Effect of Different Sprouting Conditions on Alpha Amylase Activity, Functional Properties of Wheat Flour and on Shelf-Life of Bread Supplemented with Sprouted Wheat

by

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ABSTRACT

EFFECT OF DIFFERENT SPROUTING CONDITIONS ON ALPHA AMYLASE ACTIVITY, FUNCTIONAL PROPERTIES OF WHEAT FLOUR AND ON SHELF-LIFE OF BREAD SUPPLEMENTED WITH SPROUTED WHEAT

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In this study sprouting two different wheat cultivars under various environmental conditions revealed that varietal variation is the most important factor affecting α-amylase quantity as well as quality to modify flour functionality significantly, followed by pre-soaking duration and temperature. Sprouted wheat flour post five days germination was utilized at different rates to prepare 100 g composite breads. There was an improvement in baking quality and shelf life of breads containing 1% and 5% sprouted flour resulting in a significantly increased loaf volume, better texture, and less retrogradation during 7 days post baking than the control. This study presents opportunities for industry to fortify baked products with sprouted wheat flour to yield functional whole grain products that are nutrient dense and naturally shelf-stable.
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# TABLE OF CONTENTS

Acknowledgements........................................................................................................ iii

Table of Contents........................................................................................................ iv

List of Tables ................................................................................................................ ix

List of Figures ............................................................................................................... x

Chapter One  Introduction ............................................................................................. 1

Chapter Two  Litreature Review .................................................................................. 4

2.1 Sprouting Overview ................................................................................................. 4

2.1.1 Sprouted Grains Health Benefits ....................................................................... 8

2.1.2 Sprouted Grains Quality Issues .......................................................................... 10

2.2 Physiochemical Changes during Sprouting ............................................................. 13

2.2.1 Starch Degradation ............................................................................................. 15

2.2.1.1 Alpha-Amylase ............................................................................................. 19

2.2.1.1.1 Structure .................................................................................................. 19

2.2.1.1.2 Isoforms .................................................................................................. 20

2.2.1.1.3 Action Mechanism .................................................................................. 20

2.2.1.1.4 Products .................................................................................................. 22

2.2.1.1.5 Role during Germination ........................................................................ 22

2.2.2 Protein Degradation ........................................................................................... 24

2.2.3 Fiber Degradation ............................................................................................... 27
Chapter Three  
Effect of wheat sprouting under various environments on alpha-amylase activity and phyiochemical properties of flour .......................................................... 38

3.1 Abstract ................................................................................................................. 38

3.2 Introduction ............................................................................................................. 39

3.3 Materials and Methods .......................................................................................... 44

3.3.1 Plant Material ..................................................................................................... 44

3.3.2 Sprouting Conditions .......................................................................................... 44

3.3.3 Drying and Milling .............................................................................................. 45

3.3.4 Mositure Content ............................................................................................... 45

3.3.5 Determination of α-amylase Activity .................................................................. 46

3.3.6 Zymography ....................................................................................................... 46

3.3.7 Flour and Dough Quality Analysis ....................................................................... 47

3.3.7.1 RVA ................................................................................................................. 47

3.3.7.2 Farinograph ..................................................................................................... 48

3.3.7.3 GlutoPeak Tester ............................................................................................ 48

3.3.7.4 Electrophoresis ............................................................................................... 49
3.3.7.5 Protein Content.................................................................49
3.3.7.6 Insoluble and Soluble Dietary Fiber Content...............................50
3.3.7.7 Arabinoxylan Content.........................................................50
3.3.8 Statistics.................................................................................51
3.4 Result and Discussion..................................................................51
3.4.1 Sprouting Conditions and α-Amylase Activity.................................51
3.4.2 Sprouting Conditions and Flour-Dough Functional Properties...........56
  3.4.2.1 Effect on Gelatinization Ability.............................................56
  3.4.2.2 Effect on Dough Rheology....................................................59
  3.4.2.3 Effect on Gluten Aggregation Kinetics.....................................64
  3.4.2.4 Effect on Protein Molecular Weight Distribution.......................68
  3.4.2.5 Effect on Protein Content.....................................................70
  3.4.2.6 Effect on Dietary Fiber Content.............................................71
  3.4.2.7 Effect on Arabinoxylan Content............................................72
3.3 Conclusion....................................................................................74

Chapter Four Investigating Physiochemical and Staling Behaviour of Breads Fortified with Sprouted Wheat Flour.............................................................75
4.1 Abstract.......................................................................................75
4.2 Introduction...................................................................................76
4.3 Materials and Methods.................................................................81
4.3.1 Plant Material

4.3.2 Sprouting Conditions

4.3.3 Drying and Milling

4.3.4 Determination of α-Amylase Activity

4.3.5 Bread-Making

4.3.5.1 Composite Flour Preparation

4.3.5.2 Water Absorption Capacity of Composite Flour

4.3.5.3 Dough Processing

4.3.5.4 Baking and Storage

4.3.6 Quality and Shelflife Analysis of Bread

4.3.6.1 Volume

4.3.6.2 Texture

4.3.6.3 RVA Profiles

4.3.6.4 DSC Analysis

4.3.7 Statistics

4.4 Results and Discussions

4.4.1 Alpha-amylase Activity

4.4.2 Effect on Water Absorption of Composite Flours

4.4.3 Effect of Sprouted Wheat Fortification on Bread Quality
4.4.4 Effect of Sprouted Wheat Fortification on Bread Shelf-life

4.4.4.1 Starch Retrogradation

4.4.4.2 Enthalpy of Retrogradation

4.5 Conclusion

Chapter Five Conclusions

References Cited
LIST OF TABLES

Table 3.1: Abbreviation used for all sprouted wheat samples. .............................. 45
Table 3.2: Effect of various temperratures and steeping duration on Peak Viscosity (cP) and Peak Temperature (˚C) for 5 days of sprouting in wheat.......................... 58
Table 3.3: Effect of various temperatures and steeping duration on the % Protein Content (w/w) dry basis for 5 days of sprouting in wheat.......................................................... 70
Table 3.4: Effect of various temperatures and steeping duration on the % Insoluble Dietary Fiber (IDF), Soluble Dietary Fiber (SDF) and Total Dietary Fiber (TDF) Content (w/w) dry basis for 5 days of sprouting in wheat ............................. 71
Table 4.1: Composite flour ratios and subsequent α-amylase activity units (Ceralpha Units) in 100 g of breads.................................................................................. 83
Table 4.2: Change in Peak Viscosity (cP) and Final Viscosity (cP) of Composite Breads during 7 days of Storage.......................................................... 91
Table 4.3: Change in Enthalpy (J/g of starch) and Transition Temperature (˚C) of Composite Breads during 7 days of Storage................................. 94
LIST OF FIGURES

Fig. 2.1.1: Representative Germinated Wheat Kernel ................................................. 6
Fig. 2.1.2: Representative Parts of a Spoueed Wheat Kernel ........................................ 7
Fig. 2.2.1: Wheat Kernel Anatomy ............................................................................... 14
Fig. 2.2.2: Schematic representation of two forms of starch: Amylose & Amylopectin .. 16
Fig. 2.2.3: Schematic representation of different structural levels of a starch granule .. 17
Fig. 2.2.4: Schematic representation of differences in action of different amylases .... 18
Fig. 2.2.5: Schematic representation of the double displacement mechanism and the for-
mation of a covalent intermediate by which retaining glycosylhydrolases act .......... 22
Fig. 2.2.5: Schematic representation of gluten development ........................................ 25
Fig. 2.2.6: Schematic representation of Arabinoxylan ............................................... 28
Fig. 2.3.1: Schematic representation of gluten matrix during kneading ....................... 30
Fig. 2.3.2: Schematic representation of gas bubble expanding causing gluten network to
stretch .............................................................................................................................. 30
Fig. 2.3.3: Starch granules embedded in a continuous protein matrix ......................... 32
Fig. 3.1: Effect of various temperatures and steeping durations on α-amylase activity
(Ceralpha Units/g of flour) for 5 days of sprouting in wheat .................................... 54
Fig. 3.2: Zymogram pattern of α-amylase isoforms in wheat sprouted under various
temperatures and steeping durations for 5 days and Control (C) ......................... 56
Fig. 3.3a: Peak Dough Consistency (BU) of wheat flours sprouted under various
temperatures and steeping durations for 5 days and Control (0) ......................... 62
Fig. 3.3b: Peak Dough Development Time (min) of wheat flours sprouted under various
temperatures and steeping durations for 5 days and Control (0) ......................... 63
Fig. 3.4a: Peak Torque (BE) of wheat flours sprouted under various temperatures and
steeping durations for 5 days and Control (0)………………………………………….66
Fig. 3.4b: Peak Torque Time (min) of wheat flours sprouted under various temperatures
and steeping durations for 5 days and Control (0)………………………………………..67
Fig 3.5: Effect of various temperatures and steeping durations on protein molecular
weight distribution for 5 days of sprouting in wheat……………………………………..69
Fig 3.6: Effect of various temperatures and steeping durations on Arabinoxylan content
(g/100g) for 5 days of sprouting  in wheat …………………………………………………73
Fig 4.1: Specific volume (cm$^3$/g) of composite breads…………………………………87
Fig 4.2: Firmness (g) of composite bread during 7 days of storage…………………..89
CHAPTER ONE – INTRODUCTION

Wheat is a major staple crop to billions of people around the globe and is used in a wide variety of food products such as bread, breakfast cereal, flatbreads, tortillas, cookies, pie crusts, soup thickeners, noodles and gravies. It is generally agreed that wheat was one of the first grains to be cultivated as it has been commercially cultivated around the Eastern Mediterranean and Mesopotamia for at least 5000-6000 years (Lev-Yadun et al., 2000). In addition to being a fundamental source of calories and nutrients, wheat is an economically important crop in Canada and around the globe.

In past, sprouting of wheat was considered a negative aspect due to increased hydrolytic and proteolytic activity, degrading storage carbohydrate and protein reserves into smaller fractions, to support the metabolic need of developing seedlings while decreasing the functional quality of grain as food. Recently, it has been shown that cereals sprouts are more nutritious in terms of vitamins, minerals, and phenolic compounds when compared with their native counterparts because grain’s mobilized energy reservoir is readily available in its active form (Plaza et al., 2003; Koehler et al., 2007; Hung et al., 2001, 2011). However, the chemical composition of the sprout is affected by sprouting conditions such as temperature, humidity, soaking duration and light. For this reason, determination of optimal sprouting conditions is considered crucial to yield nutrient dense yet reasonably functional flour for baked products.

Research Goals, Objectives, and Hypothesis

The goal of this research project is to gain further insight into the qualitative and quantitative production of α-amylase activity during sprouting process of wheat, as well as to investigate the functional and rheological properties of this sprouted wheat flour and then study the role of sprouted wheat flour as an alternative to commercial anti-staling agent in bread.
Although many studies have investigated various sprouting conditions and how they relate to amylase enzyme activity especially for barley based on interest from malting industry, the rheological properties of sprouted wheat flour and dough have not been comprehensively characterized. There are only few studies regarding germination’s effect either on starch or on gluten but no one complete study on changes in these two important constituents of wheat flour all together has been established so far. The objective of this study is to observe changes in functional properties of starch-gluten matrix paralleling α-amylase activity with various sprouting conditions in two different varieties of wheat and try to shed more light on the application of germinated whole wheat flour in food industry.

Furthermore, based on the demand from the consumers for the natural, additive-free and nutrient dense food products, this research project proposes to evaluate functional properties of sprouted wheat in baked products as a replacement for commercial enzymes and additives while enhancing the nutritional profile. It will characterize sprouting of different varieties of wheat at various conditions and investigate the germination process to find the variables that optimize the activities of α-amylase enzyme necessary to prevent staling in bread, naturally. By extending shelf-life, manufacturers can simplify warehousing and logistics, while also reducing waste. In brief, by characterizing the sprouting of wheat, it will allow for expansion of healthier whole grain products that are naturally shelf-stable for consumers.

Specifically, the objectives of this research project are to investigate:

1. The effect of sprouting extent, temperature, steeping duration and humidity on α-amylase activity.
2. The effect of this enzymatic activity on the functional and rheological properties of flour and dough during processing.
3. The application of sprouted wheat flour as a source of anti-staling agent in bread made with different inclusion rates as well as the structural and functional characteristics of bread made with sprouted wheat.

Therefore, the goal of the present study was to identify factors that lead to optimal α-amylase activity in wheat. The obtained results would assist in developing functional yet nutrient dense baked products that are naturally resistant to staling.
CHAPTER TWO - LITERATURE REVIEW

2.1 Sprouting Overview

Seeds are important constituent of the world’s diet. Cereal grains, about 90% of all cultivated seeds contribute up to half of the global per capita energy intake. Seeds themselves are a very nutritious form of food because they contain proteins, carbohydrates, vitamins, minerals, and oils that a beginning plant needs to grow. Recently, to further enhance the nutritive values of these seeds, a natural processing method known as sprouting is getting very popular based on the scientific evidences linking sprouting to increased health benefits. This method has been known from ancient times in the Eastern countries such as China and Japan, and is a simple and inexpensive process to carry out.

Seed germination, at microscopic level, is a complex process involving many individual reactions and phases including protein hydration, subcellular structural changes, respiration, macromolecular syntheses, and cell elongation, all important events during this process but none of them is itself unique to germination (Bewley, 1997). Furthermore, several environmental factors such as water, oxygen and temperature are required at an ideal state for the germination to progress. Firstly, wheat genotypes vary in resistance to sprouting and frequently, red-seeded genotypes are relatively more resistant to sprouting than white-seeded wheat (Gfeller & Svejda, 1960; Mares, 1994). Initial moisture required for the grain to sprout varies among varieties but generally wheat has a critical moisture content of 40% for germination to occur (Bewley & Black, 1994). A triphasic pattern of water uptake has been demonstrated during germination process. Phase I occurs in dead or live seeds due to matric forces and their attraction for water molecules inside the seed and an immediate release of gases is observed. During Phase II, there is a lag period in water uptake, but seed undergoes many processes essential for germination.
Finally in Phase III, primary root (radicle) elongation is observed and as it becomes functional it is responsible for the increased water uptake noted in Phase III (Fig. 2.1.1). Shortly after, two seedlings (seminal) roots emerge from the seed, developing equally so that there is no main root and root hairs grow on the upper regions (Fig. 2.1.2). At the same time, plumule grows straight up and through the fruit wall, but the growing point and first leaves are protected by a sheath, the *coleoptile*, with a hard, pointed tip. Once above the soil the first leaves burst out of the coleoptile which remains as a sheath round the leaf bases.

Temperature and initial moisture are the two most important environmental factors influencing the induction and progression of sprouting in wheat, playing a critical role on germination percentage and thus ultimately on the enzyme activities especially of amylases. Furthermore, with greater access to oxygen germination process then accelerates, provided sufficient moisture remains available. The starch in the grain is broken down into less complex sugars and ultimately into glucose, which is used as an energy source for growth of the primary root, one or two pairs of smaller roots and then the shoot. At this point, the non-fibrous carbohydrate (NFC) content of the grain falls and its neutral detergent fiber (NDF) and crude protein (CP) contents increase.
Fig 2.1.1 – Representative Germinated Wheat Kernel

Adopted from www.wheatbp.net
Fig 2.1.2 – Representative Parts of a Sprouted Wheat Kernel

Adopted from www.umn.edu
2.1.1 Sprouted Grains Health Benefits

Most recently there has been an increasing concern and awareness of the effect of diet on health and disease. Changes in eating patterns, food choices and methods of food preparation have decreased the nutritive value of our diets. Generally, a typical diet in developed countries is lower in dietary fiber, vitamins, minerals and antioxidants. Academy of Nutrition and Dietetics recommends to consume more whole grains, accounting for one half of an adult’s six to eight daily grain servings (AND, 2010). Cereal sprouts fulfill the nutritional principles of whole grains with added benefits of higher bioavailability of vitamins, minerals and soluble fiber as recently proved by some researchers studying the effect of sprouting on nutritional quality of grains, especially of wheat (Plaza et al., 2003; Koehler et al., 2007; Hung et al., 2001, 2011).

In a study, biosynthesis of ascorbic acid (vitamin C) was recognized as one the most important factors in the sprouting seeds, increasing its content by 1.5 fold. In the same study minerals such as Cu, Fe, K and Zn and phytoestrogens (Daidzein and Genistein) were also reported to have significant increased during sprouting (Plaza et al., 2003).

Hung and colleagues concluded that germinated waxy wheat variety had better nutritional composition, such as higher dietary fiber, free amino acids and total phenolic compounds content, than the ungerminated seeds (2012). Another study showed 2 fold increase in \( \alpha \)-tocopherol content and an average 2-3 fold increase in minerals especially Mg, Ca, Fe, Na, K and P in two different varieties of wheat (Ozturk et al., 2011). Koehler and others has reported a time- and temperature-dependent increase of total folate during germination. A maximum 3.6-fold concentration was obtained after 102 h of germination at 20 and 25˚C (2007).

According to Yang and co-workers, vitamin C and E and \( \beta \)-carotene were barely detectable in the dry wheat grains but upon germination the concentration of these antioxidant vitamins
steadily increased with increasing germination time, reaching their peaks after 7 day at 550μg/g for vitamin C, 10.92μg/g for α-tocopherol, and 3.1μg/g for β-carotene. Concentrations of ferulic and vanillic acids were also increased, reaching their maxima after 7 day at 932.4μg/g and 12.9μg/g, respectively (2001).

Nevertheless, along with nutritional benefits, whole wheat flours contain significant amount of undesirable compounds, such as phytates that forms complexes with minerals such as Ca⁺², Fe⁺³, Zn⁺², and Mg⁺² reducing their bioavailability (Lopez et al., 2000; 2001). Germination has been shown to reduce the content of phytic acid in wheat due to increased phytase activity, converting it to inositol and orthophosphate, phosphate in readily bioavailable form (Wu et al., 2009). In a recent study, at eighth day of germination, phytase activity increased to 6 fold, significantly reducing phytate while increasing total phosphorous content of wheat grains (Azeke et al., 2011).

In general, germination reduces the amount of dry weight by catabolizing carbohydrates to CO₂ and H₂O, thus concentrating the total mineral, vitamin, fiber and protein content of the flour (Lorenz, 1980; Plaza et al., 2003). At the same time, several studies mentioned above have shown that biosynthesis of Vitamin C, E, β-carotene, folate and phenolic acids takes place during sprouting process.

Furthermore, bread fortified with germinated wheat seedlings has been shown to positively affect fasting and postprandial glucose levels compared to a control wheat bread, evoked by fiber compounds as well as by non-fiber compounds such as phenolic acids (Anderson, 2008). Intake of flavonoids is associated with reduced risk of type 2 diabetes, as administration of plum showed an anti-hyperglycemic effect in rats (Utsunomiya et al., 2005), confirming that phenolic compounds exhibit positive impact on glucose and insulin metabolism (Knekt et al., 2002).
These findings lead to consider that other grain compounds than fiber may also positively affect glucose regulating factors.

Based on these evidences, sprouted grains are getting popular in the consumer market. According to the Center for Culinary Development’s 2011 Culinary Trends Mapping report, sprouted grains are at a stage two trend. CCD uses a five-step model to follow food products on their journey from emerging trends to main-stream consumer items. As a stage two trend, sprouted grains are being featured in consumer media ranging from Food Network to *Bon Appetit* magazine. They are also carried by retailers catering to culinary professionals and serious chefs (CCD, 2012).

Many of the health benefits associated with sprouted grains are due to the fact that they are produced from whole grains. Whole grain products include the entire grain, in comparison to refined flour products, which lack the bran and germ. Whole grain products are being consumed in greater quantities as nutritional and medical studies repeatedly link diets with whole grains to improved health such as reduced heart disease, cholesterol, type II diabetes, obesity and improved bowel function (Plaza et al., 2003). Therefore, health conscious people are taking the advice of Academy of Nutrition and Dietetic to incorporate more whole grains serving in their daily diet. This recent trend of consuming more whole wheat bread, whole wheat pasta and whole wheat noodles has driven the food industry to come up with whole wheat products. As consumers continue seeking for healthier grains, products made from sprouted whole grains should be considered.

### 2.1.2 Sprouted Grains Quality Issues:

Despite the benefits that sprouted wheat has to offer, it is recently being consumed only as salad, juice, and tablet for its vitamins, minerals, and phenolic contents, because it comes with...
the problem of inferior quality flour that leads to processing problems and unsatisfactory end-products during food manufacturing. Sprouting effects the physiochemical and functional properties of wheat, as flour extraction, ash and protein content and falling number decreases while α-amylase and proteolytic activity increases with sprouting. Sprouted wheat flours are ill suited for dough and bread making and use of 100% flour from sprouted flour yields bread with poor characteristics, wet, sticky dough, darker crumb color and inferior texture (Edwards et al., 1989; Ariyaman & Khan, 1990; Beleia and Grossmann, 1990; Cabrera et al., 1995; Yamauchi et al., 1998).

In a study by Singh and colleagues, effect of sprouting conditions (soaking duration, sprouting temperature and soaking duration) on functional and dynamic rheological properties were studied, demonstrating that with increased sprouting time, falling number and water absorption index (WAI) decreased while water solubility index (WSI) increased (2001). Elasticity of dough decreased with increase in soaking and spraying duration, while viscosity showed an increase with the increase in soaking duration and a decrease with the increase in sprouting duration. It is important to note that dough elasticity and viscosity are the two main rheological properties for the effective utilization of wheat in different products.

Lorenz and Valvano investigated the functional characteristics of sprout-damaged soft white wheat flours in cakes and cookies (1981). They concluded that longer sprouting times had a detrimental effect, not only on cake volume, but also on external and internal cake characteristics as well, with a dip in the center, a coarse grain and a rather dense and firm texture. Similarly, sprouted flour cookies had higher spread factors and grain scores than for cookies baked from the sound flour. The extensive damage of starch in the sprouted wheat flour, caused by high amylase activity is responsible for these poor baking characteristics because starches
from sprout-damaged cereal grains exhibit decreased swelling power and they gelatinize at a lower temperature and over a narrow temperature range than starches from sound grain (Lorenz & Kulp, 1981; Noda et al., 2003). Since the most important function of starch during baking is gelatinization, baking quality of sprouted flour’s starch is compromised for cookies and cakes.

Sprouting has been reported to adversely affect the rheological properties of wheat due to enzymatic hydrolysis of starch, protein and fiber catalyzed by the increased activity of various enzymes especially of alpha-amylase and protease in the germinated wheat (Edwards et al. 1989). They observed severe product defects in Cantonese and Korean noodles, while lesser but still deleterious effects in Arabic flat-breads. However, the quality of pan breads made from sprouted grains was better than that from corresponding untreated wheat, as water absorption capacity of sprouted flours was higher. This was in contrast to most published reports at that time where baking quality was inferior due to sprouting. It was concluded that the relative levels of protease and amylase activities in the sprouted flours were optimal in the baking system than those in the control flours, leading to believe that increased amylase and protease activity of the sprouted samples acted as an effective gluten softener thus reduced mixing time and improved loaf volumes.

Similarly, an earlier study by Morad and Rubenthaler had also postulated that amylolytic activities are important factors affecting bread crumb firmness (1983). It was indicated that insufficient or excess amylase activity increases crumb firmness and staling rate of bread.

These findings were later supported by many different studies and are further discussed in section 3.


2.2 Physiochemical Changes during Sprouting

The life cycle of a cereal grain has two phases, development and germination, separated by a period of dormancy. In the germination process, seed plays the role of a reproductive unit and is a thread of life that assures survival of all plant species. Wheat seed or kernel can be divided into three distinct morphological parts: endosperm, bran layer also called peripheral layer which is composed of aleurone layer, nucellus tegument, testa and pericarp layer, and finally germ that includes the embryo and scultellum (Evers & Bechtel et al., 1988; Tasleem-Tahir et al., 2011).

During germination, most of the stored nutrients such as carbohydrates, protein and fat are mobilized to supply energy necessary for the growth of the emerging seedling by the action of their endogenous enzymes. These enzymes are already present in the seed or produced upon initiation of sprouting. The location of enzymes within the kernels is not uniform but they are predominantly located in the outer aleurone and bran layers of the kernel, and in the germ (Poutanen, 1997). For instance, alpha-amylase is mainly located in the pericarp with small quantities in the aleurone layer and the seed coat. Proteases are concentrated in the endosperm, germ and aleurone layer. The scutellum and embryo are rich in lipoxygenase, whereas, polyphenol oxidase and peroxidase are predominant in bran layers (Rani et al., 2001). Action of these enzymes, based on the specificity of their substrate, causes major anatomical as well as physiochemical changes in the morphology of the seed.
Fig 2.2.1- Wheat Kernel Anatomy

Adopted from www.bakeinfo.co.nz
2.2.1 Starch Degradation:

Starch accounts for the about 65-70% of the total dry weight of wheat grain and is important for determining the processing and eating quality of various products from wheat flour. Starch consists of two components: amylose, which is a linear glucose polymer having α-1,4 linkages, and a larger, branched polymer, amylopectin, that has linear chains of α-1,4 glucose residues interlinked by α-1,6 linkages. The amylose/amylopectin ratio differs between wheat genotypes but typical levels of amylose and amylopectin are 25-28% and 72-75%, respectively (Colonna & Bule’on, 1992).

Starch is present as intracellular water-insoluble granules of different sizes and shapes. The native starch granule usually shows three forms of crystalline structures A, B and C type according to X-ray diffractometry (Zobel, 1988) which play a critical role not only in the starch granule architecture but also in physiochemical characteristics such as susceptibility to enzymes and swelling power (Naguleswaran, 2012). The mature endosperm of wheat contains both large A-type (10-40 μm) and small B-type granules (2-10 μm) with a bimodal distribution (Evers, 1973) as both granules possess differential compositions, molecular/granular structures, and physical (swelling, gelatinization, and pasting) properties.

Native starch granules are birefringent when viewed under polarized light, indicating a degree of order in the granule and an orientation of the macromolecules perpendicular to the surface of the granule (Bule’on et al., 1998). At the lowest structural level, starch granule is defined in terms of alternating amorphous and semi-crystalline growth rings. Amorphous areas are less dense and contain amylose and amylopectin branching regions (not crystalline) while the semi-crystalline areas are made up by amylopectin double helices packed in a parallel fashion (Fig 2.2.3).
Fig. 2.2.2 – Schematic representation of two forms of Starch: Amylose & Amylopectin

Adopted from www.alevelnotes.com
Fig 2.2.3 – Schematic representation of different structural levels of a starch granule

Adapted from Donald et al. (1997) and Buleon et al. (1998)
Amylases are the enzymes that hydrolyze starch and three major types of amylase based on their mode of action are endoamylases (α-amylases), exoamylases (β-amylase, glucoamylases, α-glucosidases), and debranching enzymes (isoamylases and limit dextrinase). Presence of these enzymes in wheat explains starch degradation during maturation, storage and processing of food.

Fig 2.2.4- Schematic representation of differences in action of different amylases

Adopted from Goesaert et al., 2009
2.2.1.1 **Alpha-Amylase:**

Alpha-amylase (1,4-D-glucan glucanohydrolase, E.C. 3.2.1.1) is not present in resting cereal grains with the possible exception of maize and sorghum (Beck & Ziegler, 1989) but upon germination a phytohormone called gibberellic acid stimulates their synthesis during Phase I and II of sprouting along with the presence of cytoplasmic or even exogenously added Ca²⁺ ions. (Bewling & Black, 1994). On the other hand, abscisic acid has been proven to inhibit this enzyme’s production (Beck & Ziegler, 1989; Muralikrishana & Nirmala, 2005).

2.2.1.1.1 **Structure:** Alpha-amylases are small proteins with a molecular weight of 20-55 kDa. Several studies have shown that α-amylase polypeptides are made up of more than one folding unit or domain and they are metalloenzymes containing covalently bound Ca²⁺ which acts as an allosteric activator (Nirmala & Muralikrishana, 2003). The polypeptide chain is folded into three domains belonging to the (β/α)₈-barrel protein family. The A-domain consists of a highly symmetrical fold of eight parallel β-strands arranged in a barrel encircled by eight parallel α-helices, with an extra helix inserted after the sixth β-strand. The β-strands and helices alternate along the polypeptide chain and are linked together by irregular loops. Amino acid residues situated on the loops join the C-terminal end of each β-strand to the N-terminal end of the following helix, resulting in characteristic βα/βα/….connectivity (Matsuura et al., 1984; van der Maarel et al., 2002). Nakajima and colleagues clearly pointed out the existence of four highly conserved regions, especially in the catalytic and substrate binding regions in 11 different α-amylases (1986). The active site has amino acid residues in loops and projects outwards from the C-terminal end of the β-strands. The B-domain of this enzyme protrudes between β-sheet no. 3 and α-helix no. 3, ranging in length from 44 to 133 amino acid residues, and plays a role in substrate or Ca²⁺ binding (Nakajima et al., 1986). In the subfamily of the (β/α)₈-barrel protein,
the third barrel forms a small, separate Ca²⁺ stabilized structural domain that participates in substrate binding (Brena et al., 1996). Besides the A- and B- domains, nine other domains have been identified in this enzyme.

2.2.1.1.2 Isoforms: There exist multiple forms of this enzyme known as isoenzymes, defined as all proteins that catalyze the same reaction and occur naturally in a single species. The number of isozymes depend on the specific cultivars and the on the sensitivity of the resolving methods used (Muralikrishana & Nirmala, 2005). It has been well documented that geminated wheat contains two main groups of α-amylase isozymes (Kruger & Thachuk, 1969; Tkachuk & Kruger 1974; Macgregor, 1988). Based on the chemical, physical and immunochemical properties, they have divided them into two groups. Alpha-AMY-1 enzymes, also termed the ‘malt’ or ‘germination’ enzymes, are the primary product of germination and may also form without visible sprouting (Lunn et al., 2001). They have pI values between 6.0 and 6.5 and therefore sometimes are referred as the basic pI group (Thachuk & Kruger, 1974). Alpha-AMY-2 are also known as ‘pericarp’ or ‘green’ enzymes because they are found primarily in high concentrations in the pericarp of immature grains. They are progressively degraded in grain development and are below detectable levels in the pericarp at maturity. However, if the grain goes through germination they are expressed by the embryo in the later stages of germination (Lunn et al., 2000). They have pI values between 4.5 and 5.1 classifying them as the acidic pI group. Both of these enzymes were compared for their ability to hydrolyze a variety of starch substrate such as β-limit dextrin, amylopectin and amylose and no difference was detected in terms of their enzymatic activity (Thachuk & Kruger, 1974).

2.2.1.1.3 Action Mechanism: The generally accepted catalytic mechanism of α-amylase is that of the α-retaining double displacement. Two catalytic residues in the active site are a
glutamic acid as an acid/base catalyst and an aspartate as the nucleophile (van der Maarel et al., 2002). These are the five steps of the action mechanism:

1. After the substrate binds in the active site, glutamic acid in the acid form donates a proton to the glycosidic bond oxygen (the oxygen between two glucose molecules at the subsites -1 and +1) and the nucleophilic aspartate attacks the $C_1$ of glucose at subsite -1.

2. An oxocarbonium ion-like transition state is formed after the formation of a covalent intermediate.

3. The protonated glucose molecule at subsite +1 leaves the active site while a water molecule or a new glucose molecule comes into the active site and attacks the covalent bond between the glucose molecule at subsite -1 and the aspartate.

4. An oxocarbonium ion-like transition state is formed again.

5. The base catalyst glutamate accepts a hydrogen from an incoming water or the newly entered glucose molecule at subsite +1 while the oxygen of the newly entered molecule at subsite +1 replaces the oxocarbonium bond between the glucose molecule at subsite -1 and the aspartate forms a new hydroxyl group at the $C_1$ position of the glucose at subsite -1 (hydrolysis) or a new glycosidic bond between the glucose at subsite -1 and +1 (transglycoxylation) (Fig. 2.2.5).
Fig 2.2.5 – Schematic representation of the double displacement mechanism and the formation of a covalent intermediate by which retaining glycosylhydrolases act.

2.2.1.1.4 Products:

The most reliable method to differentiate between different amylases is by their product characterization. Alpha-amylases are endoenzymes randomly hydrolyzing interior α-1,4-glycosidic bonds of amylose, amylopectin and related polysaccharides to smaller oligosaccharides and glucose with a DP of 2-12 and their quantity depends on substrate concentration and the amount of enzyme (Dunn, 1974). Therefore, α-amylases are considered the most important enzymes for starch metabolism in developing as well as germinating grain.

2.2.1.1.5 Role during Germination:

According to most studies, 30% of the total protein synthesized during germination is α-amylase, either in the aleurone layer or scutellum (Bisenbaev 1992; Muralikrishana & Nirmala, 2005). Therefore, germination has a profound effect on starch properties because α-amylase degrades starch granules, lowering swelling power and peak viscosity profile of the flour.

Noda and co-workers investigated the physiochemical properties of starch isolated from three wheat cultivars at an extremely late harvest and concluded that this partially digested starch
has low swelling power, peak viscosity and high digestibility. However, the germination process in wheat grain led to no or minor change in the amylose content, mean size of granules, thermal properties by DSC and distribution of amylopectin chain length (2003). It is assumed that the sprout-induced α-amylases first partially digest starch causing a less-ordered surface structure of granules that now have higher susceptibility to in-vitro hydrolysis by all the enzymes produced in-situ during germination especially amylases when heated in the RVA.

SEM studies have suggested that enzymatic hydrolysis of starch granules initiates from surface by generating pits, size enlargements of existing pores and penetration into granule interior, producing a honeycomb like structure with compromised gelation and pasting ability (Naguleswaran et al., 2012). Blazek and Gilbert observed different susceptibility of semicrystalline and amorphous growth rings toward in-vitro digestion by α-amylase, with damage more obvious in large granules (2010). Li and co-workers (2012) have reported that at day 1 post laboratory germination, wheat granule surfaces were smooth and from day 2 holes and equatorial grooves at surface appeared that were more susceptible to the enzymatic hydrolysis than the flat surfaces, while the granule size gradually decreased with sprouting. At day 4 after germination, part of the outer layer was hydrolyzed and large channels extending into the granule interior along the equatorial grooves of A-type were observed that aided the enzymatic attract.

Similar effects have been reported due to field sprouting in winter wheat as there was a significant decrease in Amylograph peak viscosities while water-binding capacity was slightly higher for sprouted samples than for control (Kulp et al., 1983; Lorenz et al., 1983), attributed to increased damage starch in the sprouted flours, which has more water holding capacity. In brief, the hydrolytic activity of α-amylases during germination of cereal grains causes structural
changes in the endosperm due to native starch granule degradation leading to inferior grain quality.

2.2.2 Protein Degradation:

Cereals storage proteins are a rich reservoir of nitrogen, sulfur and carbon. Wheat proteins contain albumins, globulins, gliadins and glutenins that have varied solubility in different solvents. As far as practical utilization and commercial benefit for industries, two of these proteins, gliadins and glutenins, collectively known as gluten proteins are of maximum value in terms of food processing and quality. They are about 80-85% of total wheat proteins and are found in the endosperm of the mature wheat grain where they form a continuous matrix around the starch granules. Gluten proteins are largely insoluble in water or dilute salt solutions (Shewry et al., 1986; 2002)

Wheat gluten is formed upon hydration of these proteins with quality and quantity depending on the gliadin/glutenin ratio as well as on the amino acid composition, structure and size of its glutenin fraction, which then directly relates to the baking properties of the flour. Fig 2.2.5 represents the structures of glutenins and gliadins contributing to the development of gluten.

There is one important difference between gliadins and glutenins, that is, gliadins have intra-molecular disulfide linkage giving it compact and globular shape while glutenins have both inter- and intra-molecular disulfide linkages, making it linear with relatively higher molecular weight 80,000 to several million compared with the molecular weight of gliadins 30,000-80,000 (Shewry et al., 1986).
Since most of these proteins are insoluble in water and they can be utilized only after degradation to soluble proteins (Capocchi et al., 2000). In order to accomplish this, cereal seeds contain a large number of proteolytic enzymes, each with its unique properties and specifications. These proteolytic enzymes are either already present in the dry seeds or synthesized de novo during germination (Muntz et al., 2001).

Numerous previous studies have also shown that protease activity parallels the α-amylase activity of germinating cereals. Rheological properties of wheat are also affected during sprouting due to increased proteolytic enzyme activity that rapidly hydrolyzes endosperm storage proteins, gliadins and glutenins which are about 75% of the total protein content of wheat. Different proteolytic enzymes break these high molecular weight proteins into smaller sub-
fractions as well as in free amino acid groups, thus affecting flour functionality during food production due to decreased elasticity and strength of dough (Hwang & Bushuk 1973; Bushuk and Lukow 1987; Capocchi et al. 2000; Barbeasu et al. 2006).

Hwang and Bushuk found a 17-fold increase in proteolytic activity after eight day’s germination in a Canadian hard red spring (HRS) wheat (1973), while another study showed that the extent of storage protein hydrolysis and release of free amino acid plus peptide nitrogen in the durum and HRS variety appeared to depend more on the rates of increase in in-situ endoproteolytic activity and less on levels of exoproteolytic and endoproteolytic activity initially present in the ungerminated seed (Preston et al. 1978).

More recently, it has been reported that the bulk of proteolytic activity (approximately 90%) during wheat germination is due to cysteine proteinase (Bigiarini et al., 1995) and that the gliadin, sub-fraction of gluten, is completely hydrolyzed by cysteine proteinase after 15 hr of incubation (Dunaevsky et al., 1989). Capocchi and co-workers purified cysteine proteinase at the fourth day germination of wheat seeds, which was extremely effective for in-vitro digestion of vital gluten in short times (2000). This study further demonstrated that storage protein mobilization brings a loss of elasticity to the polymeric network of gluten, particularly due to hydrolysis performed by cysteine proteinase versus aspartic proteinase and carboxypeptidase, as studied by spin-labelling EPR.

All these studies point out the fact that minor sprout damage can lead to significant reduction in gluten strength and end use quality of wheat. Therefore, it is very important to germinate wheat at optimal level in order to successfully use sprouted flour as an ingredient in the baked products.
2.2.3 Fiber Degradation:

Besides starch and gluten, bran also plays an important role, structurally and functionally, in cereal grains. In bread wheat, 2-7% of endosperm cell wall materials consist of Non Starch Polysaccharides (NSP) of which pentosans are major components. Wheat pentosans predominately consists of arabinoyxlan (about 70%), (1→3)(1→4)-β-D-glucans (20%), glucomannans (6%) as well as some cellulose (4%) by weight. In wheat bran, arabinoxylans are water-unextractable and mainly comprise arabinose, xylose and methylated glucuronic acid while in endosperm tissues, both water-extractable and water-unextractable arabinoyxylans are present (Mare & Stone, 1973). Structurally, cereal arabinoyxylans form a very heterogeneous group, in which the ratio of arabinose to xylose, the pattern of arabinose substitution, the feruloyl group content and the degree of polymerization can vary significantly (Izdorczky & Biliaderis 1993a, 1993b). This polysaccharide has a linear backbone of (1→4) - β-D-xylopyranosyl in which the Arabinose substitutes are linked through O-2, O-3, or O-2, 3 residues (Fig. 2.2.6).
Due to the abundance of arabinoxylan, it affects wheat grain and whole meal flour functionality during processing and breadmaking. In germinating grains, arabinoxylans degrading enzymes are also produced causing structural changes in the cell wall components, thus affecting arabinoxylans’ physiochemical properties in solution and their impact on food systems (Beaugrand 2004; Courtin & Delcour, 2001). Most studies have discovered 3 major NSP enzymes from germinating wheat i.e., α-L-arabinosidases, endoxylanases and β-xylosidases. In a study, α-L-arabinosidases were purified from germinating wheat grains and showed a 13 fold increase in activity after 5 days sprouting (Grant et al., 2000).

Since arabinoxylans play critical role in dough rheology and breadmaking due to their viscosity, water binding capacity and oxidative gelling abilities (D’Appolonia et al., 1970), their quantification and structural modification during sprouting need special attention as well.
2.3. Bread-Making

Wheat is a unique raw material for the production of leavened foods such as bread and pastry, mainly attributed to the gluten protein and starch. Bread making is an art and various parameters determine the bread quality including bread volume, crumb firmness, gas retention and baking absorption. A series of chemical reactions take place throughout the process of turning flour into bread. Upon kneading of wheat flour, main components i.e., gluten protein and starch give rise to a visco-elastic network (Fig 2.3.1) capable of retaining fermentation gases. Mechanical work stretches the gluten into sheets due to CO₂ production (Fig 2.3.2).

In bread-making, quality of flour is very important and is determined by its ability to produce bread with high loaf volume, smooth texture, light crumb color and also longer shelf life. Therefore, wheat flour composition is very important for farmers, bakers and consumers. Wheat flour mainly consists of starch (70-75%), water (14%), proteins (10-12%), non-starch polysaccharides (2-3%) in particular arabinoxylans, and lipids (2%); all percentages by weight (Fincher & Stone, 1986). However, this composition can vary in soft and hard, white and red varieties as well as due to seasonal and geographical differences.
Fig 2.3.1 – Schematic representation of gluten matrix during kneading

Adopted from www.rsc.com

Fig 2.3.2 – Schematic representation of gas bubble expanding causing gluten network to stretch

Adopted from www.rsc.com
During bread-making process, biochemical and physical transformations, mechanical mixing, chemical reactions (including enzyme-catalyzed reactions) as well as thermal conditions (baking time and temperature), all are important factors influencing textural, sensorial and shelf-life attributes of the final products.

The addition of various elements enhances these traits by either manipulating and competing for water with other ingredients present or by physically and chemically modifying these ingredients, especially protein and starch. The beneficial effects are dependent on optimum concentrations of these dough conditioners in the bread system, which relates to the type of flour and processing technology used. In other words, rheological knowledge of wheat flour and water mixture is essential to control the processing of food manufacturing and to produce high quality final products.

The first step in bread making is hydration of protein fraction, gliadin and glutenin which when combined with water form gluten to build the continuous matrix to cover the surface of the starch granules (Fig 2.3.3).
Fig 2.3.3- Starch granules embedded in a continuous protein matrix

Adopted from http://www2.mpip-mainz.mpg.de/~vilgis/Food/Research/Dough.html
During baking, starch granules gelatinize and swell due to the combination of heat, moisture and time and a small amount of amylose is leached into the inter-granular space. Upon cooling, the solubilized amylose forms a continuous network, in which swollen and deformed starch granules are embedded and interlinked, and because of this rapid retrogradation happens, a determining factor for initial loaf firmness (Eliasson and Larsson 1993). During storage, bread gradually loses its freshness and stales.

2.3.1 Bread Staling and use of Enzymes

All baked products undergo a complex process of staling after baking and during storage. Staling is defined as all changes, except microbiological spoilage, that occur during storage of a baked product, making it less acceptable for the consumers. It involves loss of aroma, changes in mouth feel, loss of crumb softness and development of crumbliness. These changes take place at different rates and intensities after removal of the bread from oven and these serial changes are not, as commonly believed, simply a drying-out process due to evaporation.

Staling cannot be explained by a single effect, and includes loss of moisture content, moisture migration between crumb and crust at macroscopic level while between the amorphous and crystalline region at microscopic level, amylopectin retrogradation as well as reorganization of polymers within the amorphous region (Martin & Hoseney, 1991), however full mechanism of bread firming is still much debated. It is thought that 3% of the production of bread is returned for problems of staling as unsaleable bread (Zobel & Kulp, 1996).

Bread has two well-distinguishable bulk phases, crust and crumb as both of them are affected due to textural changes upon aging. In the fresh state, crust is relatively dry, crisp and brittle due to the glassy state of the two principal texturogens, starch and protein while the crumb, best described as porous material with flexible elastic cell walls, is less firm because
starch and protein are present in the rubbery state due to high levels of water as plasticizer (Hug-Iten et al., 2003). On staling, water migrates from crumb to crust leading to a glass to rubber transition of the two textural components in the crust making it soft and leathery while crumb increases in firmness and crumbliness.

In most staling models, it is generally agreed that during storage of bread, rigidity of bread crumb increases and resilience deteriorates due to water migration and transformations in the starch fractions resulting in amylopectin retrogradation in particular the formation of double helical structures and crystalline regions (Gray and Bemiller 2003; Kulp and Ponte, 1981; Schoch and French 1947; Zobel and Kulp 1996), while Gerrard and co-workers believe that staling is a result of increased interactions between swollen starch granules and gluten network (1998). Since amylose is already retrograded in the bread after cooling, it is considered to have little contribution to crumb firming. The amylopectin side-chains reorganization leads to an increased rigidity of the swollen granule. Gary and Bemiller reported that amylopectin retrogradation plays a significant, but not the only, role in the staling process. Retrogradation is the process of reassociation (crystallization) of linear starch molecules as they crystallize out of solution in the interstitial spaces between starch granules (2003).

Baking industry deals with the problem of bread staling by using a variety of anti-staling ingredients including enzymes (amylases, proteases and xylanases), fatty acids (mono and diglycerides) and different emulsifiers. The intentional inclusion of enzymes in bread formulation dates back to more than a century. Enzymes are better alternative to chemical agents, as they are generally recognized as safe (GRAS) and do not have any potential health hazards. Furthermore, they do not remain active in the final product after baking and therefore, need not to appear on the label, which is an additional commercial advantage. Currently,
microbial enzymes (fungal and bacterial \(\alpha\)-amylases) are the most popular anti-staling agents in commercial baking to improve dough handling properties as well baking characteristics such as increased bread volume, improved crumb structure and increased shelf-life.

As mentioned previously, \(\alpha\)-amylase partially hydrolyzes amylose and amylopectin to glucose, maltose and oligosaccharides which are less prone to retrogradation and these low molecular weight polymers also interfere with amylopectin recrystallization. It has also been suggested that the dextrins formed due to amylosis interfere with the interaction between the swollen starch granules and the continuous protein network in the bread (Defloor & Delcour 1999; Akers and Hoseney, 1994) which is somewhat contradictory with the observation that amylase supplemented breads often have a higher degree of crystallinity (Hug-Iten et al 2003; Zobel and Senti 1959). Therefore, it is believed by some researchers that the dextrins formed by amylase are only an indication of starch modification with no significant role in retrogradation interference (Duedahl-Olesen et al 1999; Gerrard et al 1997) and that the anti-staling effect of amylases is due to the modified starch structure which has different starch properties. Zobel and Senti have suggested that \(\alpha\)-amylases cleave the long starch chains that link different crystalline regions, causing network structure to be weak and less rigid, resulting in softer bread (1959).

Davidou and colleagues have further suggested that due to amylases action, cleavage of bonds in amylose and amylopectin promotes the formation of inclusion complexes with both added (if any) and endogenous wheat polar lipids, that are less prone to retrogradation and thus cause bread to stale at a slower rate (1996).

Since the extent of amylases activity during the baking process mainly depends on their specificity, degradation products, their activity at the rather low water content of dough and on
their temperature stability in the oven, it is very important to carefully dose to avoid sticky bread crumb and overproduction of dextrins during baking.

2.3.2 Bread Staling and Sprouted Wheat

Nowadays there is increasing interest in Western world in sprouted grains due to consumer demand for the minimally processed, additive-free yet more natural, nutritional and healthy foods. Since during germination process, alpha amylase activity is increased, it is proposed that baked products made with sprouted wheat flour would be less prone to staling naturally. Several studies have shown that sprouting conditions such as initial hydration status of the grain, temperature during sprouting and most importantly sprouting duration affect alpha-amylase’s content and activity in the wheat (Fleming et al. 1960). Therefore, it is important to find the optimal conditions for this enzyme’ production to use sprouted wheat in end-products that are naturally shelf-stable.

It has been reported that using sprouted wheat flour to produce white bread appear to improve quality of bread as loaf volume increased significantly by substituting 5% of sprouted wheat flour (Ranhotra et. al. 1997). Seguchi and co-workers showed that bread making properties (bread height and volume) were gradually enhanced by blending the germinated outer bran layers at 10% by weight, with maximum specific volume after 5 days of germination (2010). However, the improvement was lost after 8 days of germination and it was suggested that α-amylase and xylanase activities in the outer bran layers are highly related to the enhancement of bread making properties. However, an excessively high concentration of α-amylase in sprouted wheat has a negative effect on the loaf volume and other internal and external properties of bread (Hwang & Bushuk 1973; Finney et al., 1980). Therefore, in order to
achieve anti-staling benefits from the sprouted wheat, optimal sprouting conditions leading to optimal enzymes production must be determined.

Finally, during germination process antioxidants as well as fat degrading enzyme lipases and lipoxygenases are produced which prevent oxidation of fatty acids in the grains, minimizing rancidity and providing longer shelf-life (Kubicka et al., 2000).

2.4 Conclusion

Sprouting effect on the nutritional and bioactive constituents of seeds as well as the on functional and dynamic rheological property of flour and dough vary with the time of germination, light, moisture, temperature, seed varieties and the kind of dry processes employed with the seeds. As sprouting progresses, levels of starch and protein hydrolyzing enzymes increase, different cultivars as well as different environmental conditions can have different moments with highest activity of these enzymes. Therefore, it is important to determine when this moment happens and how it affects the rheological properties of wheat. In order to make acceptable as well as functional products from the sprouted wheat, optimal sprouting conditions should be determined such as to obtain high quality, nutrient dense baked goods with sprouted wheat flour as an ingredient or as whole.

Therefore, in this project, we are proposing to develop a strategy to germinate wheat, *Triticum aestivum*, in a controlled manner to deliver nutrients while minimizing the negative impacts on product quality. This project also has the potential for increasing omega-3 fatty acids content in the end-products prepared from the sprouted wheat flour through SMARTGRAIN Technology of the sponsor organization.
CHAPTER THREE
EFFECT OF WHEAT SPROUTING UNDER VARIOUS ENVIRONMENTS ON ALPHA AMYLASE ACTIVITY AND PHYSIO-CHEMICAL PROPERTIES OF FLOUR

3.1 Abstract

Germination is the process that initiates seed growth and like seeds of other cereals, wheat seeds are dormant and must first be subjected to the appropriate environmental conditions to activate hormones within the grain to initiate growth. These hormones, in turn, regulate production and release of the enzymes governing the metabolic processes involved in growth. During germination, enzyme activity, most notably that of alpha-amylase, increases rapidly. Extent of these enzymes’ activity depends on the duration of sprouting time, moisture, temperature, seed varieties and the kind of dry processes employed with the seeds. The aim of this study was to assess and quantify α-amylase activity during different sprouting conditions. Wheat cultivars var. Ava (soft white winter) and var. Wentworth (hard red winter) were steeped for 3 and 24 hours before sprouting at 70% humidity and 12°C or 20°C for 5 days. Endogenous α-amylase activity increased rapidly in the 20°C samples as compare to 12°C for both varieties. 3 h steeping time resulted in greater enzymatic activity than 24 h in Ava but not for Wentworth. Several isoforms of α-amylase were detected by zymography. Wentworth demonstrated a greater response overall than Ava. Results were consistent among GPT, farinograph, RVA and α-amylase activity tests, showing greater changes in functional and rheological properties of sprouted wheat flour with increased α-amylase activity for both varieties. It was shown that genotype, temperature and pre-soaking duration, all are important factors affecting α-amylase quantity as well as quality and thus modifying wheat flour functionality significantly during sprouting process.

Key words: wheat sprouting, α-amylase, zymogram
3.2 Introduction

Germination or sprouting is the process that initiates seed growth. Bewley and Black defined germination as a process initiated by water uptake by the quiescent dry seed and is terminated with the elongation of the embryonic axis. Visible sprouting is the emergence of radicle around the embryo of seed, a visible sign that germination is complete (1994). All seeds are dormant and must first be subjected to the appropriate environmental conditions (temperature and moisture) to activate hormones within the germ to initiate growth. These hormones, in turn, regulate production and release of the enzymes governing the metabolic processes involved in growth. During germination, enzyme activities, most notably that of alpha-amylase, increase rapidly (Dunn 1974; Kruger 1976; Corder and Henry 1989). These enzymes mobilize storage reserves of starch and protein by breaking them into smaller fractions to meet the increased need of energy for seedling growth, since most of the storage components are insoluble in water, and their utilization by the growing embryo is only possible after their degradation to soluble products.

Seeds can be sprouted in a controlled manner by stimulating environment such as moisture, temperature and humidity in the laboratory or can germinate in the field prior to harvest, a process known as preharvest sprouting (PHS). In either case, sprouting results in increased amounts of important nutrients such as soluble dietary fiber, vitamins, minerals, antioxidants and phytochemicals while having negative impact on grain quality compared to their fresh products (Yang et al. 2001; Plaza et al. 2003; Koehler et al. 2007; Hung et al. 2011, 2001).

Hung and colleagues concluded that germinated waxy wheat variety had better nutritional composition, such as higher dietary fiber, free amino acids and total phenolic compounds.
content, than the ungerminated seeds (2012). Another study showed 2 fold increase in α-tocopherol content and an average 2-3 fold increase in minerals (Ozturk et al., 2011) while a 3.6-fold increase in folate was obtained after 102 h of germination at 20 and 25°C (Koehler et al., 2007).

According to Yang and co-workers, vitamin C and E and β-carotene were barely detectable in the dry wheat grains but upon germination the concentration of these antioxidant vitamins steadily increased with increasing germination time, reaching their peaks after 7 day at 550μg/g for vitamin C, 10.92μg/g for α-tocopherol, and 3.1μg/g for β-carotene. Concentrations of ferulic and vanillic acids were also increased, reaching their maxima after 7 day at 932.4μg/g and 12.9μg/g, respectively (2001).

In general, germination reduces the amount of dry weight by catabolizing carbohydrates to CO₂ and H₂O, thus concentrating the total mineral, vitamin, fiber and protein content of the flour (Lorenz, 1980; Plaza et al., 2003). At the same time, several studies mentioned above have shown that biosynthesis of Vitamin C, E, β-carotene, folate and phenolic acids takes place during sprouting process.

Many of the health benefits associated with sprouted grains are due to the fact that they are produced from whole grains. Whole grain products include the entire grain, in comparison to refined flour products, which lack the bran and germ. Whole grain products are being consumed in greater quantities as nutritional and medical studies repeatedly link diets with whole grains to improved health such as reduced heart disease, cholesterol, type II diabetes, obesity and improved bowel function (Plaza et al. 2003). Academy of Nutrition and Dietetic recommends making at least half of an adult’s six to eight grains serving to be whole grain each day (AND 2012). This recent trend of consuming more whole wheat bread, whole wheat pasta and whole
wheat noodles has driven the food industry to come up with whole wheat products. As consumers continue seeking for healthier grains, products made from sprouted whole grains should be considered.

Furthermore, bread fortified with germinated wheat seedlings has been shown to positively affect fasting and postprandial glucose levels compared to a control wheat bread, evoked by fiber compounds as well as by non-fiber compounds such as phenolic acids (Anderson, 2008). Intake of flavonoids is associated with reduced risk of type 2 diabetes, as administration of plum showed an anti-hyperglycemic effect in rats (Utsunomiya et al., 2005), confirming that phenolic compounds exhibit positive impact on glucose and insulin metabolism. These findings lead to consider that other grain compounds than fiber may also positively affect glucose regulating factors.

Despite the benefits that sprouted wheat has to offer, it is recently being consumed only as salad, juice, and tablet for its vitamins, minerals, and phenolic contents, because it comes with the problem of inferior quality flour that leads to processing problems and unsatisfactory end-products during food manufacturing. Sprouting affects the physiochemical and functional properties of wheat, as flour extraction, ash and protein content and falling number decreases while α-amylase and proteolytic activity increases with sprouting. Sprouted wheat flours are ill suited for dough and bread making and use of 100% flour from sprouted flour yields bread with poor characteristics: wet, sticky dough, darker crumb color and inferior texture (Edwards et al., 1989; Ariyaman & Khan, 1990; Beleia and Grossmann, 1990; Derrick and Every, 1990; Cabrera et al., 1995; Yamauchi et al., 1998).

In a study by Singh and colleagues, effect of sprouting conditions (soaking duration, sprouting temperature and soaking duration) on functional and dynamic rheological properties
were studied, demonstrating that with increased sprouting time, falling number and water absorption index (WAI) decreased while water solubility index (WSI) increased (2001). Elasticity of dough decreased with increase in soaking and sprouting duration, while viscosity showed an increase with the increase in soaking duration and a decrease with the increase in sprouting duration. Lorenz and Valvano concluded that longer sprouting times had a detrimental effect, not only on cake volume, but also on external and internal cake characteristics as well, with a dip in the center, a coarse grain and a rather dense and firm texture (1981). Similarly, sprouted flour cookies had higher spread factors and grain scores than for cookies baked from the sound flour.

Sprouting adversely affects the rheological properties of wheat due to enzymatic hydrolysis of starch, protein and fiber catalyzed by the increased activity of various enzymes especially of alpha-amylase and protease in the germinated wheat, as severe product defects in Cantonese and Korean noodles, while lesser but still deleterious effects in Arabic flat-breads have been reported (Edwards et al., 1989). However, the quality of pan breads made from sprouted grains was better than that from corresponding untreated wheat, as water absorption capacity of sprouted flours was higher. This was in contrast to most published reports at that time where baking quality was inferior due to sprouting. It was concluded that the relative levels of protease and amylase activities in the sprouted flours were optimal in the baking system than those in the control flours, leading to the belief that increased amylase and protease activity of the sprouted samples acted as an effective gluten softener thus reduced mixing time and improved loaf volumes.

Similarly, an earlier study by Morad and Rubenthaler had also postulated that amylolytic activities are important factors affecting bread crumb firmness (1983). It was indicated that
insufficient or excess amylase activity increases crumb firmness and staling rate of bread. Alpha-amylase catalyzes the random hydrolysis of the interior α-1,4-glycosidic bonds of amylose, amylopectin and related polysaccharides to smaller oligosaccharides and glucose, thus playing a very important role in the starch metabolism in developing as well as germinating grain.

This study aims to investigate the role of these environmental factors on α-amylase quantity as well as quality in sprouted wheat, as well as to investigate the functional and rheological properties of this sprouted wheat flour to see if this flour rich in nutrients can be utilized in bread as an ingredient. Although many studies have investigated various sprouting conditions and how they relate to amylase enzyme activity especially for barley based on interest from malting industry, the rheological properties of sprouted wheat flour and dough have not been comprehensively characterized. There are only few studies regarding germination’s effect either on starch or on gluten but no one complete study on changes in these two important constituents of wheat flour all together has been established so far. The objective of this study is to observe changes in functional properties of starch-gluten matrix paralleling α-amylase activity with various sprouting conditions in two different varieties of wheat and try to shed more light on the application of germinated whole wheat flour in food industry.

Furthermore, based on the demand from the consumers for the natural, additive-free and nutrient dense food products, this research project proposes to evaluate functional properties of sprouted wheat flour. It will characterize sprouting of different varieties of wheat at various conditions and investigate the germination process to find the variables that optimize the activities of α-amylase enzyme necessary to prevent staling in bread, naturally. By extending shelf-life, manufacturers can simplify warehousing and logistics, while also reducing waste. In
brief, by characterizing the sprouting of wheat, it will allow for expansion of healthier whole grain products that are naturally shelf-stable for consumers.

3.3 Materials and Methods

3.3.1 Plant Material

Two wheat varieties [Ava - soft white winter (SWW) and Wentworth – hard red winter (HRW)] were kindly provided by Hyland Seeds (Blenheim, ON).

3.3.2 Sprouting Conditions

Wheat samples were stored at room temperature before germination. Any broken, damaged and off-color wheat kernels were removed. They were disinfected in a 2% (v/v) H₂O₂ solution for 3 h at room temperature and washed with several changes of distilled water. Then these seeds were steeped for an additional 21 h for the 24 h steeping treatment and considered as day1 sprouts. After either 3 h or 24 h of steeping, wheat seeds were germinated sandwiched between two synthetic mesh screens at 12°C and 20°C while relative humidity was kept at 70%. After 24, 48, 72, 96 and 120 h of initial contact with water, 150 g of wheat samples were frozen at -80°C to halt sprouting. Samples were hydrated every 8-10 h and scrambled to prevent matting. Abbreviations used for all sprouted samples in this report are listed in Table 3.1.
<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wentworth at 20˚C with 24h steeping</td>
<td>WW 20˚C/24h</td>
</tr>
<tr>
<td>Wentworth at 12˚C with 24h steeping</td>
<td>WW 12˚C/24h</td>
</tr>
<tr>
<td>Wentworth at 20˚C with 3h steeping</td>
<td>WW 20˚C/3h</td>
</tr>
<tr>
<td>Ava at 20˚C with 24h steeping</td>
<td>Ava 20˚C/24h</td>
</tr>
<tr>
<td>Ava at 12˚C with 24h steeping</td>
<td>Ava 12˚C/24h</td>
</tr>
<tr>
<td>Ava at 20˚C with 3h steeping</td>
<td>Ava 20˚C/3h</td>
</tr>
</tbody>
</table>

Table 3.1 – Abbreviation used for all sprouted wheat samples

3.3.3 **Drying and Milling**

After exactly day 1, 2, 3, 4 and 5 samples were frozen at -80˚C and then freeze-dried to inactivate enzyme activity. Upon drying any vegetative parts (roots and shoots) were removed by scrubbing and then screened. Each sample was milled in the UDY Cyclone Mill (UDY Corp., Fort Collins, CO) equipped with a 0.5mm mesh screen. Ungerminated flour of each variety was also freeze-dried and milled to be used as control sample. All samples were stored at -34 ºC until analysis.

3.3.4 **Moisture Content**

Knowing the moisture content of flour is prerequisite for many flour quality tests. Standard flour test of 2 min at 180˚C on moisture balance (MB 45 OHAUS) was used to find the moisture content of each sample in duplicate and average was calculated to be considered the actual moisture of the flour.
3.3.5 Determination of α-amylase Activity

Alpha-amylase activity was measured by the Amylazyme method (AACC 22-05.01) using Amylazyme-Red tablet by Megazyme (Megazyme International, Ireland). It is specifically designed to measure cereal alpha-amylase activity to quantify this enzyme in the sprouted flour malted under variable conditions. 1.00 g flour was stirred with Sodium maleate buffer (100mM pH 6.0) for 15 min and then centrifuged at 1,500 g for 10 min. 0.2 mL of the supernatant was diluted appropriately with Sodium maleate buffer and then 0.5 mL aliquots was incubated with Amylazyme Red tablet for exactly 10 min at 40°C. Reaction was terminated by adding 10 mL of Trizma base solution (2% w/v, pH 8.5) and then filtered. Absorbance of the filtrate was measured against a reaction blank at 510nm and α-amylase activity was determined by reference to the appropriate standard curve of the purified barely malt to convert absorbance to Ceralpha Units of activity. Each test was performed in duplicate. Alpha-amylase activity in each sample is reported as Ceralpha units per gram of flour (Fig 3.1)

3.3.6 Zymography

Zymography is an electrophoretic technique that includes a substrate co-polymerized with the polyacrylamide gel for the detection of enzymes and their activity. Samples are prepared without denaturing the active enzymes present in them. Following electrophoresis, gel is placed in an enzyme activation buffer which allows the enzymes present in the sample to become active and digest the substrate co-polymerized in the gel. The gel is subsequently stained and the areas of enzyme activity and digestion become visible as clear bands.
To detect α-amylase activity and its isoforms present in the sprouted wheat, a modified procedure from Belay and Furtura was used (2001). Aliquot was extracted by mixing 1 g flour with 2 ml of a buffer containing 62.5 mM TRIS-HCl, pH 6.8, 10% (w/v) glycerol and 0.001% (w/v) bromophenol blue. After centrifugation at 14,000 g for 20 min, supernatant was incubated at 70°C for 15 min to inactivate other enzymes especially β-amylase. Protein content in each sample was determined by BSA standard curve to load 300 μg of protein on 8% (w/v) polyacrylamide native gels. Electrophoresis was carried out at 4°C at 100-130 V for 60-90 min or until samples reached the bottom. These gels were then transferred on to 8% (w/v) polyacrylamide native gels copolymerized with 2% (w/v) amylopectin (Sigma-Aldrich). Gels were then incubated with buffer containing (20 mM MES-NaOH, pH 6.6, mM Na-citrate 45mM Glc-1-P, 2.5mM AMP, 1 mM Na₂-EDTA and 1mM DTT) for 2-3 hr at 28°C in a shaking incubator. After incubation gels were washed with water and were developed with Lugol’s solution and visualized immediately. Results are presented in Fig 3.2.

3.3.7 Flour and Dough Quality Analysis

Starch and gluten quality and functionality in flour and dough were analyzed by various methods.

3.3.7.1 RVA:

Rapid visco analyzer has been widely used for determining the pasting properties of different starches with high α-amylase activity. While the wheat flour and water slurry is heated in this instrument, starch granules are degraded by endogenous α-amylase; the flour viscosity determined reflects the level of this enzyme present in the flour. Rapid Visco™ Analyser (RVA-4, Newport Scientific, Warriewood, Australia) equipped with
Thermocline for Windows (TCW3) soft-ware was used to heat 4.0 g whole wheat flour in 25.0 g distilled water corrected to 0% water basis. Water and flour slurry was heated from 35˚C to 95˚C at a rate of 2˚C/min with 160 rpm shear. All tests were performed in duplicate and average raw curves showing peak viscosity of each sample with heating profile are presented in Table 3.1.

3.3.7.2 Farinograph:

This test gives a measure of the dough consistency during its formation. Consistency is given in Brabender Units (BU) and represents resistance to mixing when the dough is mixed at constant speed. In this study, dough was prepared in a Farinograph-E (C.W. Brabender Inc., Hackensack, NJ, USA) using a 10 g mixing bowl at 30˚C. As mixing proceeds, consistency increases due to gluten network development, reaches to a peak when all the flour particles are hydrated, and then decreases. Water absorption to reach 500 BU for the control sample was determined and water absorption from the control sample was used for each sprouted sample to perform farinograph test. Peak consistency and development time results are reported in Fig 3.3a and 3.3b.

3.3.7.3 GlutoPeak Tester:

Gluto Peak Tester is a rapid shear-based method to discriminate gluten quality and records the time to reach peak torque on the formation of a gluten network. It is a good measure of gluten functionality in dough and was performed using the procedure described by Chandi & Seetharaman (2011). 8.5 g flour in 9.5 g 0.5M CaCl₂ solvent was subjected to 1,900 rpm shear at 34˚C in Brabender GPT (Brabender, Duisberg, Germany). Peak torque and peak time are presented in Fig 3.4a and 3.4b.
3.3.7.4 Electrophoresis:

Sodium dodecyl sulfate polyacrylamide gel electrophoresis is a technique widely used to separate proteins according to their electrophoretic mobility. The binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. One dimensional SDS-PAGE analysis in this study was performed according to the method of Laemmli (1970), using 10% Tris Glycine resolving gel (MP TGX, 10%, 10W, 30 μL by Bio-Rad Laboratories, Canada). 25mg sample of flour was dissolved with 1ml of sample buffer prepared by 1:1 of water to Laemmli sample buffer (Bio-Rad Laboratories, Canada) with 0.772% DTE or DTT w/v. These aliquots were shaken at 60°C for 15 min and then centrifuged at 5 rpm for 5 min. 15μL of this aliquot along with 10μL of prestained protein standard were injected onto the resolving gel and ran in a 10x Tris/Glycine/SDS electrophoresis buffer (Bio-Rad Laboratories), diluted to 1 in10, at 120-150 V and 40 mAmp for 45 min or until proteins reached bottom. Gels were stained with Coomassie Brilliant Blue G-250, scanned and presented in Fig 3.5.

3.3.7.5 Protein Content:

It is critical to know the total protein content of each sample and how it varies with different sprouting conditions. An automated Dumas protein analysis system (LECO-FP-52 Instruments Ltd., Mississauga, ON, Canada) was used to perform Combustion Nitrogen Analyses (CNA) method to determine crude protein concentration in each flour sample (AACC 46-30.01). Protein was calculated by % N * 5.7 factor and is reported in Table 3.2 based on dry basis (w/w).
3.3.7.6 Insoluble and Soluble Dietary Fiber Content:

Fiber content directly relates to flour’s water absorption capacity, important aspect of flour’s performance and economy during food processing, therefore, it is necessary to track changes in soluble and insoluble fiber with various sprouting conditions. Any changes in insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) content indicate the presence of Non Starch Polysaccharide hydrolyzing enzymes causing modification in flour’s nutritional as well as functional profile. It was determined by the AOAC method (1998) and reported in Table 3.3.

3.3.7.7 Arabinoxylan Content:

Arabinoxylan content of day5 sprouted flour for all treatments was determined by Megazyme D-xylose assay procedure kit (Megazyme International, Ireland). If in a polysaccharide, the ratio of D-xylose to other sugars is known, then the amount of the polysaccharide can be quantified, for example wheat flour arabinoxylan has D-xylose content of 62%. 100 mg of flour was incubated with 1.3 M HCl for 1 h at 100°C and then neutralized with 5mL of 1.3 M NaOH. An aliquot of 0.1 mL was assayed by first diluting to 15 mL and then filtering. Assay was performed in 1cm light path plastic cuvette at room temperature by pipetting 2.00 mL distilled water, 0.10 mL sample, 0.40 mL buffer, 0.40 NAD*/ATP and 0.02 mL Hexokinase suspension and absorbance (A1) was read at 340 nm after 5 min against air. Second reaction was started by adding 0.05 mL of XDH/XMR solution and absorbance (A2) was read after 6 min. Absorbance for blank was also determined by substituting 0.10 mL of sample volume with distilled water in the above procedure. Absorbance difference (A2-A1) for both blank and sample was determined and absorbance difference of the blank was subtracted from the absorbance
difference of the sample to obtain $\Delta A_{D-xylose}$. The concentration of D-xylose was calculated by multiplying $\Delta A_{D-xylose}$ with 0.7076 and this result was multiplied by the dilution factor and initial flour weight to yield content of D-xylose as g per 100g of flour. Finally to obtain arabinoxylan content per 100 g of flour, content of D-xylose was divided by 62 and multiplied by 100.

3.3.8 Statistics

All analyses were performed at least in duplicate and the mean values are reported. Analysis of variance was performed using IBM SPSS Statistics 20 software. Significant difference ($p<0.05$) among means were detected using the Tukey’s multiple range test at a fixed level of $\alpha = 0.05$.

3.4 Results and Discussion

3.4.1 Sprouting Conditions and $\alpha$-Amylase Activity: Alpha-amylase activity in Ceralpha Units per gram of flour increased from day 0 to day 5 of germination, as expected (Fig 3.1). In general, at higher sprouting temperature (20°C vs. 12°C), higher $\alpha$-amylase activity was observed in both wheat varieties, in agreement with previous conclusion by Fleming et al. (1960). In terms of steeping duration, 3 h soaked samples had higher $\alpha$-amylase activity than 24 h in Ava but not for Wentworth, as longer steeping time (24 h vs. 3 h) yielded higher $\alpha$-amylase activity for Wentworth.

Analysis of variance showed a significant ($p<0.05$) increase in $\alpha$-amylase activity at day 3 for all treatments in both varieties. For Ava, a rapid increase at day 3 for 20°C/3h was observed, reaching its peak at day 5 (1712 Units/g) while it was only 308 Units/g for 12°C/24h and 329 Units/g for 20°C/24h treatment. For the Wentworth, $\alpha$-amylase activity increased
rapidly at day 3 for all treatments and reached a maximum at day 5 (1913 Units/g for 20°C/24h, 1460 Units/g for 20°C/3h and 1236 Units/g for 12°C/24h treatment). Highest activity was observed for WW 20°C/24h at day 5, 1913 units/g while second highest for day 5 of Ava 20°C/3h, 1712 units/g. In brief, for Wentworth, 20°C/24h led to highest α-amylase production for all five days and interestingly there were no significant differences between WW20°C/3h and WW12°C/24h treatment for all 5 days. On the other hand, Ava 20°C/3h yielded the highest α-amylase activity through all five days compare to Ava 20°C/24h and Ava 12°C/24h treatments. Overall, Wentworth (HRW) had higher α-amylase activity per gram of flour than Ava (SWW) when exposed to the same sprouting conditions. Three hours of steeping was enough for Ava to achieve optimal hydration for sprouting while Wentworth required longer steeping time.

This difference in enzyme activity was related to the percentages of kernels sprouted under same environmental conditions, which in turn was directly related to the rate of water uptake of the kernels, affecting their hydration status. Huang and co-workers have showed previously that white wheat varieties exhibit looser integument structure and greater separation between the seed coat and tube cells of the inner pericarp than the red wheat varieties (1983). Therefore, Wentworth being hard red wheat took longer time than Ava which is soft white wheat to reach optimal hydration for GA3 synthesis which in turns signaled α-amylase production. Wheat kernel’s seed coat color is generally associated with dormancy as red wheat varieties are more resistant to sprouting while white seeded varieties are nondormant or weakly dormant and therefore susceptible to preharvest sprouting (Gfeller & Svejda, 1960; Mares, 1994). Weidner and Paprocka have proposed that dormancy might be partially controlled by the high levels of free phenolic acids in red seed through their
inhibitory effect on germination and cell division (1997). In another study by Ueno and Takahashi, red-coloured wheat with 46% germination rate after 8 days of steeping proved to be more tolerant to flooding than white seeded wheat with only 23% average germination (1997). However, they indicated that wheat seed germination significantly relates to accumulation of ethanol rather than seed coat color as wheat is unable to germinate under anoxia. Excessive water treatment negatively affects enzymatic activity in germinating seed because aleurone layers do not respond to GA3 (Perata et al., 1992; Perata & Alpi, 1993; Guglielminetti et al. 1995). Therefore, Ava, soft white wheat readily absorbed optimal water within 3 h from the surroundings, initiating aerobic metabolism for energy production to activate enzymes while steeping for 24 h led to anaerobic conditions, producing energy through the fermentative pathway with ethanol as a waste by-product, affecting its ability to germinate. On the other hand, Wentworth being a red seed coat required longer duration to uptake optimal water to break through dormancy.

All these results indicate the importance of initial hydration status of wheat kernel as well as environmental temperature for the progression of sprouting process. Furthermore, it can be concluded that hard and soft wheat varieties based on the differences in their anatomical structure, have different rate of moisture uptake and thus enzyme production under same environmental conditions.
Fig 3.1- Effect of various temperatures and steeping durations on α-amylase activity (Ceralpha Units/g of flour) for 5 days of sprouting in wheat

Increased α-amylase activity with increasing sprouting duration under different environmental conditions was indicated by zymography as well, demonstrating that with ideal sprouting conditions and increased sprouting time different isoforms of α-amylases are produced (Fig 3.2). With increased sprouting time, quality and quantity of α-amylase isoforms enhanced represented by clear bands against darkly stained background. At day 2 for all treatments in both
varieties clear bands representative of the malt (α-Amylase I) isoforms appeared while at day 4, except for Ava 12°C/24h treatment, bands representing the green (α-Amylases II) activity were present. By analyzing these zymograms, it can be concluded that for Ava 20°C/24h treatment five isoforms of α-Amylase I and two isoforms of α-Amylase II were produced at day 4 and day 5, represented by seven individual clear bands while Ava 12°C/24h treatment produced only five isoforms of α-Amylase I and no isoforms of α-Amylase II were detected. For rest of the samples, α-Amylase I bands at day 4 and day 5 were very bright such that no individual clear band can be accounted while two clear α-Amylase II isoforms were detected responsible for higher α-amylase activity at day 4 and day 5 as assayed by Amylazyme method. Overall, for all sprouting conditions in both varieties, five α-Amylase I isoforms and two isoforms of α-Amylase II were produced in all samples, except for Ava 12°C/24h. Machaiah and Vakil (1984) have previously reported that in the 4th day of wheat flour sprouted at 25°C, 15% of the total activity was present in α-amylase I that was resolved into 3 isoforms while the rest in α-amylase II that was separated into 4 isoforms. While Belay and Furuta (2001) observed a total of 13 bands, 8 from the malt and 5 from the green components in a number of tetraploid wheat landrace collection from Ethiopia, sprouted at 20°C for 6-7 days. This discrepancy between the numbers of isoforms can be due to differences in sprouting conditions, sprouting time, α-amylase extraction and electrophoretic techniques and most importantly due to different wheat genotypes as both Ava and Wentworth are hexaploid.

In brief, variety was the most important parameter for α-amylase activity, followed by temperature and steeping duration, furthermore, there exist an interaction between variety, temperature and initial moisture of wheat kernel.
Fig 3.2 – Zymogram pattern of $\alpha$-amylase isoforms in wheat sprouted under various temperatures and steeping durations for 5 days and Control (C)

3.4.2 Sprouting Conditions and Four-Dough Functional Properties:

3.4.2.1 Effect on Gelatinization Ability:

Table I shows the effect of different sprouting conditions on the RVA profiles of Ava and Wentworth. With increased sprouting time peak viscosity and gelatinization temperature decreased rapidly in both varieties, with no gelatinization at all for day 4 and day 5 for all sprouted flour samples. For Wentworth, peak viscosity was significantly lower ($p<0.05$) only after one day of sprouting for all treatments than non-sprouted flour, dropping from 2867 cP for the control to 2517 cP (WW 12°C/24h), 1922 cP (WW 20°C/3h) and 1571 cP (WW20°C/3h).
However, for soft white wheat peak viscosity increased significantly for day 1 of Ava 12°C/24h (4227 cP) from the non-sprouted Ava (3027 cP). This increase in viscosity may be due to anatomical changes in the kernel leading to increased swelling capacity of the starch granules and also because no α-amylase activity was detected for this sample. However, for the other two treatments, peak viscosity dropped to 1956 cP (Ava 20°C/24h) and 1660 cP (Ava 20°C/3h) at day 1. It can be generalized that peak viscosity (cP) is inversely proportional to the α-amylase activity (Units/g) for all sprouting treatments in both varieties.

It was also observed that with higher α-amylase activity, gelatinization (peak) temperature and the time to reach this gelatinization temperature (peak time), not shown in the Table I, for all sprouted samples dropped gradually. For all treatments of both varieties there was a 25% drop in the peak temperature only after day 2, except for Ava 12°C/24h with only 1.3% drop, reaching a 30% drop at day 5 for all sprouted flour sample.

Break down of starch by α-amylase is time and temperature dependent and the overall effect is to reduce water-holding capacity. This loss of viscosity was mainly due to two factors, firstly starch granules were hydrolyzed in-situ by the amylases produced during sprouting and then further degraded by these enzymes during pasting, as partially digested starch has a less-ordered surface structure of granules, indicating higher enzymatic susceptibility.

Since peak viscosity is indicative of the extent of the endosperm modification leading to reduced water-binding capacity of the starch in the flour, it can be concluded that with ideal sprouting conditions leading to higher α-amylase activity, flour loses water-binding and swelling power, affecting the final product quality.
In brief, sprouting of wheat kernels negatively impacted peak viscosity, gelatinization temperature and peak time, due to decrease in the degree of starch polymerization, all important factors that affect flour’s functionality during processing and baking. RVA results were correlated to α-amylase activity.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ava</th>
<th>Wentworth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12°C/24 h</td>
<td>20°C/24 h</td>
</tr>
<tr>
<td>Peak Viscosity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3027 ± 41</td>
<td>2867 ± 14</td>
</tr>
<tr>
<td>Day 1</td>
<td>4227 ± 36</td>
<td>1956 ± 76</td>
</tr>
<tr>
<td>Day 2</td>
<td>1301 ± 8</td>
<td>793 ± 10</td>
</tr>
<tr>
<td>Day 3</td>
<td>327 ± 8</td>
<td>157 ± 10</td>
</tr>
<tr>
<td>Day 4</td>
<td>70 ± 2</td>
<td>39 ± 8</td>
</tr>
<tr>
<td>Day 5</td>
<td>21 ± 4</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>Peak Temp (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>91.4 ± 0.14</td>
<td>92.5 ± 0.1</td>
</tr>
<tr>
<td>Day 1</td>
<td>92.3 ± 0.1</td>
<td>94.6 ± 0.2</td>
</tr>
<tr>
<td>Day 2</td>
<td>90.2 ± 0.3</td>
<td>70.1 ± 0.1</td>
</tr>
<tr>
<td>Day 3</td>
<td>69.6 ± 0.3</td>
<td>67.4 ± 0.1</td>
</tr>
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<td>Day 4</td>
<td>67.4 ± 0.2</td>
<td>66.3 ± 0.1</td>
</tr>
<tr>
<td>Day 5</td>
<td>66.9 ± 0.1</td>
<td>65.2 ± 0.1</td>
</tr>
</tbody>
</table>

Table 3.2 – Effect of various temperatures and steeping durations on Peak Viscosity (cP) and Peak Temperature (°C) for 5 days of sprouting in wheat
3.4.2.2 Effect on Dough Rheology:

Fig 3.2a and 3.2b show the summarized results of farinograph profiles for different sprouting conditions in both varieties. As expected, Wentworth, hard wheat with higher protein content required more water to reach 500 BU compared to Ava soft wheat (72.5% and 60% respectively). Soft wheat dough reached its maximum peak at 3.5 min with low stability, while hard wheat dough achieved peak at 5.75 min and exhibited higher stability. For Wentworth, peak dough consistency of the control (513 BU) dropped sharply only after day 1 of sprouting (288 BU for WW 20°C/24h, 323 BU for WW 12°C/24h and 443 BU for WW 20°C/24h) and remained consistently lower for rest of the days for all treatment (p<0.05). Previous studies have also reported these changes in dough after sprouting due to enzymatic hydrolysis of starch-gluten matrix, which gets broken down, losing its ability to bind water leading to wet and stickier dough. All these changes in dough consistency due to lower water binding capacity were accompanied by changes in peak dough development time. For all three treatments, peak time which indicates when the dough has reached is maximum viscosity before gluten strands begin to break down, increased at day 1 of germination, 34% (12°C/24h), 35% (20°C/3h) and 42% (20°C/24h), before dropping at day 4, while it remained consist with non-sprouted sample for all day 2 and day 3 germinated samples. Therefore, it can be concluded that after day 3 of germination, sprouting time when α-amylase activity increased significantly, gluten and starch matrix was disrupted by enzymes including amylases as well as proteases, such that gluten quality and functionality was impacted significantly.

However, that was not the case for Ava as for 20°C/3h treatment, peak consistency significantly increased from that of control flour, 515 BU water absorption, during first three days of germination process, reaching a peak (635 BU) at day 2 before dropping at day 4 (468
BU), indicating a higher water absorption capacity for these samples, due to changes in structure and composition of spouted flour. Furthermore, for all five day treatments for Ava 12°C/24h no significant changes in peak dough consistency were observed at 60% water absorption as well as for first four days of Ava 20°C/24h except at day 5 for which dough consistency dropped to 471 BU. In terms of peak dough development time, no significant changes (p<0.05) were observed at day 1 for all three treatments and day 2 of 12°C/24h, however it decreased gradually for rest of the samples dropping 63% (12°C/24h), 69% (20°C/24h) and 71% (20°C/3h) at day 5, positively correlated to α-amylase activity in these samples.

These results can be explained by the differences in kinetic forces existing in soft and hard wheat. Jazaeri and colleagues have recently suggested that hydrophobic interactions play a more important role in soft wheat gluten network formation, while hard wheat dough is dominated by disulfide linkages (2013). Soft wheat hydrophobic interactions led to a compact gluten structure that was not easily hydrolyzed by the action of protein degrading enzymes produced during sprouting. Formation of dry and hard dough for Ava after sprouting can be explained by the fact that proteases modified gluten proteins and the hydrophobic interactions existing between them such that gluten now had enhanced water binding capacity. On the other hand, disulfide linkages in hard wheat gluten matrix were easily broken down by the action of proteases, causing a drop in dough consistency leading to wet and sticky dough.

Higher water binding capacities of starches isolated from sprouted grains have been reported by Lorenz and Valvano (1981) and linked due to changes after sprouting from crystalline to amorphous regions in the starch granules which are more hygroscopic and therefore cause water-binding capacity to increase.
It was also noticed from the raw curves of farinograph, that after reaching a peak, tails ran downward on an angle which increased with increased sprouting time and temperature for both varieties. Increased $\alpha$-amylase activity broke down starch causing a drop in viscosity while protease activity affected gluten quality and functionality, causing gluten stands to break down easily and faster.

For Wentworth, peak time for all three treatments increased significantly ($p<0.05$) at day 1 (34%, 35% and 43% for 12°C/24h, 20°C/3h and 20°C/24h, respectively) and day 2 (42% for 20°C/24h) and then dropped for rest of days for all treatments. However, with increasing sprouting time and temperature, soft wheat sprouted flour dough reached its maximum viscosity earlier than non-sprouted flour as peak time decreased significantly ($p<0.05$) at day 2 (62% and 47% for 20°C/24h and 20°C/3h, respectively) while at day 3 (54% for 12°C/24h) than the control. It has been previously reported that minor pre-harvest sprouting led to significant reductions in gluten strength of winter wheat as falling number predicted sprout damage in only 6 wheat lines while farinograph data indicated that gluten strength of all 17 flours was significantly reduced (2005).
Fig 3.3a – Peak Dough Consistency (BU) of wheat flours sprouted under various temperatures and steeping durations for 5 days and Control (0)
Fig 3.2b – Peak Dough Development Time (min) of wheat flours sprouted under various temperatures and steeping durations for 5 days and Control (0)
3.4.2.3 Effect on Gluten Aggregation Kinetics:

Results for Peak Maximum Torque (BE) and Peak Development Time (min) are listed in Fig 3.3a and 3.3b. For Wentworth, at day 1 and day 2 for 12°C/24h no significant change in peak torque was observed while for 20°C/24h peak torque dropped 16% only after day 1 (40.0 BE from 47.8 BE) and 20% drop at day 1 for 20°C/3h (38.1 BE from 47.8 BE) (p<0.05). No or only minor change in maximum torque required for gluten aggregation was seen between day 3 and day 4 of all spouting treatments before dropping significantly at day 5. Maximum drop 41% (28 BE from 47.8 BE) was seen at day 5 of 20°C/24h. Overall, time to reach peak torque dropped gradually with increasing sprouting duration and was more significant for higher temperature treatment and longer steeping time, indicating that gluten has been aggregating faster in sprouted wheat. Maximum decrease was for day 4 and day 5 of 20°C/24h treatment, dropping from 0.60 min to 0.20 min, a 67% decline followed by day5 of 12°C/24h (0.25 min), 58% decline and day 5 of 20°C/3h (0.27 min), 55% drop.

For Ava, no significant changes were observed in peak torque until day 4 of 20°C/3h, 29% decrease (25.44 BE from 35.9 BE), and day5 of 20°C/24h, 21% drop (28.5 BE from 35.9 BE). Ava 12°C/24h treatment did not have any effect on peak torque to develop gluten, suggesting no significant modification in protein quality when sprouted at these conditions. As in the case of Wentworth, there was also a general downward trend for peak gluten development time in Ava for all sprouting treatment conditions. At day 5, 67%, 69% and 73% drop in time for gluten aggregation was observed for 20°C/24h, 20°C/3h and 12°C/24h, respectively.
In general, it was noticed that areas under the raw GlutoPeak Tester curves, (not shown here) for all sprouted samples of both varieties decreased gradually with increasing sprouting duration, reflecting an overall decrease in gluten quality and functionality.

All these results point out the fact that soft wheat gluten proteins have lesser impact on their quality and functionality after sprouting as compared to hard wheat due to differences in the kinetic forces existing between different fractions.
Fig 3.3a – Peak Torque (BE) of wheat flours sprouted under various temperatures and steeping durations for 5 days and Control (0)
Fig 3.4b – Peak Torque Time (min) of wheat flours sprouted under various temperatures and steeping durations for 5 days and Control (0)
3.4.2.4 **Effect on Protein Molecular Weight Distribution:**

Modification in protein quality during sprouting process was also evident by SDS-PAGE analysis, presented in Fig (3.4), represented by bands (subunits) ranging from 100 to 15 kDa. The most intense band for both varieties appeared at 100 kDa and was completely hydrolyzed by day2 for all sprouted samples in both Wentworth and Ava. At day3 bands larger than 75 kDa and at day5 larger than 65 kDa disappeared for all three treatments in Wentworth while in Ava no subunits larger than 50 kDa appeared at day4 for any of the sprouting conditions. In general, high MW subunits (100 to 30 kDa) reduced in intensity as sprouting progressed while low MW subunits (<30 kDa) increased in intensity, with 20°C/3h sprouting condition showing the most intense of this SDS-PAGE pattern in both varieties. It is generally agreed that as sprouting progresses, the albumin and glutenin fractions increase while the globulin and residue protein fractions decrease and the gliadin fraction remains relatively constant (Ariyama & Khan 1990; Hwang & Bushuk 1973). This qualitative and quantitative difference in sprouted flour protein due to protease activity has detrimental effect on physiochemical and functional properties of wheat flour.
Fig 3.4 - Effect of various temperatures and steeping durations on protein molecular weight distribution for 5 days of sprouting in wheat.

M (molecular weight standard), Control (C) and Sprouted Samples (Day 1, 2, 3, 4 and 5)

- (a) WW20°C/24h, (b) WW20°C/3h, (c) WW 12°C/24h, (d) Ava 20°C/24h, (e) Ava 20°C/3h and (f) Ava 12°C/24h.
3.4.2.5 **Effect on Protein Content**:

Results are reported in Table 3.3. For Ava, protein content reached a maximum at day 2 of 20°C/3h (7.87% w/w) and day 4 of 20°C/24h (7.74% w/w) than non-sprouted flour (7.40% w/w) while no significant changes were seen for rest of the sprouted wheat samples. However, for Wentworth variety, protein content dropped significantly for all five days of 20°C/24h as well as for day 3, 4 and 5 of 12°C/24h but no changes for any of the days for 20°C/3h (p<0.05).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ava</th>
<th></th>
<th></th>
<th>Wentworth</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12°C/24 h</td>
<td>20°C/24 h</td>
<td>20°C/3 h</td>
<td>12°C/24 h</td>
<td>20°C/24 h</td>
<td>20°C/3 h</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Control</td>
<td>7.40±0.07</td>
<td></td>
<td>9.95±0.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Content</td>
<td>Day 1</td>
<td>7.42±0.04</td>
<td>7.49±0.03</td>
<td>7.63±0.00</td>
<td>9.45±0.04</td>
<td>9.30±0.08</td>
<td>9.83±0.05</td>
</tr>
<tr>
<td>(%w/w)</td>
<td>Day 2</td>
<td>7.65±0.06</td>
<td>7.69±0.04</td>
<td>7.87±0.11</td>
<td>9.82±0.01</td>
<td>9.17±0.02</td>
<td>9.88±0.11</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>7.49±0.01</td>
<td>7.79±0.07</td>
<td>7.87±0.13</td>
<td>9.19±0.07</td>
<td>8.90±0.01</td>
<td>10.0±0.07</td>
</tr>
<tr>
<td></td>
<td>Day 4</td>
<td>750±0.01</td>
<td>7.77±0.03</td>
<td>7.39±0.08</td>
<td>9.89±0.12</td>
<td>9.45±0.06</td>
<td>9.69±0.06</td>
</tr>
<tr>
<td></td>
<td>Day 5</td>
<td>7.55±0.08</td>
<td>7.44±0.06</td>
<td>7.24±0.15</td>
<td>9.61±0.04</td>
<td>9.11±0.04</td>
<td>9.94±0.04</td>
</tr>
</tbody>
</table>

Table 3.3 – Effect of various temperatures and steeping durations on the % Protein Content (w/w) dry basis for 5 days of sprouting in wheat
3.4.2.6 Effect on Dietary Fiber Content:

With increased sprouting time and temperature, no significant changes were observed for the total dietary fiber (TDF), insoluble fiber (IDF) or soluble fiber (SDF). Results are reported in Table 3.4.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ava</th>
<th>Wentworth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IDF</td>
<td>SDF</td>
</tr>
<tr>
<td>Control</td>
<td>9.47 ± 0.35</td>
<td>4.41 ± 0.20</td>
</tr>
<tr>
<td>12°C/24 h</td>
<td>9.44 ± 0.19</td>
<td>3.87 ± 0.34</td>
</tr>
<tr>
<td>20°C/24 h</td>
<td>9.21 ± 0.22</td>
<td>4.38 ± 0.10</td>
</tr>
<tr>
<td>20°C/3 h</td>
<td>9.83 ± 0.38</td>
<td>5.03 ± 0.11</td>
</tr>
</tbody>
</table>

Table 3.4 – Effect of various temperatures and steeping durations on the % Insoluble Dietary Fiber (IDF), Soluble Dietary Fiber (SDF) and Total Dietary Fiber (TDF) Content (w/w) dry basis for 5 days of sprouting in wheat
3.4.2.7 Effect on Arabinoxylan Content:

Results are reported in fig (3.5). For both Ava and Wentworth, total arabinoxylan content at day 5 increased significantly (p < 0.05) for sprouting conducted at 20°C treatment regardless of steeping duration, indicating that temperature was the most important factor for increase in arabinoxylan content. At day 5 of germination, highest increase was noted for Ava 12°C/3h (5.3g/100g) from non-sprouted sample (6.2g/100g), a 17% increase while 9% increase for WW 20°C/24h (6.6g to 7.2g for 100g). Previously, Coutrin and Delcour have reported that endo-β-1,4-xylanase (EC 3.2.1.8) are produced during germination altering arabinoxylan physiochemical properties. They convert water-unextractable arabinoxylan (WE-AX) into solubilized arabinoxylan (S-AX) and degrade water-extractable arabinoxylan (WE-AX) and S-AX to lower molecular weight, thereby lowering viscosity inducing properties and partially impairing gelling capacity (2000).
Fig 3.6 – Effect of various temperatures and steeping durations on Arabinoxylan content (g/100g) at day 5 and Control
3.3 Conclusion

It can be concluded that variety difference is the most important factor affecting $\alpha$-amylase activity during sprouting followed by temperature and initial moisture status of the grain and there exist an interaction between these three factors as hard and soft wheat responded differently to the same environmental conditions. Furthermore, results of this quantitative and qualitative measure of $\alpha$-amylase activity under various sprouting conditions correspond to the effect on sprouted flour’s functionality. The detrimental effect on gelatinization power is due to excessive $\alpha$-amylase activity affecting starch in-situ and during processing. While the changes in water holding capacity, dough consistency and its development time as well as on gluten development and its aggregation time are due to in situ damage of starch and gluten by amylases and proteases. These are all important parameters for the baking quality and performance of flour during dough processing and therefore needs to be closely monitored if sprouting wheat to utilize as an ingredient in food production. A significant increase in Arabinoxylan was also observed at day5 of sprouting in both varieties for all treatments. Further work will focus on the physiochemical and staling behavior of the breads fortified with different levels of $\alpha$-amylase activity by addition of sprouted flour as an ingredient.
CHAPTER FOUR

INVESTIGATING PHYSIOCHEMICAL AND STALING BEHAVIOR OF BREADS FORTIFIED WITH SPROUTED WHEAT FLOUR

4.1 Abstract

Bread staling is associated with sensorial changes such as loss of flavor, loss of crispness in the crust and increased crumb firmness. The effect of laboratory sprouting of a commercial blend of Canadian western hard red spring wheat as an ingredient on textural attributes and shelf-life of bread were examined. Sprouted wheat flour post five days germination having $2.2 \times 10^3$ units $\alpha$-amylase activity per gram of flour was utilized at 1%, 5%, 10% and 15% rate to prepare composite flour containing $2.2 \times 10^3$, $11 \times 10^3$, $22 \times 10^3$ and $33 \times 10^3$ unit of $\alpha$-amylase activity in 100 g bread, respectively. After baking, the staling parameters of breads were monitored over 7 days of storage by mechanical (compression measurements), physiochemical (DSC) and rheological (RVA) methods. There was an improvement in baking quality and shelf life of 1% and 5% composite breads resulting in a significantly increased loaf volume, better texture, and less retrogradation during 7 days post baking than the control as well as than the composite breads with 10% and 15% sprouted wheat flour. These results should help to identify optimal $\alpha$-amylase activity in sprouted wheat for the development of functional whole grain products that are naturally shelf-stable and more nutritionally dense.

Key words: sprouted wheat flour, $\alpha$-amylase, anti-staling, DSC, RVA
4.2 Introduction

Cereal grains form a major source of dietary nutrients for all people in the developed and developing countries. It is generally agreed that wheat was one of the first grains to be cultivated, as early 9600 BC. It has been cultivated around the Eastern Mediterranean and Mesopotamia for at least 5000-6000 years (Lev-Yadun et al., 2000). Today wheat is extensively grown on more land area than any other commercial food all around the world. In 2011-2012, world production of wheat was estimated to be 696 million tons making it the second most-produced cereal after maize (877 million tons) (IGC, 2013). The importance of wheat is mainly due to the fact that its seed can be ground into flour and semolina, which form the basic ingredients of bread, bakery products, noodles and pasta, providing the main source of nutrients to the most of the world population.

Bread is one of the most important foods consumed in the modern world, with an average of about 50 kg of bread per capita per year in Europe, and an important constituent of a balanced health diet (FOB, 2013). All baked products undergo staling after baking and during storage, which is a complex process responsible for huge economic losses to both baking industry and consumers. It is thought that 3% of the total bread produced is wasted due to the problem of staling as it becomes unsaleable (Zobel and Kulp, 1996). Staling is defined as all changes, except microbiological spoilage, that occurs at different rates and intensities after removal from the oven and during storage of a baked product, making it less acceptable for the consumers. It involves loss of aroma, changes in mouth feel, loss of crumb softness and development of crumbliness. These serial changes cannot be explained by a single effect, and includes loss of moisture content, moisture migration between crumb and crust at macroscopic level while between the amorphous and crystalline region at microscopic level, amylopectin retrogradation.
as well as reorganization of polymers within the amorphous region (Martin and Hoseney 1991; Morgan et al., 1997). However, full mechanism of bread firming is still unknown.

Baking industry deals with the problem of bread staling by using a variety of anti-staling ingredients including enzymes (amylases, proteases and xylanases), fatty acids (mono and diglycerides) and different emulsifiers. The intentional inclusion of microbial enzymes in bread formulation dates back to more than a century (Fox & Mulvihill, 1982). Enzymes are better alternative to chemical agents, as they are generally recognized as safe (GRAS) and do not have any potential health hazards. Furthermore, they do not remain active in the final product after baking and therefore, need not to appear on the label, which is an additional commercial advantage. Currently, microbial enzymes (fungal and bacterial \( \alpha \)-amylases) are the most popular anti-staling agent in commercial baking to improve dough handling properties as well baking characteristics such as increased bread volume, improved crumb structure and increased shelf-life.

Dragsdorf and Varriano-Marston indicated that the addition of amylase from different sources to bread formulations reduces the firming of stored bread. This reduction in bread firmness has been attributed to the enzymatic breakdown of starch into dextrins fragments that interfere with the crystallization of starch (1980). A decreasing degree of starch crystallinity was observed in bread with bacterial \( \alpha \)-amylase, cereal \( \alpha \)-amylase, fungal \( \alpha \)-amylase and unsupplemented bread, respectively.

During baking \( \alpha \)-amylase partially hydrolyzes amylose and amylopectin to glucose, maltose and oligosaccharides which are less prone to retrogradation and these low molecular weight polymers further interfere with amylopectin recrystallization during storage. It has been suggested that the dextrins formed due to starch hydrolysis interfere with the interaction between the swollen starch
granules and the continuous protein network in the bread, making it less prone to staling (Defloor and Delcour 1999; Akers and Hoseney, 1994). Furthermore, \( \alpha \)-amylases’ anti-staling properties have been attributed to the changed crystallization behavior of the residual amylose and amylopectin populations (Zobel & Kulp, 1996; Hug-Iten et al., 2003). Alpha-amylase mainly hydrolyze the internal bonds of the starch polymers due to their endo-acting nature, reducing the levels of long chains connecting different junction zones and thus different starch networks are weakened, resulting in a decreased crumb firmness.

Staling is not the only challenge that baking industry has to deal with to satisfy consumers. Most recently there has been an increasing concern and awareness of the effect of diet on health and disease. Changes in eating patterns, food choices and methods of food preparation have decreased the nutritive value of our diets. Generally, a typical diet in developed countries is lower in dietary fiber, vitamins, minerals and antioxidants. Health conscious consumers are taking the advice of Academy of Nutrition and Dietetics that recommends to consume more whole grains, accounting for one half of an adult’s six to eight daily grain servings (AND 2013). Therefore, based on the demand from the consumers for wholesome, additive-free and nutrient dense food products, market opportunities for value-added wheat-based products such as bread have been grown rapidly over the past few decades.

Sprouted wheat flour fulfills the nutritional principles of whole grains with added benefits of higher bioavailability of nutrients as recently, it has been shown that sprouted grains are more nutritious in terms of vitamins, minerals, fiber and phenolic compounds when compared with their native counterparts because grain’s mobilized energy reservoir is readily available in its active form (Plaza et al. 2003; Koehler et al. 2007; Hung et al. 2011). Whole wheat bread contains substantially more vitamins, minerals, antioxidants and other nutrients than refined
wheat flour and sprouting, a simple, natural and inexpensive method, further improves the
nutrition value.

Despite the benefits that sprouted wheat has to offer, it is recently being consumed only as salad,
juice, and tablet for its vitamins, minerals, and phenolic contents, because it comes with the
problem of inferior quality flour that leads to processing problems and unsatisfactory end-
products during food manufacturing. Sprouting effects the physiochemical and functional
properties of wheat, as flour extraction, ash and protein content and falling number decreases
while α-amylase and proteolytic activity increases with sprouting. Sprouted wheat flours are ill
suited for dough and bread making and use of 100% flour from sprouted flour yields bread with
poor characteristics: wet, sticky dough, darker crumb color and an inferior texture that is sticky
and gummy (Ariyaman & Khan, 1990; Beleia and Grossmann, 1990; Cabrera et al., 1995;
Yamauchi et al., 1998).

However, fully acceptable with a good volume and crumb texture bread was prepared by
blending sprouted wheat flour with sound flour as amylases enzymes synthesized during
germination are important factors affecting bread crumb firmness (Ranhotra et al, 1977; Morad
& Rubenthaler 1983). Later it was reported that the quality of pan breads made from sprouted
grains was better than that from corresponding untreated wheat, as water absorption capacity of
sprouted flours was higher. This was in contrast to most published reports at that time where
baking quality was inferior due to sprouting. It was concluded that the relative levels of protease
and amylase activities in the sprouted flours were optimal in the baking system than those in the
control flours, leading to believe that increased amylase and protease activity of the sprouted
samples acted as an effective gluten softener thus reduced mixing time and improved loaf
volumes (Edwards et al., 1989). These results were confirmed by another study where using
sprouted wheat flour to produce bread appeared to improve quality of bread as loaf volume increased significantly by substituting 5% of sprouted wheat flour (Ranhotra et al. 1997) while Seguchi and co-workers showed that bread making properties (bread height and volume) were gradually enhanced by blending the germinated outer bran layers at 10% by weight, with maximum specific volume obtained after 5 days of germination (2010). However, the improvement was lost after 8 days of germination and it was suggested that α-amylase and xylanase activities in the outer bran layers are highly related to the enhancement of bread making properties.

Since during germination process, alpha amylase activity is increased (Geddes et al. 1941; Kneen et al. 1942; Fleming et al. 1960; van der Maarel et al. 2002), it is proposed that baked products made with sprouted wheat flour would be less prone to staling naturally. It is generally agreed that wheat flour is the main ingredient that influences the processing characteristics as well as the textural and quality attributes of the bread. Since studies have shown that sprouting conditions such as initial hydration status of the grain, temperature during sprouting and most importantly sprouting duration affect alpha-amylase’s content and activity in the wheat, it is important to find the optimal enzyme’ activity levels to use sprouted wheat in end-products that are naturally shelf-stable as insufficient or excess amylase activity increases crumb firmness and staling rate of bread.

Furthermore, during germination process antioxidants as well as fat degrading enzyme lipases are produced which prevent oxidation of fatty acids in the grains, minimizing rancidity and providing longer shelf-life (Kubicka et al., 2000).

Therefore, it can be concluded that sprouted wheat flour can be blended with sound flour at an optimal ratio to yield naturally shelf stable bread with acceptable volume and crumb texture with
added benefits of sprouting. The purpose of this study was to determine the effect of sprouting on the baking quality of breads made from composite flours with different ratios of sprouted Canadian western hard red wheat as well as to determine the effect of germinated wheat on the staling rate of bread crumb during seven days of storage post-baking. This project will help formulate bread fortified with sprouted wheat to extend shelf life while enhancing nutritional value with minimum commercial additive, having positive impact on baking industry economy by offering scientific basis to develop functional wheat products as well as consumers’ health and their satisfaction. The study involved preparing composite breads and the staling properties were tested by measuring their firmness. Rheological properties were measured by RVA viscosity profiles of bread crumb while starch retrogradation enthalpies were determined by DSC analysis.

4.3. Materials and Methods

4.3.1 Plant Material

Canadian Western Red Spring (Commercial hard wheat blend) was kindly provided by P & H Milling Group (Hanover, ON).

4.3.2 Sprouting Conditions

Wheat samples were stored at room temperature before germination. Any broken, damaged and off-color kernels from the wheat samples were removed. Seeds were washed with distilled water and disinfected in a 2% (v/v) H₂O₂ solution for 3 hours, rinsed again with distilled water and then steeped in distilled water for an additional 21 hours. Wheat samples were germinated in Sanyo Growth Chamber (MLR-351H) at 20°C with 70% relative humidity 5 days, post water contact. Samples were hydrated every 4 hours and scrambled to prevent matting.
4.3.3 Drying and Milling

Germinated wheat kernels were frozen at -80°C to stop sprouting, and then freeze-dried. Upon drying any vegetative parts (roots and shoots) were removed by scrubbing. Then, wheat samples were milled with UDY Cyclone mill with a 0.5 mm mash screen and kept at -34°C for analysis.

4.3.4 Determination of α-Amylase Activity

Alpha-amylase activity was determined by the Amylazyme-Red tablet (Megazyme International Ireland Ltd.) specifically designed to measure cereal α-amylase activity (AACC 22-05.01). 1.00 g flour was stirred with Sodium maleate buffer (100mM pH 6.0) for 15 min and centrifuged at 1,500 g for 10 min. 0.2mL of the supernatant was diluted with 10 mL Sodium maleate buffer and 0.5 mL of diluted aliquots were incubated with Amylazyme Red tablet for exactly 10 min at 40°C. Reaction was terminated by adding 10 mL of Trizma base solution (2% w/v, pH 8.5) and then filtered. Absorbance of the filtrate was measured against a reaction blank at 510 nm and α-amylase activity was determined by reference to the appropriate standard curve for purified barely malt to convert absorbance to Ceralpha Units of activity. Alpha-amylase activity was reported as Ceralpha Units per gram of flour. All tests were performed in duplicates.

4.3.5 Bread-Making

4.3.5.1 Composite Flour Preparation: Commercial refined hard wheat flour was blended with different percentages of sprouted wheat flour to yield 100 g composite flour as listed in Table 4.1.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Sprouted Flour (%)</th>
<th>α-amylase (Ceralpha Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1% Composite</td>
<td>1%</td>
<td>2.2x10^3</td>
</tr>
<tr>
<td>5% Composite</td>
<td>5%</td>
<td>11x10^3</td>
</tr>
<tr>
<td>10% Composite</td>
<td>10%</td>
<td>22x10^3</td>
</tr>
<tr>
<td>15% Composite</td>
<td>15%</td>
<td>33x10^3</td>
</tr>
</tbody>
</table>

Table 4.1 – Composite flour ratios and subsequent α-amylase activity units (Ceralpha Units) in 100 g of breads

4.3.5.2 Water Absorption Capacity of Composite Flours: Physical dough characteristics and water absorption capacity of composite flour samples were tested with farinograph according to the approved method (AACC, International Method 54-21.02). Dough was prepared in Brabender farinograph, using a 10 g mixing bowl at 30˚C and water absorption to reach 500 BU for all composite flours was determined in duplicates and average was used to make bread.

4.3.5.3 Dough Processing: An optimized straight dough process (AACC 10-10.03) was performed using the following ingredients (% on the flour basis): 100% flour, 6% sugar, 5.3% yeast, 3% shortening and 1.5% salt and 61%, 59%, 57%, 55% and 55% water for control, 1%, 5%, 10% and 15% sprouted flour supplemented composite breads respectively, based on their flours’ water absorption capacity. All dry ingredients were incorporated in the initial mixing step and fermented doughs were obtained after two-step bulk fermentation and proofing for 123 min, with first punch after 52 min and second punch after 25 min.
4.3.5.4 Baking and Storage: Loaves were baked at 215°C for 24 min in a gas oven. After 1 h cooling, loaves were packed in polyethylene bags and stored for 1, 3, 5 and 7 days at 22 ± 2°C. Moisture content test, water activity test, texture, DSC and RVA analysis of bread were performed after 1, 3, 5 and 7 day of storage. Duplicates loaves were made each treatment.

4.3.6 Quality and Shelf life Analysis of Bread

4.3.6.1 Volume: After cooling for 1 h, baked breads were weighed and loaf volume was measured by Rapeseed Displacement method according to AACC method (10-05.01). At least two measurements were made on each loaf and averaged. Specific volume was calculated from the weight and volume and results are reported in Fig 4.1.

4.3.6.2 Texture: Crumb texture was determined with a Texture Analyzer TA-XT Plus (Texture Technologies Corp, New York) provided with the software “Texture Exponent 32” using the AACC method (74-10-02). Bread slices with dimension of 20 mm x 25 mm x 15 mm were compressed. Slices were compressed with an aluminum 21mm diameter cylindrical probe with the rate of compression set at 1.7mm/s and firmness was the ‘force’ (g) required to compress the slice 40%. At least two measurements on slices from each loaf were taken and average is represented in Fig 4.2.

4.3.6.3 RVA Profiles: Rapid Visco™ Analyser (RVA-4, Newport Scientific, Warriewood, Australia) equipped with Thermocline for Windows (TCW3) software was used to study gelatinization and retrogradation of starch in the bread. Freeze-dried bread samples were ground in a coffee grinder and sieved through a 425-μm screen. Ground samples of 4 g (14% Moisture basis) were added to 25 g distilled water and samples were heated from 50°C to 95°C within 3.5 min and then held at 95°C for 2.5 min. It was subsequently cooled to 50°C within 3.5 min and then held at this temperature for 2 min. The peak viscosity and setback viscosity were
determined from the RVA plots. At least two replicates were done and averages are reported in Table 4.1.

4.3.6.4 DSC Analysis: Differential scanning calorimeter model DSC-Q 200 (Dupont TA instrument, USA) was used to measure the retrogradation enthalpies of starch in breads during storage. 5 mg of freeze-dried bread crumb samples were loaded into the pans and water was added at 1:2 (w/v, sample: water ratio). The pans were hermetically sealed and kept at room temperature for 30 min. Then the samples were scanned by DSC from 10 to 90°C at a heating rate of 10°C/min. Starch especially of amylopectin recrystallization was measured from an endothermic melting enthalpy corresponding to a peak between 50 to 60°C range. ∆H is expressed as J/g of starch and was calculated by using a starch content of 63% for the control bread and 61% for the composite breads as calculated by Total Starch Assay Kit (Megazyme International Ireland Ltd.). At least two replications were done.

4.3.7 Statistics:

All analyses were performed at least in duplicate and the mean values are reported. Analysis of variance was performed using IBM SPSS Statistics 20 software. Significant difference (p<0.05) among means were detected using the Tukey’s multiple range test at a fixed level of α = 0.05.

4.4. Results and Discussion

4.4.1 Alpha-amylase Activity: This enzyme’s activity increased during germination as expected and in agreement with all previous studies (Fleming et al., 1960; Belay & Furuta, 2001). A rapid increase after day 2 was observed (243 Ceralpha units/g of flour) reaching its peak at day 5 (2197 Ceralpha units/gram of flour).
4.4.2 Effect on Water Absorption of Composite Flours: By adding spouted water flour water absorption decreased, composite flour samples containing 10 and 15 percent sprouted flour had the lowest water absorption (54.3% and 54.5%) compared with control flour (59.75%). Lower water absorption is due to anatomical as well as physiochemical changes in structure and composition of sprouted wheat flour. As mentioned previously, enzymatic hydrolysis of starch leads to significant changes in starch granules such that their water holding capacity drops.

4.4.3 Effect of Sprouted Wheat Fortification on Bread Quality:

Specific loaf volume of composite breads and control bread is shown in Fig 4.1. The volume of 1% and 5% composite breads were significantly higher than control (18% and 12% higher, respectively) as well as than the 15% supplemented bread (19% and 15% higher, respectively) (p<0.05). These two bread loaves had a finer and more homogenous crumb structure with larger air cells than all other loaves. A more open grain structure with higher loaf volume is due to increased amounts of fermentable sugars resulting from starch degradation enhanced by presence of amylases. This reaction caused a concurrent increase in gas production and thus more open grain, larger cell size and higher loaf volume.

There was no significant difference in specific volume for 10% and 15% composite breads than the bread with no α-amylase activity. Surprising, only 1% sprouted wheat flour having 2.2x10³ units of α-amylase activity in 100 g bread appears to improve bread volume. These results are in agreement with previous study where both lack of α-amylase activity and high activity produced lower loaf volume (Morad & Rubenthaler 1983).

Low volume of breads made with 10% and 15% sprouted wheat flour than the control bread can be attributed to two major factors. First, gluten functionality of the sprouted flour has been affected by enzymes synthesized during sprouting as well as hydrolysis of refined flour.
gluten by the action of these enzymes during bread making. Similar, swelling capacity of starch granules in the sprouted flour as well as in the refined flour is impaired due to amylosis by α-amylase as well as by all other starch degrading enzymes. All these changes have been indicated in the previous work of RVA, Farinograph and GlutoPeak Tester results. Furthermore, increasing spouted flour ratio contributes soluble and insoluble dietary fiber, well-known ingredient for reducing bread volume.

Fig 4.1- Specific volume (cm³/g) of composite breads
4.4.4 Effect of Sprouted Wheat Fortification on Bread Shelf-life:

When staling rates of both control and composite breads were compared for the crumb firmness, sprouted flour supplementation up to a certain level had a beneficial effect during seven days of storage. Although, the firmness of both control and sprouted flour supplemented loaves increased linearly over the 7 days storage, the bread supplemented with 1% and 5% sprouted wheat flour were found to be less firm in all cases (p<0.05). 1% composite bread was significantly less firm than control as well as from 10% and 15% composite breads post 7-days baking while 5% bread was found to be less firm than other samples at day 1 and day 7 post baking. It can be assumed that α-amylase depolymerized amylopectin chains, preventing the trapping of water in the network of amylopectin and as a result the amount of free water is high and the crumb softer.

However, sprouted flour supplementation at 5% and 10% had negative effect on bread firmness, as these two bread samples were consistently more firm not only from other two composite breads but also had either similar or higher firming rate than the control bread during 7-days of storage. These results are consistent with earlier studies (Zobel & Kulp 1996; Every et al., 1998) showing an inverse relationship between bread loaf volume and firmness, probably because more entanglements and interactions occurred between the more densely packed polymer in samples derived from low volume breads.

All these results indicate that only 1 g of sprouted wheat flour in 100 g bread is enough to increase volume and reduce firmness during 7 days of storage and further addition of sprouted flour has deleterious effect on bread volume which then relates to its firmness during storage.
Table 4.1 shows the RVA profiles of peak viscosity (cP) and final viscosity (cP) of composite breads. When the bread-water dispersion was heated and subjected to shear forces, the starch granules that were not fully gelatinized due to restricted hydration, absorbed water and swelled resulting in an increase of viscosity, reaching to the point where the number of swollen-intact granules was maximum. In this study, peak viscosity of control bread increased significantly from 1058 cP to 1160 cP (9.6% increase), 1164 cP (10%) and 1186 cP (12%) for day 5, day 3 and day 7, respectively due to starch recrystallization as well as due to inter-chain association of the amylose and amylopectin fraction during storage (p<0.05).
However, with increasing α-amylase activity in the composite breads, peak viscosity dropped significantly at day 1 post baking, from the control bread (57%, 79%, 78% and 76% for 1%, 5%, 10% and 15% composite breads, respectively) with no significant changes in the peak viscosities observed during storage.

Final viscosity values among RVA data has been related to staling in literature (Thomas & Atwell 1999; Lent & Grant 2001). When gelatinized starch cools, an increase in viscosity is observed until the formation of gel due to the reordering/retrogradation of amylose chains. As expected, final viscosity of the control bread increased significantly during storage with 5%, 125 and 17% increase for day 3, day 5 and day 7, respectively, as compared to day 1. During storage, final viscosities of the α-amylase supplemented breads were lower (56% for 1% and approximately 77% for other three composite breads) than the control bread at day 1 and kept dropping consistently during 7-days of storage. At day 5 of storage, final viscosity of all composite breads dropped significantly and remained consistently lower at day 7 than day 1 and day 3. Final viscosity for 1% composite bread dropped 15% (657 cP from 758 cP), 18% for 5% (314 cP from 384 cP), 20% for 10% (315 cP from 394 cP) and 18% for (315 cP from 384 cP) for 15% supplemented bread after 5 days of storage from day 1, indicating α-amylase addition was successful in preventing starch retrogradation by affecting the extent of starch recrystallization during storage.
Table 4.2 – Change in Peak Viscosity (cP) and Final Viscosity (cP) of Composite Breads during 7 days of Storage

<table>
<thead>
<tr>
<th></th>
<th>Sprouted Flour supplemented in Bread</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peak Viscosity (cP)</strong></td>
<td>0</td>
<td>1058 ± 0.7</td>
<td>1164 ± 4</td>
<td>1160 ± 24</td>
<td>1186 ± 6</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>453 ± 11</td>
<td>513 ± 52</td>
<td>437 ± 10</td>
<td>488 ± 3</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>226 ± 8</td>
<td>252 ± 44</td>
<td>178 ± 15</td>
<td>201 ± 3</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>232 ± 3</td>
<td>228 ± 2</td>
<td>197 ± 8</td>
<td>198 ± 6</td>
</tr>
<tr>
<td></td>
<td>15%</td>
<td>251 ± 39</td>
<td>234 ± 8</td>
<td>199 ± 1</td>
<td>196 ± 1</td>
</tr>
<tr>
<td><strong>Final Viscosity (cP)</strong></td>
<td>0</td>
<td>1710 ± 14</td>
<td>1800 ± 30</td>
<td>1916 ± 11</td>
<td>2010 ± 13</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>758 ± 14</td>
<td>780 ± 20</td>
<td>657 ± 38</td>
<td>679 ± 5</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>384 ± 20</td>
<td>386 ± 4</td>
<td>314 ± 6</td>
<td>346 ± 4</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>394 ± 3</td>
<td>374 ± 8</td>
<td>315 ± 4</td>
<td>338 ± 6</td>
</tr>
<tr>
<td></td>
<td>15%</td>
<td>384 ± 5</td>
<td>388 ± 9</td>
<td>315 ± 3</td>
<td>332 ± 4</td>
</tr>
</tbody>
</table>

4.4.4.2 Enthalpy of Retrogradation:

Table 4.3 shows the melting enthalpy (\(\Delta H\) J/g of starch) of the composite breads stored for 7 days post baking. In all breads endothermic transition took place between 52 and 59°C corresponding to the amylopectin retrogradation. As expected, retrogradation enthalpy increased during the storage time for the control and composite breads. In addition, the retrogradation enthalpy increased linearly with increasing \(\alpha\)-amylase activity in all composite bread with 15% supplementation requiring highest energy input to melt recrystallized starch (5.60 \(\Delta H\)/g of starch) while control bread lowest (1.72 \(\Delta H\)/g of starch). Retrogradation of amylopectin took place when bread came out of oven and cooled to room temperature and these results indicate
that presence of α-amylase activity led to higher ΔH values due to an indirect effect on the starch molecules by increasing the amount of crystallized amylopectin, in agreement with a previous study (Purhagen et al., 2011). However, bread made without α-amylase had a significant increase in starch retrogradation enthalpy with increasing storage time, 44% at day 3 and 245% at day 5 and day 7 (p<0.05) than the breads with varying degree of α-amylase activity. All composite breads except the one with 15% sprouted wheat flour followed a similar pattern for the retrogradation enthalpy, as it increased gradually from day 1 to day 5 (11%, 34% and 49% for 1%, 5% and 10% composite breads, respectively) before dropping at day 7 of storage (3%, 25% and 27% for 1%, 5% and 10% composite bread, respectively, drop from day 5 to day 7).

For the 15% supplemented bread, melting enthalpy dropped 22% at day 3 (from 5.60 J/g starch to 4.36 J/g starch) and then increased at day 5 with no significant increase until day 7. It can be assumed from these results that α-amylase enhanced the initial crystallization of starch after baking but reduced the firming rate of bread on aging. Hug-Iten and co-workers have previously shown that freshly baked bread with no anti-staling agent had a weak birefringence which became more intense during aging while amylase containing bread exhibited strong birefringence in the amylose rich region of the granules directly after baking which did not significantly increase during aging, suggesting that the enzyme hindered the retrogradation of amylopectin (2001). Therefore, it can be hypothesized that α-amylase activity contributed by the sprouted flour has an anti-staling effect based on the capacity to partially degrade amylopectin to hinder its recrystallization. On the other hand, they also concluded that amylases slightly degrade amylose by an endo-mechanism which promotes the rapid formation of a partly crystalline amylose network due to increased mobility in fresh bread, explaining initial increase in ΔH, and hinders amylose rearrangements during storage hence lower ΔH at day 7.
Furthermore, starch hydrolysis products resulting from amylases can be responsible for the anti-staling effect induced by sprouted flour as Jagannath and co-workers have suggested previously that enthalpy associated with staling in bread supplemented with maltodextrins is almost similar in magnitude and pattern to that of bread containing maltogenic amylase after 15 days (1997).

In brief, all composite breads had a significant (p<0.05) reduction of the staling endotherm in comparison with the control at day5 and day7 suggesting that α-amylase activity contributed by sprouted flour is effective at preventing staling of bread during storage which is indirectly related to amylopectin retrogradation.
Sprouted flour supplemented in Bread Day 1 Day 3 Day 5 Day 7
ΔH (J/g starch)
0 1.72 ± 0.03 2.47 ± 0.09 5.92 ± 0.08 5.93 ± 0.19
1% 3.04 ± 0.02 2.94 ± 0.02 3.26 ± 0.02 3.17 ± 0.19
5% 3.71 ± 0.06 4.28 ± 0.06 4.98 ± 0.01 3.72 ± 0.03
10% 3.75 ± 0.09 4.35 ± 0.07 5.59 ± 0.09 4.09 ± 0.02
15% 5.60 ± 0.10 4.36 ± 0.04 5.07 ± 0.08 5.05 ± 0.00
Tm (˚C)
0 53.3 ± 0.13 55.4 ± 0.34 52.0 ± 0.07 53.9 ± 0.06
1% 53.7 ± 0.00 52.6 ± 0.23 59.2 ± 0.08 52.6 ± 0.15
5% 57.3 ± 0.33 52.7 ± 0.01 52.5 ± 0.01 52.7 ± 0.09
10% 54.6 ± 0.22 53.8 ± 0.48 54.0 ± 0.14 56.6 ± 0.24
15% 55.7 ± 0.30 53.7 ± 0.17 55.2 ± 0.37 54.2 ± 0.33

Table 4.3 – Change in Enthalpy (J/g of starch) and Transition Temperature (˚C) of Composite Breads during 7 days of Storage

4.5 Conclusion

Analysis of starch retrogradation results revealed that the antistaling effect of α-amylase is essentially due to retardation in the starch retrogradation as indicated by final viscosity and gelatinization enthalpy. It can be concluded that control bread involves changes in the amyllopectin and amylose fractions during storage and therefore it is assumed that reorganization of both polymers contribute to the increase of firming during staling of baked products. Supplementation of sprouted flour contributes α-amylase activity which degrades amyllopectin side chains impeding its crystallization which in turn reduces firmness in bread during storage. However, day 5 sprouted flour is a rich source of multiple isoforms α-amylase with high activity, causing in-situ damage to starch and gluten as well as during bread processing, leading to
negative impact on bread quality and shelf-life. Therefore, only 1 to 5% can yield acceptable yet functional breads with added benefit of longer shelf-life without the addition of commercial anti-staling agents. Further work needs to be done to evaluate quality and shelf-life attributes of bread formulation with wheat sprouted at sub-optimal conditions.
CHAPTER FIVE

CONCLUSION

From an industrial point of view, including sprouted wheat-based formula to enrich bread and other products will yield baked products naturally resistant to staling while enhancing the health profile. Wheat sprouts fulfill the nutritional principles of whole grains with added benefits of higher bioavailability of vitamins, minerals and soluble fiber as recently proved by some researchers studying the effect of sprouting on nutritional quality of grains. While on the other hand, several studies have indicated that sprouted wheat flour incorporated at an optimal level is a good alternative to commercial anti-staling microbial amylases.

Genotype difference is the most important factor affecting α-amylase activity during sprouting followed by temperature and initial moisture status of the grain and there exist an interaction between these three factors, as hard and soft wheat responded differently to the same environmental conditions. Furthermore, results of the quantitative and qualitative measure of α-amylase activity under various sprouting conditions correspond to the effect on sprouted flour’s functionality. Starch granules’ gelatinization power is affected due to excessive α-amylase activity, damaging their structural integrity in-situ making them more susceptible to hydrolysis during processing. Furthermore, in situ damage of starch and gluten due to amylases and proteases compromise flour’s gluten-starch matrix strength. These are all important parameters for the baking quality and performance of flour during dough processing and therefore needs to be closely monitored if sprouting wheat to utilize as an ingredient in food production.

Physiochemical and staling behavior of the breads fortified with different levels of sprouted flour as an ingredient revealed that inclusion only at 1% by weight is enough to achieve
desirable quality and shelf-life attributes and further increasing sprouted flour ratio does not have any positive outcomes.
REFERENCES CITED


