (S) and maltose (M) changes in rye sourdough sample with fermentation time

4.2 LEAVENING HEIGHT CHANGES DURING FERMENTATION

During fermentation of sourdough starter, a significant change observed was the leavening height. The initial and final heights were both recorded daily for each type of sourdough starter and were plotted in a graph showing all values of them side by side to ease referral and comparison (Figure 4.11). By getting the maximum difference between initial and final heights, the relationship between height increment due to leavening and fermentation time is displayed in Figure 4.12. Referring to both of the diagrams, the wheat sourdough starter showed the greatest height increment significantly during most of the time. For wheat sourdough starter, a leavening height of 82.91% was shown on day 2 of fermentation as the greatest height increment among all types of sourdough starter as well as along the fermentation period. On the other hand, the peaks of height increment for sorghum and rye sourdough starters were 37.17% and 31.40% respectively. As concluded by Ogunsakin et al. (2015), positive leavening height achieved by sorghum sourdough starter on day 2 was 1.4 cm, which was somewhat lower than the height achieved in this research at 1.78 cm, possibly due to the difference in species of sorghum used.

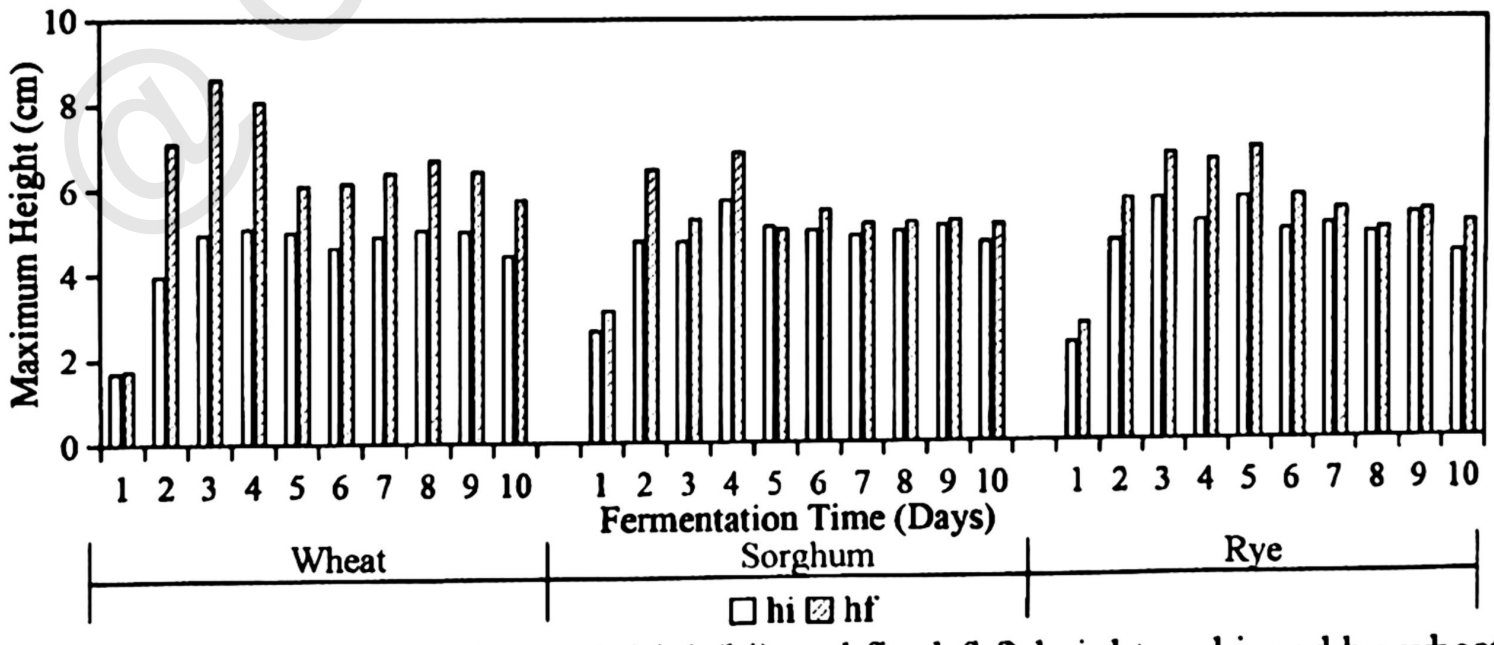

Figure 4.11: Graph of maximum initial (hi) and final (hf) heights achieved by wheat, sorghum and rye sourdough starters against fermentation time

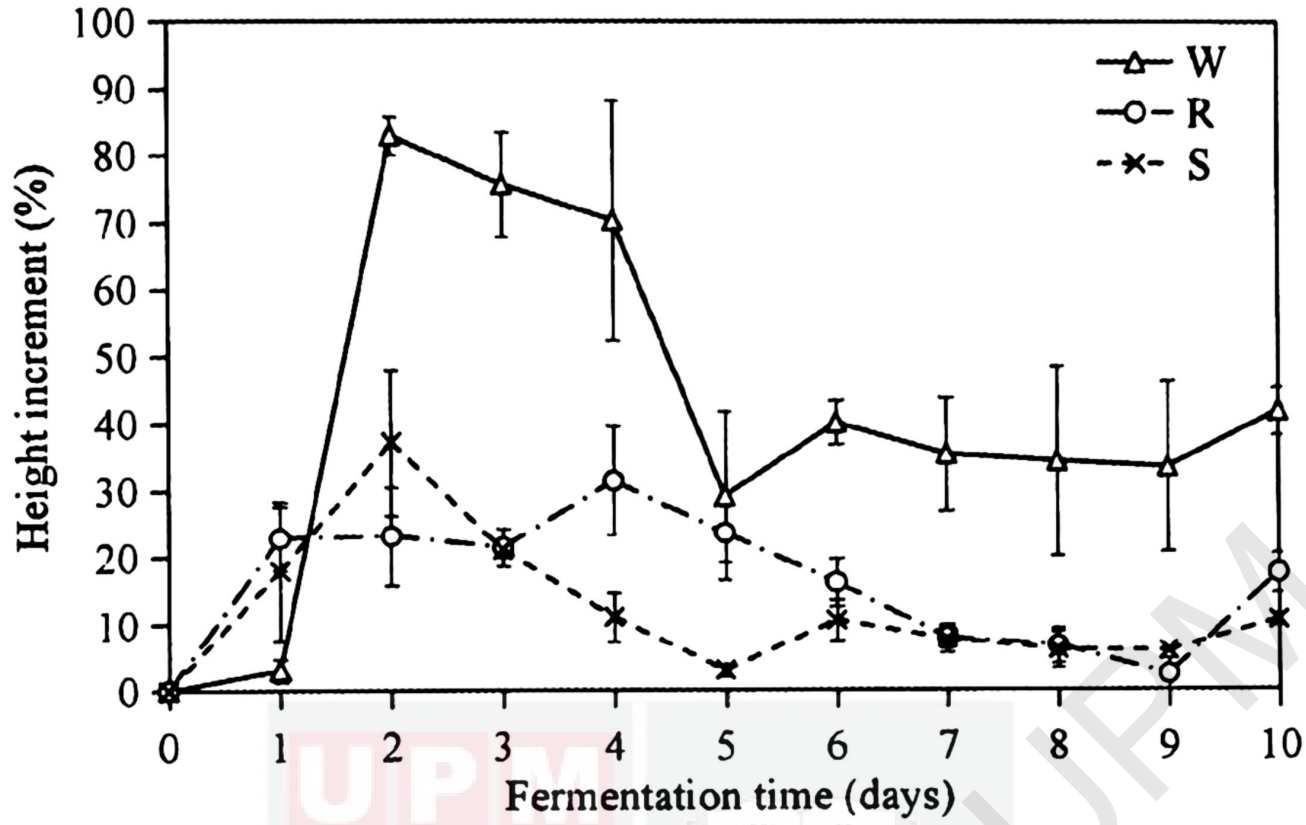

Figure 4.12: Graph of height increment in wheat (W), sorghum (S) and rye (R) sourdough starters against fermentation time

Leavening height is in fact resembling the carbon dioxide generation and retention of the sourdough starters. At the same time, the presence of heterofermentative LAB can be proven to be present in the sourdough starter as carbon dioxide generation is due to their carbohydrate metabolism activities (Batt & Tortorello, 2014). In addition, the sourdough starters should also sustain the carbon dioxide produced by the gluten network due to their viscoelastic properties in order for the height to be raised (Preedy & Watson, 2019). Thus, wheat sourdough starter gained the greatest leavening height most of the time in present research due to the highest amount of gluten in wheat flour as compared to sorghum and rye flours (De Vuyst et al., 2017). The sorghum sourdough starter whereas was unable to reach a high leavening height as it is gluten free and unable to retain the gas produced for long period of time (Ogunsakin et al., 2017).

The trend of height increment was almost identical for sorghum and wheat sourdough starter, in which the lowest leavening height reached on day 5 and no

significant changes afterwards. The lowest value of leavening height in rye sourdough starter was only found on the ninth day. It also took 2 more days than others for development until the maximum leavening height. As the free water available in rye sourdough starter is the least, the height increment process is therefore slower (Cappa et al., 2016). Ognean (2015) also supported the fact that since LAB are less active with low moisture, drier sourdough has a lower capacity in generation of carbon dioxide, which leads to smaller extent of height increment.

Through increment of leavening height, the addition of sourdough brings contribution to bigger bread volume and softer bread crumb. The high acidity of sourdough solubilises and degrades gluten in dough, thus gives the bread dough a soft and less elastic texture, followed by higher expansion (Clarke et al., 2002). However, degraded gluten is weaker in trapping carbon dioxide, whereby metabolism of exopolysaccharides is important to prevent excess drop in gas retention ability of dough (Katina, 2005). For optimum increment of bread volume, Katina (2005) claimed pH of sourdough starter was within range between 4.9 and 5.2, whereas for sourdough bread, pH should lie between 5.1 and 5.5.

For easy and rapid detection of height increment with fermentation time, statistical modelling was made in Figure 4.13 for each type of sourdough starter using curve fitting according to data recorded for this research with the general formula of $y = ax^3 + bx^2 + cx + d$, whereby y refers to height increment, x refers to fermentation time while a, b, c and d are the constants summarised in Table 4.4. To rise the accuracy of the models, models were done for each phase of microbial succession in sourdough starters by following the best-fitted curves with lowest possible root mean square error and highest possible r^2.

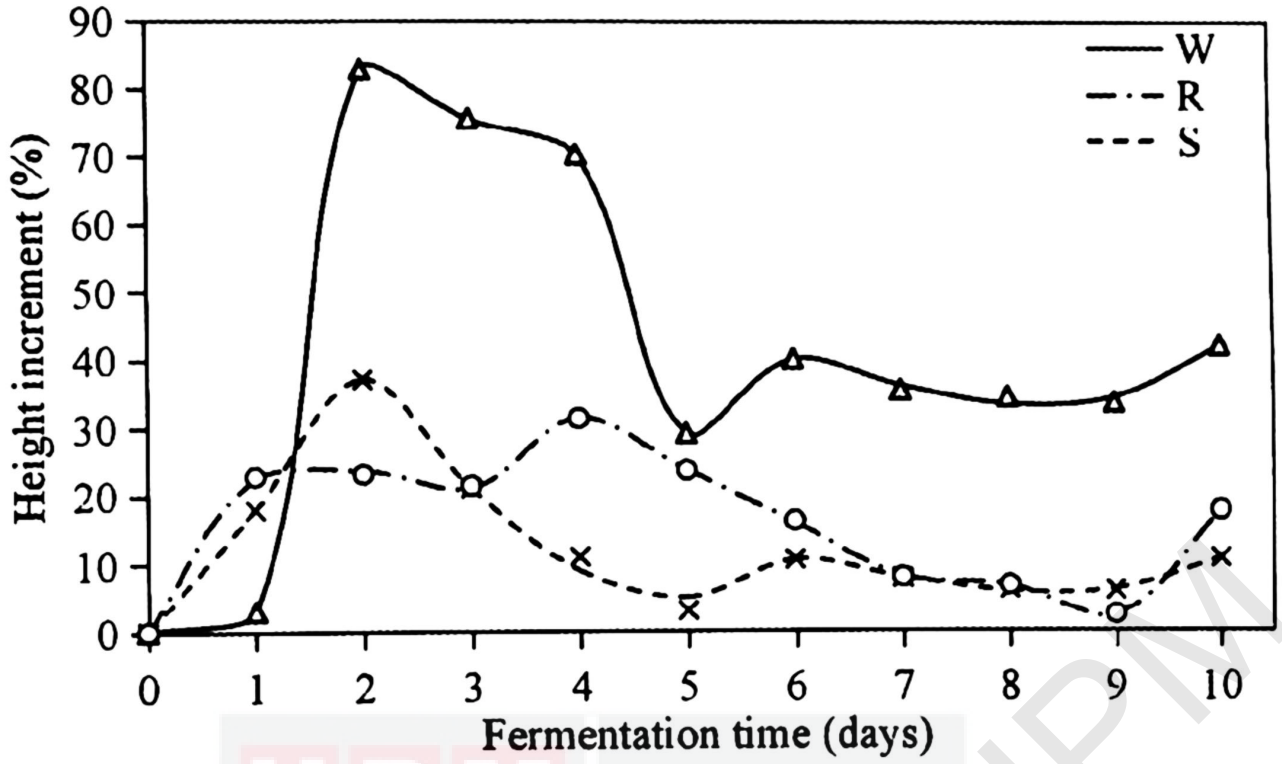

Figure 4.13: Statistical modeled curves of wheat (W), sorghum (S) and rye (R) sourdough starters for height increment with fermentation time

Table 4.4: Statistical models of different sourdough starters for height increment (y) with fermentation time (x) with general formula, $y = ax^3 + bx^2 + cx + d$

Flour	Day	a	b	c	d	r^2
Wheat	0-3	-27.357	120.540	-90.200	$6.000(10^{-12})$	1.000
	3-6	14.637	-193.550	807.930	-1001.400	1.000
	6-10	0.465	-9.292	58.028	-74.225	0.961
Sorghum	0-3	-6.115	19.008	5.032	$-2.000(10^{-12})$	1.000
	3-6	-	4.408	-43.639	112.970	0.945
	6-10	0.309	-6.223	39.127	-66.903	0.994
Rye	0-4	2.855	-19.110	38.614	0.094	0.999
	4-7	-	-	-7.798	62.643	1.000
	7-10	3.782	-92.311	744.350	-1976.500	1.000

4.3 MICROBIAL POPULATIONS IN DIFFERENT SOURDOUGH STARTERS

In this research, the mixture of flour and water had gone through spontaneous fermentation when refreshing is done constantly to supply carbohydrates and water for microbial growth. Type I sourdough starters made from different types of flour possessed different physicochemical properties, hence affecting the growth and succession of microorganisms. The microbial diversity and relative abundance for each type of sourdough starter were determined after carrying out sequence clustering and some analysis using the bioinformatics data.

The characteristic of wheat sourdough starter which had the least diverse microbial population was also observed by referring to Figures 4.14 and 4.15, portraying the microbial diversity at class and genus level each. From Figure 4.14, around 90.2% of the bacteria in wheat sample were under class of *Bacilli* while the unclassified bacteria were 7.6%. Figure 4.15 whereas displayed there were only two main types of genera found in this sample, which are *Leuconostoc* and those unclassified. The reason behind this could be the massive and conventional farming of wheat using artificial fertilizers and pesticides, leading to less diverse natural microbial population in wheat flour (Stanzer et al., 2017). The diversity of microbes in sourdough starter can be affected due to different maturity, growing environment and parts of grain used for flour milling (Minervini et al., 2015). Other possible factors which influence microbial diversity in sourdough starters are presence of antimicrobial phenolic compounds (Ripari et al., 2019), moisture content as well as interactions between LAB and other bacteria (Van der Meulen et al, 2007). On the other hand, the diversity of bacteria in both sorghum and rye sourdough starters were

found to be generally same despite the difference in respective relative abundance of class and genus.

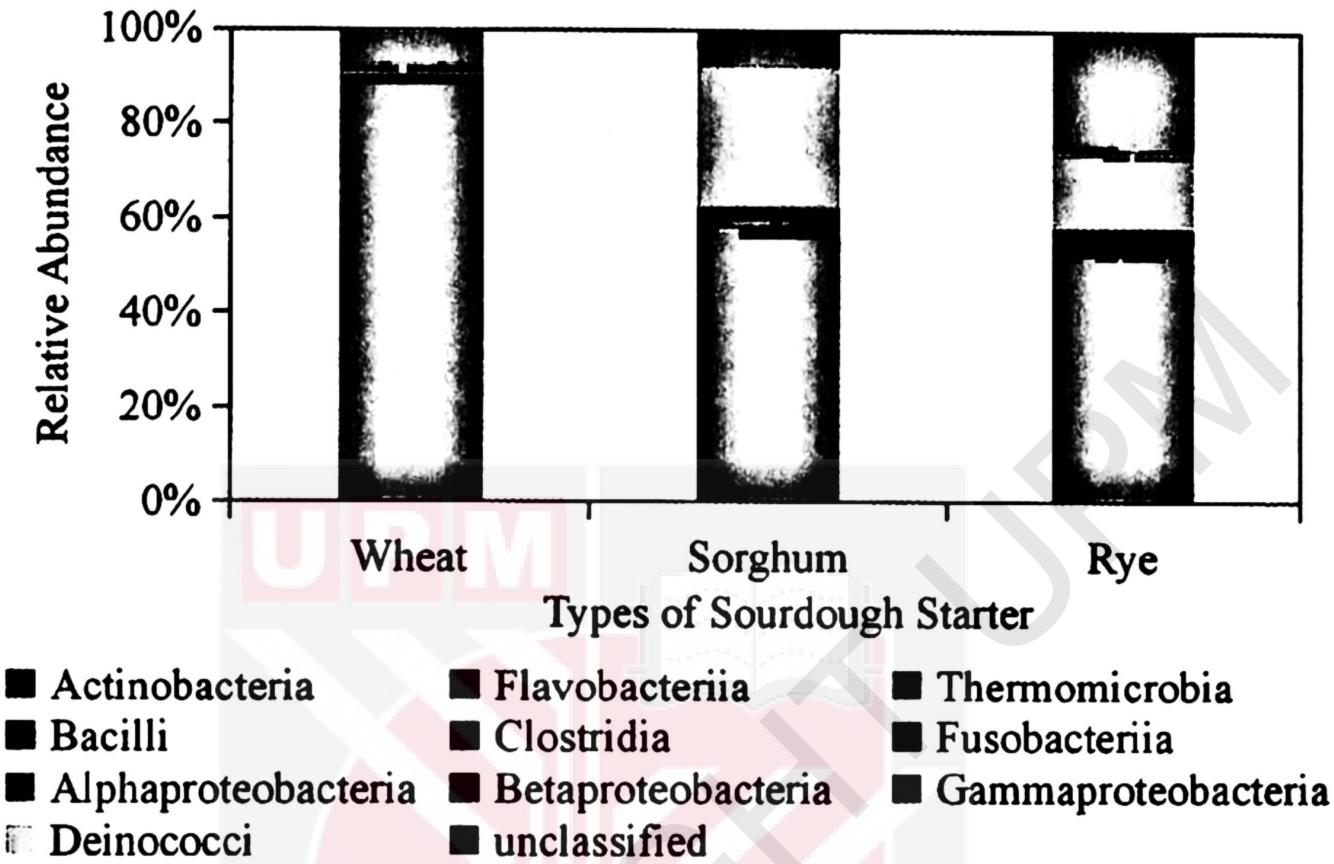

Figure 4.14: Microbial diversity and abundance for each sourdough starter at class level

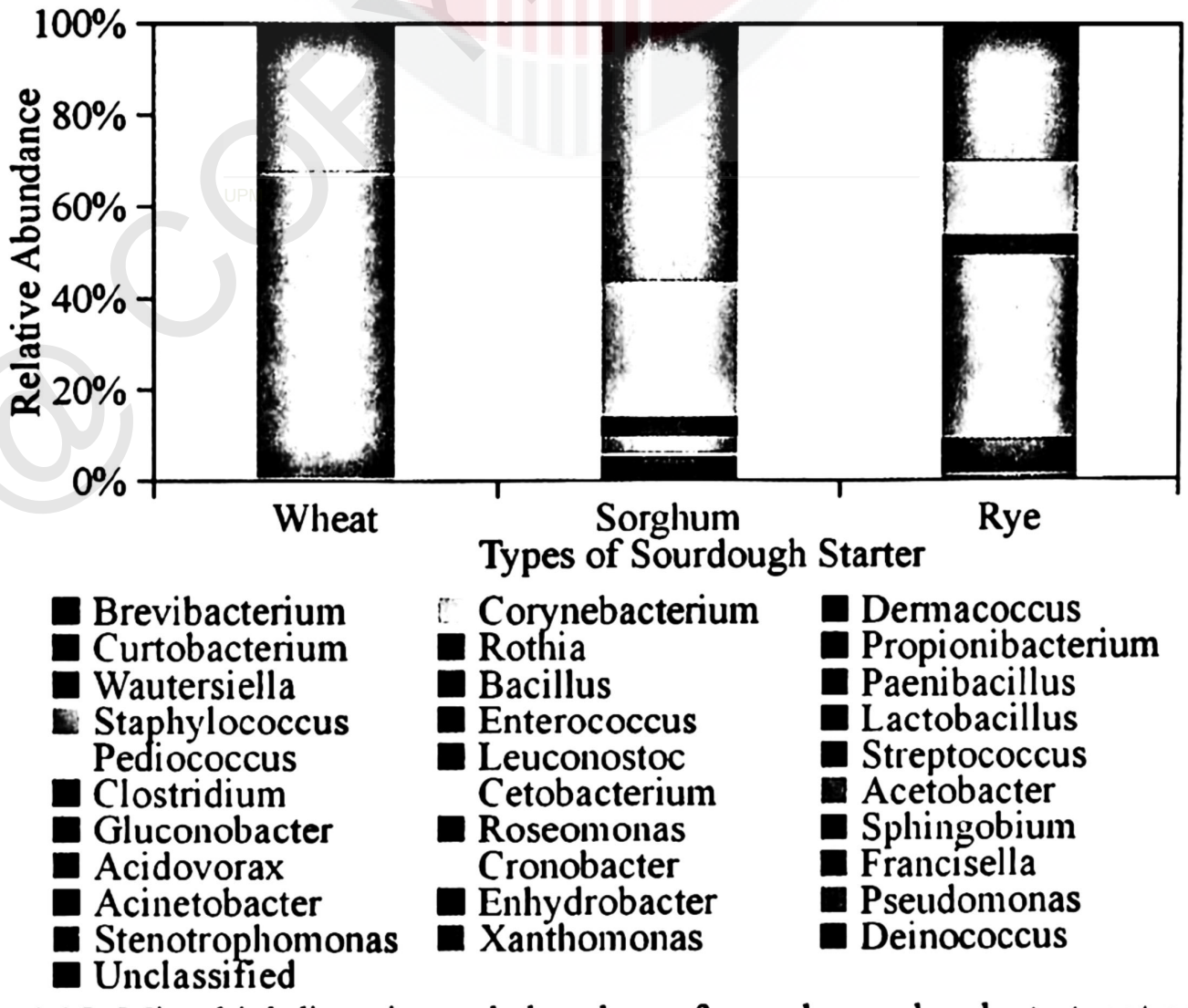

Figure 4.15: Microbial diversity and abundance for each sourdough starter at genus level

From bacterial profile shown in Figure 4.14, *Bacilli* was the dominating bacteria in all sourdough starters, wheat, sorghum and rye with a total of 90.21%, 57.93% and 53.89%, from all existed bacteria individually. Since fermentation of sourdough starter requires mainly LAB, collectively under the class of *Bacilli*, the incidence of *Bacilli* in the samples was notably high (De Angelis & Gobbetti, 2011). Relative abundance of *Gammaproteobacteria* was significantly higher in rye sourdough sample at 25.79% than in sorghum sample with the value of 8.07%, whereas for *Alphaproteobacteria*, they constituted more (29.75%) in sorghum than in rye (16.16%). The presence of the two classes were both insignificant in wheat sourdough samples. In the meanwhile, little *Actinobacteria* were identified in all samples.

Under the genus of *Lactobacillus* which belongs to class of *Bacilli*, one of the identified LAB was known as *L. brevis*, comprised of 0.135% in rye sourdough starter and 4.723% in sorghum sourdough starter. *L. brevis*, an obligately heterofermentative LAB, is found in rye and wheat sourdough (Bartkiene et al., 2020; Çakır et al., 2020; Hui & Sherkat, 2005), milk, cheese, fermented vegetables as well as other fermented food products (Teixeira, 2014). It can grow in cheese under conducive temperature and salt concentration (De Angelis & Gobbetti, 2011). Being an antifungal LAB, *Lactobacillus brevis* was competent in tolerating the conditions with low pH as well as presence of sodium chloride and potassium sorbate (Çakır et al., 2020). Low pH conditions in sourdough starters, due to production of lactic acid and acetic acid by *L. brevis*, are not favourable for many types of undesirable fungi and bacteria. Hence, the excellent potential in extending shelf life of sourdough bread contributes to the manufacture of freeze-dried *L. brevis* which allows rapid and convenient sourdough bread baking (Teixeira, 2014).

Lactobacillus paraplantarum, constituted 0.010% and 0.008% of rye and sorghum sourdough starters respectively, was identified from various food products, such as sauerkraut, a type of traditionally fermented white cabbage (Peñas et al., 2017), white brined cheese (Özer, 2014) and sourdough (Çakır et al., 2020; Settanni et al., 2005). *L. paraplantarum* undergoes facultatively heterofermentation which generates equimolar of lactic acid and acetic acid if gluconate is not present (Chavan & Chavan, 2011). *L. paraplantarum* SF9 is collectively known as probiotics, living microbes which brings positive health effects towards the host when being administered, with *L. brevis* SF15. They are able to survive in the acidic environment and at the same time, dominate over the pathogens competitively along the gastrointestinal tract (Peñas et al., 2017). Çakır et al. (2020) discussed the importance of *L. paraplantarum* in sourdough as it showed more than 90% of survival rate under pH of 2.5 for 24 hours, sodium chloride concentration of 6% and presence of potassium sorbate since sourdough bread is usually low in pH and added with sodium chloride or potassium sorbate as preservatives or flavouring. Antibacterial property towards *B. subtilis* together with antifungal effects against *Penicillium carneum*, *Aspergillus favus*, and *Aspergillus niger* molds were both observed from research of Çakır et al. (2020) too.

5.692% of rye sourdough starter was the homofermentative *Lactobacillus helveticus*. It is thermophilic yet limited to sodium chloride concentration of around 5% (Donnelly, 2014). Although usually being outcompeted in microbiota of sourdough starter, *L. helveticus* was the dominating LAB under genus of *Lactobacillus* for rye sourdough starter in present research. Besides, Viiard et al. (2013) pointed out *L. helveticus* as the LAB species with the highest abundance too in rye sourdough starter, while Banu and Aprodu (2012) concluded that higher

acidity was detected by inoculation of *L. helveticus* in rye sourdough starter when fermentation was done at higher temperature, around 40 °C. Apart from sourdough, *L. helveticus* is found more often in metabolising galactose from cheese and yogurt (Donnelly, 2014; Stanley, 2003).

4.3.1 Summary

By referring to the bacterial profiles, wheat sourdough starter had the lowest microbial diversity, in which around 90.2% of the bacteria were under class of *Bacilli* while the rest were mostly unclassified. Besides wheat sourdough sample, rye and sorghum sourdough samples also primarily comprised of *Bacilli* at 53.89% and 57.93% respectively. Relative abundance of *Gammaproteobacteria* and *Alphaproteobacteria* classes were both significant in rye and sorghum sourdough samples too. The major genus under class of *Gammaproteobacteria* was *Enterobacteriaceae*. In the meanwhile, the genus which dominated the microbial profile in sorghum sourdough starter was *Acetobacter*, from class of *Alphaproteobacteria*, which is under the category of acetic acid bacteria (AAB), thus contributes to acidification and leavening of sourdough starter together with crumb softness improvement.

Some important LAB found in the sourdough starters were *L. brevis*, which is commonly used in bakery industry for extension of shelf life due to its high acid tolerance, *L. paraplantarum*, playing the role as probiotics with antibacterial properties and antifungal effects, together with the thermophilic and homofermentative *Lactobacillus helveticus*.

CHAPTER 5

CONCLUSIONS & RECOMMENDATIONS

All the analysed properties of sourdough starters in this research, which are pH, total titratable acidity (TTA) and concentration of water-soluble free sugars, leavening height as well as ecological community of bacteria, are interconnected and interdependent. During fermentation, pH of all sourdough starters decreases with time. The lowest pH was achieved by wheat sourdough starter at 3.23 while the greatest plunge was observed in sorghum sourdough starter by pH of 2.43 (41.94%). Besides, the longer the fermentation time, the higher the TTA as pH decreases. The highest TTA value at the end of fermentation was reached by wheat sourdough starter, whereby 14.8 ml of 1.0N sodium hydroxide was required to titrate 10 grams of sample. Sorghum sourdough starter whereas showed the largest TTA rise by 13.3 ml or 324%.

During fermentation, the concentrations of maltose and sucrose were generally lower (less than 0.5g/100g sample) than fructose and glucose. The amount of fructose, glucose, sucrose and maltose peaked on day 2 at 2.011g, 2.205g, 0.370g and 0.136g respectively in 100g of wheat sourdough sample. The abundances for maltose and glucose increased significantly on day 6 to 0.161g/100g sample and 0.580g/100g sample respectively. Fructose concentration also rose notably on day 7 up to 0.534g/100g sample. Sorghum sourdough starter consisted of the least concentration of sugars, which ranged between 0 and 0.936 g/100g sample for each of them, and exhibited almost similar trend of changes in sugar concentration for other samples. As the greatest height increment among all types of sourdough starter and along the whole fermentation period, a leavening height of 82.91% was shown on day 2 of fermentation for wheat sourdough starter. The peaks of height increment for sorghum and rye sourdough starters were 37.17% and 31.40% respectively.

Among the microbiological ecosystem in sourdough starters, wheat sourdough starter had the lowest microbial diversity, with only one significant class of *Bacilli* (90.2%) while the rest were mostly unclassified. Sorghum and rye sourdough samples were also significantly comprised of *Enterobacteriaceae* (under class of *Gammaproteobacteria)* and *Acetobacter* (under class of *Alphaproteobacteria)* genera besides having *Bacilli* as the major class. There were some essential and beneficial LAB found in the sourdough samples, for example, *L. brevis*, *L. paraplantarum* and *Lactobacillus helveticus*.

The values of properties at any time within the fermentation period can be estimated easily and rapidly by applying the statistical models for the specific type of sourdough starter. However, statistical modelling done by best-fitting the experimental data had a lower accuracy as the possibility of having errors was high.

The models with cubic equations, which only applicable for some of fermentation period were troublesome too. Hence, it is recommended to have further researches conducted to determine the fundamental models.

To further enhance the results from this research, some improvements can be done for future researches. For example, properties of sourdough starters can be determined under different fermentation period, culture inoculation, temperature. The results for spontaneous fermentation can be compared with performance of industrial freeze-dried sourdough starters too. Researchers also can monitor the growth of different targeted LAB during fermentation of sourdough under different controlled conditions.

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APPENDIX

Table A1: pH changes of wheat, sorghum and rye sourdough starters along 10-day-long fermentation time

	pH		
Day	Wheat	Sorghum	Rye
0	4.20 ± 0.0465	5.79 ± 0.0150	6.01 ± 0.0545
1	3.80 ± 0.0918	4.62 ± 0.0824	5.85 ± 0.2962
2	3.52 ± 0.1684	4.71 ± 0.0264	5.45 ± 0.0605
3	3.67 ± 0.1807	4.86 ± 0.0943	5.27 ± 0.1189
4	3.37 ± 0.1958	3.74 ± 0.1690	5.13 ± 0.2481
5	3.78 ± 0.3055	3.38 ± 0.0508	4.80 ± 0.6080
6	3.47 ± 0.0071	3.28 ± 0.0071	3.98 ± 0.0141
7	3.27 ± 0.021	3.30 ± 0.0071	3.91 ± 0.0778
8	3.26 ± 0.0543	3.32 ± 0.0963	3.79 ± 0.0640
9	3.25 ± 0.0245	3.32 ± 0.0660	3.75 ± 0.0931
10	3.23 ± 0.4031	3.36 ± 0.0692	3.86 ± 0.0407

Table A2: Total titratable acidity (TTA) changes of wheat, sorghum and rye sourdough starters along 10-day-long fermentation time

	TTA		
Day	Wheat	Sorghum	Rye
0	4.8 ± 0.5657	4.1 ± 0.1414	3.1 ± 0.3536
1	7.8 ± 0.5972	10.4 ± 0.7616	6.2 ± 0.9465
2	13.5 ± 1.3000	11.2 ± 0.8832	11.1 ± 0.9777
3	10.1 ± 1.5706	10.1 ± 0.6602	12.3 ± 0.6898
4	7.9 ± 1.1165	14.6 ± 0.4787	13.2 ± 1.0720
5	10.9 ± 0.6801	14.5 ± 0.7365	11.1 ± 1.1177
6	11.4 ± 0.8485	14.9 ± 0.4243	11.7 ± 0.1414
7	15.0 ± 0.2828	17.4 ± 0.8485	11.3 ± 0.1414
8	15.8 ± 0.5058	15.4 ± 0.4031	15.1 ± 1.4728
9	15.8 ± 1.5927	15.1 ± 0.5033	14.9 ± 0.9215
10	14.8 ± 0.4899	14.6 ± 1.2500	13.9 ± 0.6602

Table A3: Leavening height of wheat, sorghum and rye sourdough starters along 10-day-long fermentation time

	Leavening Height (%)		
Day	Wheat	Sorghum	Rye
0	0.00 ± 0.0000	0.00 ± 0.0000	0.00 ± 0.0000
1	2.99 ± 3.4469	17.92 ± 20.7547	22.83 ± 9.6407
2	82.91 ± 5.6136	37.17 ± 21.7891	23.16 ± 14.6866
3	75.63 ± 15.4968	21.05 ± 2.4309	21.40 ± 5.7712
4	70.30 ± 35.7351	10.96 ± 7.2158	31.40 ± 16.2729
5	29.15 ± 25.0787	2.96 ± 1.9704	23.58 ± 8.6164
6	40.00 ± 6.4865	10.50 ± 5.9722	16.16 ± 7.1900
7	35.38 ± 16.9464	7.69 ± 4.2288	7.88 ± 3.2177
8	34.33 ± 28.4410	6.03 ± 5.4432	6.67 ± 5.3948
9	33.50 ± 25.3706	5.88 ± 0.0000	2.35 ± 4.6948
10	41.81 ± 7.0263	10.58 ± 1.7280	17.61 ± 5.9772

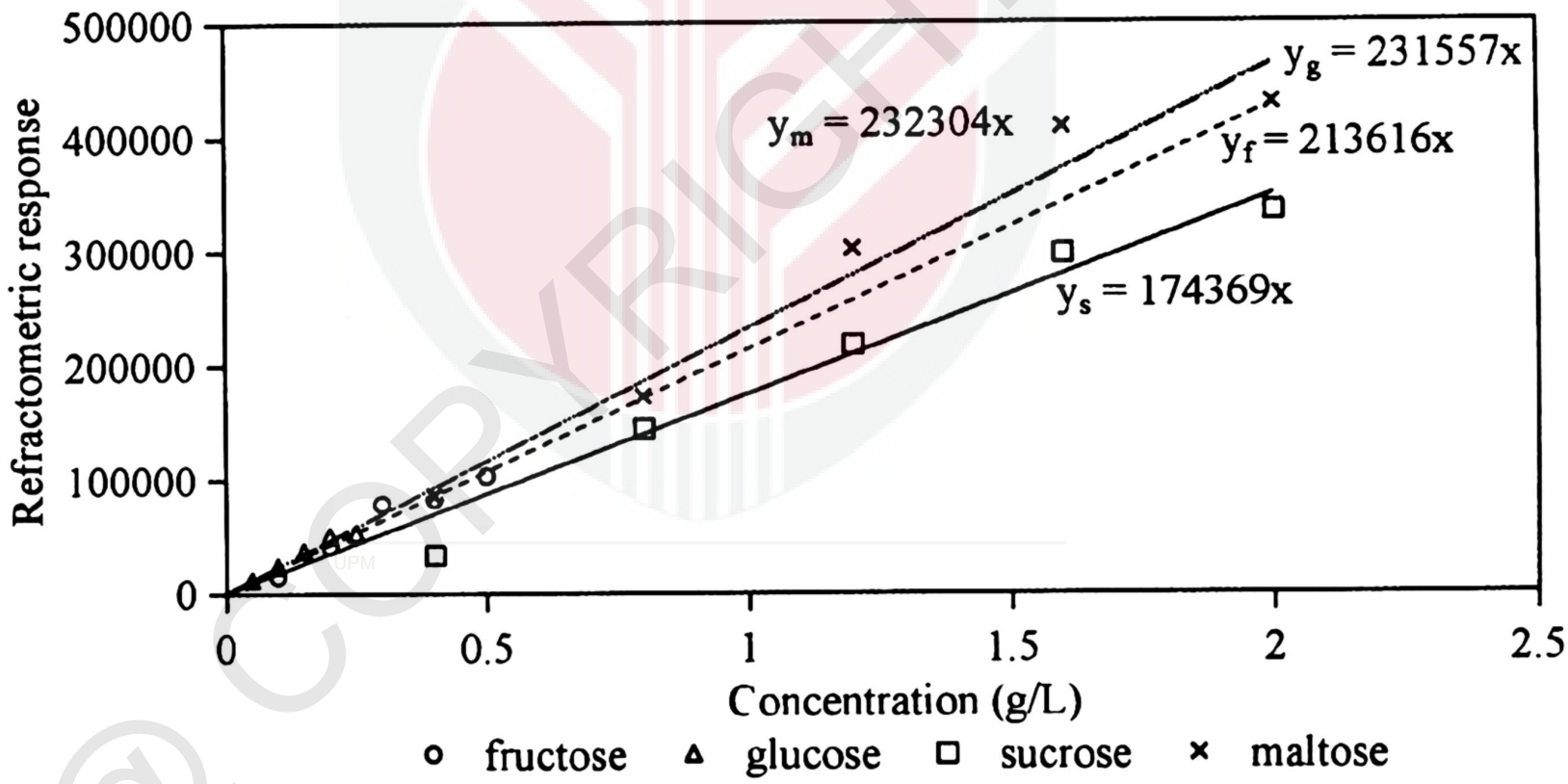

Figure A1: Calibration curves for water-soluble free sugars under refractometric (RI) detection

Table A4: Taxonomic identities of microbiota in rye (R), sorghum (S) and wheat (W) sourdough starters and their relative abundance

Taxonomic Identity							Relative abundance		
Kingdom	Phylum	Class	Order	Family	Genus	Species	R (%)	S (%)	W (%)
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Brevibacteriaceae	Brevibacterium	aureum	0.000	0.008	0.000
				Corynebacteriaceae	Corynebacterium	-	0.000	0.000	0.004
						stationis	0.000	0.000	0.014
				Dermacoccaceae	Dermacoccus	-	0.010	0.000	0.014
				Microbacteriaceae	Curtobacterium	-	0.000	0.017	0.000
				Micrococcaceae	Rothia	terrae	0.007	0.000	0.000
				Propionibacteriaceae	Propionibacterium	acnes	0.000	0.008	0.000
	Bacteroidetes	Flavobacteriia	Flavobacteriales	Weeksellaceae	Wautersiella	-	0.000	0.000	0.007
	Chloroflexi	Thermomicrobia	JG30-KF-CM45	-	-	-	0.000	0.008	0.000
	Cyanobacteria	-	-	-	-	-	0.000	0.000	0.007
	Firmicutes	Bacilli	-	-	-	-	0.000	0.000	0.090
			Bacillales	Bacillaceae	Bacillus	-	0.317	0.104	0.000
				Paenibacillaceae	Paenibacillus	-	0.925	0.038	0.011
				Staphylococcaceae	Staphylococcus	-	0.232	0.000	0.047
				-	-	-	0.000	0.000	0.004
			Lactobacillales	Enterococcaceae	Enterococcus	-	0.000	0.017	0.000
				Lactobacillaceae	-	-	4.389	48.086	23.234
					Lactobacillus	-	1.536	0.276	0.000
						brevis	0.135	4.723	0.000
						helveticus	5.962	0.000	0.000
						paraplantarum	0.010	0.008	0.000
					Pediococcus	-	0.000	0.679	0.735
				Leuconostocaceae	-	-	0.000	0.155	0.004
					Leuconostoc	-	40.381	3.830	66.177
				Streptococcaceae	Streptococcus	-	0.000	0.013	0.000
		Clostridia	Clostridiales	Clostridiaceae	Clostridium	-	0.031	0.000	0.000

					butyricum	3.981	4.192	0.000
Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium	somerae	0.079	0.000	0.018
-	-	-	-	-	-	0.000	0.000	7.462
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacter	-	16.145	29.672	0.441
				Gluconobacter	-	0.007	0.038	0.000
				Roseomonas	mucosa	0.000	0.008	0.000
		Rickettsiales	-	-	-	0.000	0.021	0.011
			Anaplasmataceae	-	-	0.010	0.000	0.000
		Sphingomonadales	Sphingomonadaceae	Sphingobium	-	0.000	0.008	0.000
	Betaproteobacteria	-	-	-	-	0.052	0.000	0.000
		Burkholderiales	Comamonadaceae	Acidovorax	-	0.000	0.000	0.022
		Rhodocyclales	Rhodocyclaceae	-	-	0.000	0.000	0.011
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	-	-	25.517	8.031	1.627
				Cronobacter	sakazakii	0.010	0.000	0.000
		Legionellales	Francisellaceae	Francisella	-	0.027	0.000	0.000
		Pseudomonadales	Moraxellaceae	Acinetobacter	guillouiae	0.159	0.000	0.000
				Enhydrobacter	-	0.012	0.000	0.014
			Pseudomonadaceae	Pseudomonas	veronii	0.000	0.000	0.007
					viridiflava	0.000	0.000	0.011
		Vibrionales	Pseudoalteromonadaceae	-	-	0.033	0.044	0.025
		Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	-	0.029	0.000	0.000
				Xanthomonas	campestris	0.000	0.000	0.004
WPS-2	-	-	-	-	-	0.000	0.017	0.000
Thermi	Deinococci	Deinococcales	Deinococcaceae	Deinococcus	-	0.005	0.000	0.000