Department of Food and Environmental Sciences

Use of bio-enriched yeast and stability of its vitamin D$_2$ in wheat dough baking

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Helsinki, 2015
Preference

This study was conducted at the department of Food and Environmental Sciences, Nutritional Science division, Faculty of Agriculture and Forestry of the University of Helsinki.

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Helsinki 2015

Tolosa, Bogale
List of abbreviations

RDA ........... Recommended daily allowance
UVB ....................... Ultraviolet B
25(OH) D .......... 25-hydroxyvitamin D
1, 25(OH)\textsubscript{2}D\textsubscript{2} .. 1, 25-dihydroxyvitamin D\textsubscript{2}
PTH ..................... Parathyroid hormone
DBP ................... Vitamin D-binding proteins
VDR .................... Vitamin D receptors
cGMP .............. Cyclic guanosine monophosphate
1-OHase ............ 1-alpha-hydroxylase
LC-MS/MS .................. Liquid chromatography tandem mass spectrometry
IGF .................. Insulin like growth factors
GH ..................... Growth hormone
PUFA .............. Poly unsaturated fatty acids
HTST ............. High temperature - short time
UHT ................ Ultra high treatment
FU ..................... Farinograph units
BU ..................... Brabender units
RH ...................... Relative humidity
SV ...................... Specific volume
SBP .................. Standard baking process
LEM ...................... Low energy mixing
HEM ..................... High energy mixing
LBT ..................... Low baking temperature
HBT ..................... High baking temperature
ANOVA .................. Analysis of variance
Mwet ................... Wet mass
Mdry .................... Dry mass
V_b ...................... Bread volume
FFQ .................... food frequency questionnaire

**Standard measurement units**

µg ........................ micro gram
IUs .......................... International Units
IU/g ........................ International Units per gram
mg ............................ Milligrams
mL ............................ milliliters
nmol/L ..................... nanomoles per liter
Kg ............................. kilogram
g ............................. gram
°F ............................. Degree Fahrenheit
°C .......................... Degree centigrade
Ø .............................. Diameter
rpm ........................ revolution per minute
Use of bio-enriched yeast and stability of its vitamin D$_2$ in wheat dough baking

Abstract

Vitamin D is one of the basic vitamins required by our body for maintaining stable health conditions and to have stronger bone structure. In countries where there is shortage of sunlight exposure (like in Northern European countries) the oral intake of vitamin D either from dietary source or from supplementing tablets is very essential.

This study aimed to analyze stability of vitamin D$_2$ originating from bio-enriched yeast in baking process and focused to investigate impact of such yeast on bread quality. Impact of mixing time (intensity), baking temperature-time combinations and effect of two different storage conditions were studied. The presence of vitamin D$_2$ containing bio-enriched yeast as an ingredient didn’t affect the quality of bread. Overall breads obtained from all designed protocols have shown low specific volume, fine and evenly distributed porosity on the crumb structure.

Variation of mixing time or baking regime did not influence on the stability of vitamin D$_2$. Furthermore, bread storage conditions, which were considered with in this study, didn’t affect the stability of vitamin D$_2$. Vitamin D$_2$ from bioenriched yeast has shown stability in wheat dough baking.

Keywords

Vitamin D, Vitamin D fortification, fortified bread, vitamin D$_2$, vitamin D$_3$

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# CONTENT

## ABSTRACT

## PREFERENCE

## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>INTRODUCTION</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>LITERATURE REVIEW</td>
<td>11</td>
</tr>
<tr>
<td>2.1</td>
<td>Vitamin D</td>
<td>11</td>
</tr>
<tr>
<td>2.2</td>
<td>Vitamin D metabolism</td>
<td>12</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Metabolic pathway difference between vitamin D(_2) and vitamin D(_3)</td>
<td>16</td>
</tr>
<tr>
<td>2.3</td>
<td>Sources of vitamin D &amp; its bioavailability</td>
<td>17</td>
</tr>
<tr>
<td>2.4</td>
<td>Vitamin D fortification and the required accuracy in fortification process</td>
<td>19</td>
</tr>
<tr>
<td>2.5</td>
<td>Stability of vitamin D in fortified foods</td>
<td>20</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Stability of vitamin D in milk and milk products</td>
<td>21</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Vitamin D stability in none milk products</td>
<td>24</td>
</tr>
<tr>
<td>2.6</td>
<td>Bread baking process</td>
<td>26</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Bread recipes</td>
<td>26</td>
</tr>
<tr>
<td>2.6.2</td>
<td>The leavening agent yeast</td>
<td>26</td>
</tr>
<tr>
<td>2.7</td>
<td>Overview of bread baking process</td>
<td>27</td>
</tr>
<tr>
<td>2.1</td>
<td>Major bread quality indicators</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>EXPERIMENTAL RESEARCHS</td>
<td>31</td>
</tr>
<tr>
<td>3.1</td>
<td>Aims</td>
<td>31</td>
</tr>
<tr>
<td>3.2</td>
<td>Materials and methods</td>
<td>31</td>
</tr>
<tr>
<td>3.3</td>
<td>Preliminary experiments</td>
<td>33</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Moisture content determination of wheat flour</td>
<td>33</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Water absorption and mixing behavior determination</td>
<td>34</td>
</tr>
<tr>
<td>3.4</td>
<td>The bread baking process</td>
<td>34</td>
</tr>
<tr>
<td>3.4.2</td>
<td>Vitamin D(_2) content determination in dough and bread samples</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>(Analytical method)</td>
<td></td>
</tr>
</tbody>
</table>
4 RESULTS ........................................................................................................................................39
  4.1 Dough mixing behavior and water absorption property .........................................................39
  4.2 Bread quality ..........................................................................................................................39
    4.2.1 Specific volume of breads .................................................................................................39
    4.2.2 Result from Image based crumb texture and porous structure .......................................42
  4.3 Vitamin D₂ content in dough and bread samples .................................................................45

5 DISCUSSIONS ...........................................................................................................................48
  5.1 Bread quality and vitamin D₂ stability ..................................................................................48
    5.1.1 Bread quality and impact of baking protocols .................................................................48
  5.2 Effect of mixing and baking parameters on the stability of vitamin D₂ ..............................50
  5.3 Stability of vitamin D₂ in different storage conditions .........................................................51

6 CONCLUSIONS ........................................................................................................................52

7 REFERENCES .............................................................................................................................54
1 INTRODUCTION

Vitamin D is one of the basic vitamins required by our body in order to maintain stable health status, good growth conditions and to have stronger bone structure. Humans can obtain sufficient amount of vitamin D from sunlight exposure. If there is no sufficient level of sunlight exposure vitamin D supply from diet and/or supplementing tablets is very important. In Northern European countries, e.g. in Finland, there is shortage of sunlight in the winter. Therefore, between November and March oral intake of vitamin D either from dietary source or from supplementing tablets is very essential. However, naturally available dietary sources of vitamin D are very limited. Fish and egg yolks contain significant amounts of vitamin D₃ and some wild mushrooms contain vitamin D₂. For optimum supply of vitamin D cholecalciferol (vitamin D₃) and/or ergocalciferol (vitamin D₂) can be utilized in the human body. Since naturally existing dietary sources of vitamin D are quite limited, fortification of food with vitamin D has been considered as valuable strategy to improve people’s vitamin D status (Wagner et al. 2008).

The oral intake of vitamin D in Finland has improved in the past few years (Helldán 2013). This is associated with availability of commercial margarine products and fortified milks. However, the trend of vitamin D intake in Europe depicts that a very low amount of average intake is observed in these region. This is related with the weather condition, personal food choices, and lack of awareness for healthy nutrition style and narrow options of vitamin D containing foods.

At the end of 1600s rickets was identified as critical health issue of children’s. In late 19th century 90% of children from modern cities of North America and Europe were a victim of rickets. In addition to the obvious rickets problem in adequate intake of vitamin D is also reported with deformed pelvis and complicating delivery of babies in young women (Holick 1994).

Unlike most European countries the recommendations of using sun screen and avoiding excessive sun exposure has brought high risk of vitamin D deficiency in different parts of the world. For example the information given about sun-safe idea has caused a rapid increase of vitamin D deficiency problem in Australia (Holick and Chen 2008).

According to International Osteoporosis Foundation (IOF) vitamin D deficiency has been reported with osteoporosis problem, hip fractures, and different skeletal and non-skeletal disquiets. Osteoporosis causes more than 8.9 million fractures annually, resulting in an osteoporotic fracture in every 3 seconds (International Osteoporosis Foundation 2014). The
recommended intake of vitamin D intake depend on varies factors. Initial vitamin D status, individuals BMI and their habitual sunlight exposure considered as basic factors that determine the intake. For individuals having sufficient sunlight exposure lower daily vitamin D intake (< 20 μg/day) is considered as optimum. However, the average intake of vitamin D in individuals with obesity problem, osteoporosis incidence, those having shortage of sunlight exposure and malabsorption problem should be up to 50 μg/day. People from non-European countries and those living in the Middle East and South Asia, or immigrants from such regions living in Europe are also victims of vitamin D deficiency. Measuring and controlling the level of serum 25(OH)D level within these people are very important (Dawson-Hughes et al. 2010).

According to Institute of Medicine (IOM) on 2010 the optimum vitamin D intake is recommended based on the age of different population groups. Based on the assumption of low sunlight exposure 15 μg/day of vitamin D intake is recommended for individuals up to age 70 and 20μg/day is recommended at the age of 71. In addition IOM also raised the adults’ safe upper limit of vitamin D intake from 50 to 100 μg/day. Children’s upper safe limit also rose from 25 to 75 μg/day. Apart the recommendations of optimum intake IOM also suggested the optimum serum 25 (OH) D concentrations to maintain balanced vitamin D status. 50 nmol/l is considered as optimum serum 25 (OH) D concentrations for 97 percent of the population to have healthy bone structure (IOM 2010). Likewise with the intention of securing acceptable vitamin D status of different individuals and to avoid the declining of serum 25(OH)D level the Nordic Nutrition Recommendations has increased the vitamin D daily intake from 5 μg /day to 7.5 μg /day for age group of 2 to 60 years (Pedersen 2008). In addition, Nordic Nutrition Recommendations added that increasing the awareness of the people about the importance of daily vitamin D intake and diversifying commercially available fortified food products can be considered as the possible ways of maintaining balanced vitamin D status of individuals. Since individuals 25(OH)D responses toward supplemental intake of vitamin D is variable, the serum 25(OH)D levels has to be retested after 3 months of intervention for checking the attainment of the desired level of 25(OH)D (Dawson-Hughes et al. 2010).

People who do not consume fish or drink fortified milk suffer the consequence of low vitamin D intake which is caused by their food choice. Trends in individuals’ lifestyle, nutritional habits and physical activity are associated with moderate or severe vitamin D deficiency resulting in health problems.
In different countries food fortification has been practiced as possible method of enriching foods with micro nutrients for a long time. For example in 2010, Finland raised the concentration of vitamin D in margarine and fluid milks to 20 µg/100 g and to 1 µg/100 g respectively. However, the current market situation within Finland implies that commercially available fortified foods are mostly enriched by vitamin D₃. From this we can see that even though the production of vitamin D₂ is more cost wise compared to the production of vitamin D₃ it has got less consideration. Furthermore novel sources of vitamin D₂ will be important options for vegans who do not use animal sourced foods.

The first part of this work is the theoretical review which reviews scientific literatures about factors influencing stability of vitamin D followed by short introduction of the baking process and quality attributes.

The second part gives brief introductions about vitamin D and reviews it’s metabolism in human body. Furthermore basic vitamin D sources and the recommended daily intake from the perspective of different target groups are also included within this part. The experimental section describes basic steps followed to conduct this research specially the bread baking process and measurement of samples vitamin D content for the stability study were included.
2 LITERATURE REVIEW

2.1 Vitamin D

Vitamin D (calciferol) is steroid hormones which contain fat soluble seco-sterols naturally existing in few foods. Vitamin D can also be photosynthesized in the skin of vertebrates under the effect of UVB radiation (Holick 1994; Glerup et al. 2000). It exist in two principal forms; namely as vitamin D$_3$ (cholecalciferol) and Vitamin D$_2$ (ergocalciferol). If there is sufficient level of sunlight exposure by our body, vitamin D$_3$ can be synthesized in the skin. Whereas, vitamin D$_2$ is obtained by UVB irradiation of plant or yeasts (Lips 2006; Bikle 2009). In addition, irradiating the “ergosterol” a 5,7-diene phytosterol in fungi and phyto plankton can yield Vitamin D$_2$ (Holick 2003). The UVB irradiation process first transforms ergosterol into pre-vitamin D$_2$ later the thermal energy around 37$^\circ$C converts pre-vitamin D$_2$ into final vitamin D$_2$. Vitamin D$_2$ has less hypercalcemic effect compared to vitamin D$_3$. For this reason vitamin D$_2$ considered as potential therapeutic agent for cancer and other easily prorogating coetaneous disorders (Slominski et al. 2006).

Ultraviolet conversion of ergocalciferol to vitamin D was started from late 1920s and later this method is licensed for pharmaceutical companies for production of vitamin D$_2$ supplement called Viosterol (Houghton and Vieth 2006). The vitamin D metabolism process in the liver converts vitamin D$_2$ into 25-hydroxyvitamin D [25(OH)D] and the metabolism process in kidney converts to 1,25-dihydroxyvitamin D (Holick 2008).

Vitamin D$_2$

Vitamin D$_2$ and D$_3$ have comparative efficiency of improving vitamin D status of the people. Taking 1250 $\mu$g/g of vitamin D$_2$ twice per week and for consecutive 5 weeks has increased the serum 25(OH)D level of individuals almost by 100% and has improved bone hip and spine mineral density (Holick 2008).

Premature birth, pigmentation of skin, inadequate sunlight exposure, obesity, improper vitamin D absorption and age are some of the factors that increase vitamin D deficiency problem in humans. Severe vitamin D deficiency is reflected by rickets or osteomalacia that associated with poor bone mineralization problem in infants or children. In addition, vitamin D deficiency also cause poor muscle strength and osteoporosis problem as a consequence of improper intestinal calcium absorption (Glerup et al. 2000). However, less severe vitamin D deficiency problem
increase the parathyroid hormone (PTH) which is the leading factor for bone resorption, osteoporosis and bone fracture problem. Optimum level of Serum 1,25(OH)\(_2\)D concentrations promotes fast calcium absorption in the gut. However, individuals with vitamin D deficiency problem suffer from low serum 1,25(OH)\(_2\)D and calcium level which drives high PTH production that lead fast bone mass loss and increased bone resorption (Lips 2006). Optimum intake of vitamin D either from dietary sources or supplementing tablets are very important for maintaining balanced level of body serum 25(OH) D concentration. Study conducted on individuals with poor sunlight exposure has shown that vitamin D intake between 20-25 μg maintained the serum 25(OH) D concentration at optimum level (Glerup et al. 2000). If there is no sufficient sunlight exposure by our body taking 25μg /g of vitamin D per day is highly recommended for maintaining balanced serum 25(OH)D concentration (Holick et al. 2008).

### 2.2 Vitamin D metabolism

Existence of structural difference between vitamin D\(_2\) and D\(_3\) caused their metabolism pathway dissimilarity. Vitamin D\(_2\) has a C\(_{24}\) methyl group and double bond at C22–C23. This structural difference caused the dissimilarity on oxidative process of side chains unlike the oxidative process of vitamin D\(_3\). However, invivo metabolic conversion of vitamins D\(_2\) and D\(_3\) are accomplished with similar hydroxylating enzymes which yield identical 24- and 25-hydroxy final derivatives (Slominski et al. 2006).

If vitamin D is obtained from the dietary source firstly it is fused into chylomicrons before its absorption through the lymphatic system. This accounts 80% of vitamin D main absorption process in small intestine (Intakes 2009). However, vitamin D which obtained from any sources should pass through double hydroxylation process that changes inactive form of vitamin D to active form (Baeke et al. 2010). Over all, both vitamin D\(_2\) and D\(_3\) yield similar final metabolites (Horst, R. L.; Reinhardt 1997). Since pre-vitamin D\(_2\) and D\(_3\) are biologically inactive, the metabolic reaction taking place in the liver and kidney changes them to their active metabolites. Vitamin D\(_2\) metabolic path way yield metabolically active hormone called 1,25-dihydroxyvitamin D\(_2\) \([1, 25(\text{OH})_2 \text{D}_2]\) (Horst, R. L.; Reinhardt 1997). During this conversion firstly the vitamin D is transported to the liver with the help of vitamin D-binding proteins (DBP). Following this enzyme catalyzed hydroxylation process takes place. The hydroxylation
process takes place at C-25 and converts inactive form of vitamin D to active 25-hydroxyvitamin D [25 (OH) D] form. The hydroxylation reaction is catalyzed by microsomal cytochrome P450 enzyme CYP2R1 and/or mitochondrial cytochrome P450 CYP27A1. In addition to these enzymes various cytochrome P450 mixed function oxidase enzymes (CYP2C11, CYP3A4, CYP2D5, and CYP2J3) are also involved in the hydroxylation process. After the first hydroxylation process 25(OH) D enters circulation system. 25(OH)D has shorter half-life which is about 2 weeks and average serum 25(OH)D concentration between 25 nmol/L to 200 nmol/L is considered as optimum level for individuals leading healthy life style (Lehmann and Meurer 2010).

The concentration of 25(OH) D₃ in healthy individuals and with proper vitamin D intake lies between 95.4- 159 nmol/L. However, < 95.4 nmol/L of 25(OH) D₃ indicates insufficient or lack of vitamin D intake. If serum 25(OH) D₃ concentration is <15.9 nmol/L the probability of suffering from severe vitamin D deficiency problem is very high. This can be reflected by osteomalacia and rickets. The second enzyme assisted hydroxylation process of in metabolic pathway takes place in kidney. This hydroxylation process is assisted by enzyme 1-α hydroxylase enzyme (CYP27B1). The hydroxylation process takes place at carbon 1 of the A ring which yields bioactive metabolite calcitriol or 1,25-dihydroxyvitamin D₃ [1,25(OH)₂ D₃] the responsible active metabolite for most biologic function of vitamin D (Baeke et al. 2010; Christakos et al. 2012).

Calcitrol or 1α, 25(OH)₂ D₃ starts its biological function in the kidney. However, it is also transported by DBP to different vitamin D receptors (VDR) a positive tissue destinations to act as genomic and nongenomic ways. The target tissue includes bone, intestine and parathyroid glands. VDR intermediate the calcitriol gene expression process within few hours. Whereas, none genomic calcitriol activity takes place with the help of some particular membrane-bound VDR in few seconds or minutes. Main non genomic effect of calcitriol includes acceleration of phosphoinositide metabolism, rising intracellular calcium and cyclic guanosine mono phosphate (cGMP) concentration, inducing intestinal calcium and phosphate flow and stimulation of protein kinase C activities (Lehmann and Meurer 2010). None binding 1α, 25(OH)₂D has shorter life for about 5 hours. When 25-hydroxylated molecules bind to DBP (transcaliciferin), none hydroxylated molecules (molecules formed at the first stage of vitamin D activation) are stored in adipose tissue (Horst, R. L.; Reinhardt 1997; Nussey, Stephen 2001).
Figure 1: Vitamin D metabolism

The diagram shows the coetaneous conversion of pro-vitamin D$_3$ (7-dehydrocholesterol) to pre-vitamin D$_3$ by UVB irradiation to form vitamin D$_3$ (cholecalciferol). After possible isomerization of pre-vitamin D$_3$ in epidermal basal layer vitamin D$_3$ is attached to vitamin binding proteins (DBP) and transported to the liver. Apart from coetaneous conversion of pro-vitamin D$_3$ vitamin D obtained from natural or fortified foods also transported to the liver by DBP. With action 25-hydroxylases (25-OHase) enzyme vitamin D$_3$ is converted to 25-hydroxycholecalciferol (25(OH) D$_3$). 25(OH) D$_3$ further hydroxylated in the kidney to yield active secosteroid called 1 α, 25(OH)$_2$D$_3$ (calcitriol). This Calcitriol is the final metabolite which acts on different target tissues. PTH positively promotes production of 1 α, 25(OH)$_2$D$_3$ from 25(OH) D$_3$ whereas Ca$^{2+}$, Pi and 1 α, 25(OH)$_2$D$_3$ negatively hinders the production of Calcitriol (Deeb, Trump, and Johnson 2007)

CYP27B1 is an enzyme alternatively called 1-alpha-hydroxylase (1-OHase) which catalyze the formation of active vitamin D or formation of 1, 25-dihydroxyvitamin D[1, 25(OH)$_2$D] (Dardenne et al. 2003). Calcium, parathyroid hormone (PTH), calcitonin, growth hormone (GH) and insulin like growth factors (IGF) positively control the activity of CYP27B1. In contrary,
phosphates, fibroblast growth factors (FGF)-23, klotho, and (1, 25(OH)₂ D₃) negatively control its activity. If serum calcium level is lower parathyroid glands fasten the release of PTH resulting high renal activity of CYP27B1 that produce more (1, 25(OH)₂ D₃). However, if the level of calcium is at optimum condition the PTH released to suppress the CYP27B1 activity. Generally 1, 25(OH)₂ D₃ controls the serum calcium level in three principal ways. These are by minimizing the renal calcium discharge, by enhancing intestinal calcium assimilation and/or enhancing maturity of osteoclast which release calcium from bones (Baeke et al. 2010). As far as vitamin D excretion process is concerned bile is the main organ responsible for the excretion process. The vitamin D metabolic pathway yields water soluble metabolites like calcitriol. This metabolite is excreted by the kidney through the urine systems (Intakes 2009).

Table 1: The associations between osteomalacia, plasma phosphate and 1,25(OH)D₂ D₃ in various clinical disorders (Kanis 1982).

<table>
<thead>
<tr>
<th>State</th>
<th>Plasma phosphate</th>
<th>Plasma 1,25(OH)D₂ D₃</th>
<th>Osteoid mineralization</th>
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<tbody>
<tr>
<td>Vitamin D deficiency</td>
<td>Low</td>
<td>Low</td>
<td>Impaired</td>
</tr>
<tr>
<td>Hyperparathyroidism</td>
<td>High</td>
<td>Low</td>
<td>Normal</td>
</tr>
<tr>
<td>Renal failure</td>
<td>Always high</td>
<td>Absent</td>
<td>Normal (in variable proportion of patients)</td>
</tr>
<tr>
<td>Vitamin D-resistant Rickets</td>
<td>Low</td>
<td>Normal</td>
<td>Impaired</td>
</tr>
<tr>
<td>Vitamin D-dependent rickets, Type I</td>
<td>Low</td>
<td>Low</td>
<td>Impaired</td>
</tr>
<tr>
<td>Vitamin D-dependent rickets, Type II</td>
<td>Low</td>
<td>Normal</td>
<td>Impaired</td>
</tr>
<tr>
<td>Phosphate deprivation</td>
<td>Low</td>
<td>High</td>
<td>Impaired</td>
</tr>
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The physiological effect of vitamin D has remained unclear for long time. The main physiological contribution of vitamin D is supplying calcium and phosphate to gut, bone and kidney. In addition, it induces production of PTH and calcitonin. Since vitamin D is inactive at physiological level it should primarily metabolized to 1,25(OH)D₂ D₃. Therefore individuals with low levels of 1,25(OH)D₂ D₃ have poor calcium absorption capacity. Even though such individuals suffer from chronic renal failure and hyperparathyroidism their health status can be reinstated by providing physiological doses of 1,25(OH)D₂ D₃. The circulation of 1, 25(OH) D₂
D₃ with in human body is much less compared to 25(OH) D₃. Its concentration with in plasma is almost 1000-fold less than 25(OH)D₃ and its physiological production rate ranges between 0.2 and 1 μg per day (Kanis 1982).

2.2.1 Metabolic pathway difference between vitamin D₂ and vitamin D₃

The side chain structural difference between forms of vitamin D (D₂ and D₃) caused slight dissimilarity in their metabolic pathway and on production of their unique biologically active final metabolites (Houghton and Vieth 2006).

The existence of unsaturation on C-22/C-23 combined with presence of methyl group at C-24 brought the difference in the hydroxylation reaction. 25-hydroxylation process is the first hydroxylation reaction for both forms of vitamin D and it is the initial step in vitamin D metabolism. The enzyme responsible for production of this metabolite is located in the liver (Horst, R. L.; Reinhardt 1997).

Following 25-hydroxylation process 25(OH)D pass through further hydroxylation reaction at C24 in the kidney and it yields 24,25(OH)₂D. Likewise 1,25(OH)₂D undergo hydroxylation process on the same C24 to yield 1,24,25(OH)₃D₂. Formation of 1,24,25(OH)₃D₂ promotes deactivation of the vitamin D₂ molecule. In contrary, the corresponding vitamin D₃ metabolite 1,24,25(OH)₃D₃ should pass through further side-chain oxidation process to be biologically deactivated. However, the binding ability of [1, 24, 25(OH)₃D] to VDR is greater than compared from 1,25(OH)₂D₃ by 40%. This enables generation of its biological activity. During metabolism of vitamin D₃ 24-hydroxylation of the side chain occurs before 25-hydroxylation process. This is not the case for vitamin D₂ metabolism pathway. The metabolism process of vitamin D₂ shows that 24-hydroxylation process is also take place in the liver which yields 20–50% 24(OH)D₂. This 24(OH)D₂ further forms 1,24(OH)₂D₂ in the kidney which has less affinity toward VDR compared to 1,25(OH)₂D₃ and 1,24(OH)₂D₃. The affinity of vitamin D metabolites binding themselves to VDR determines their biological action or function. Generally, vitamin D₃ has shown higher bioefficacy compared to vitamin D₂. This is because vitamin D₃ metabolites have higher affinities to hepatic 25-hydroxylase, DBP and VDR. That is why vitamin D₃ is not directly metabolized to 24(OH)D as is vitamin D₂ (Houghton and Vieth 2006).
2.3 Sources of vitamin D & its bioavailability

In healthy lifestyle and optimum atmospheric condition the skin can provide 80 to 100% of vitamin D for our body. However, if individuals live in environment where there is shortage of sunlight dietary or vitamin D supplemental intake will be very important (Glerup et al. 2000). Vitamin D$_3$ is primarily obtained from sunlight and under sufficient sunlight source it fulfills 90% of individuals’ daily vitamin D requirement (Biancuzzo et al. 2013).
Since most foods other than fish and marine products contain very low amount of vitamin D, fortified products can be extra food options for our daily vitamin D. For instance fish contains 3 to 12.5 μg of vitamin D₃ per 3-oz serving which is 50 to 200% of the daily recommended intake. In contrary, unfortified whole milk and cheese can only provide 1% of the daily recommended intake of vitamin D which is insufficient for proper nutrition (Holden 2009). Vitamin D₂ can be naturally obtained from oily fishes and produced from lanolin. In addition, vitamin D₂ can also exist in UVB treated mushrooms and yeasts. These vitamins also available from vitamin D enriched milk, varies dairy based foods, orange juices and cereal products. Apart from foods vitamin D₂ and vitamin D₃ also exist as a supplementing tablets (Biancuzzo et al. 2013). According to different studies vitamin D intake in different population groups is insufficient. The main source of vitamin D i.e. UVB-sunlight does not fully fulfill the whole required amount of the vitamin D for all people of the world. Environmental, cultural and physiological factors restrict UVB from fulfilling the required amount of vitamin D intake. Therefore, other dietary options of vitamin D are important. As means of fulfilling this gap food fortification process has been practiced in different countries. For example, in Canada it is compulsory to fortify milk with 2.5 μg /250mL of vitamin D and 13.25 μg of vitamin D/10g of margarine. In USA fortification of milk is not obligatory. However, breakfast cereals and fruit juices are fortified with 1 - 3.5 μg of vitamin D/ serving. Bioavailability study done on consumption of cheese fortified with 100 μg cholecalciferol/ds for consecutive 8 weeks has shown increment of the serum 25(OH)D level to ≥ 75nmol/L (Wagner et al. 2008). Study done in USA has shown that consumption of fortified orange juice has improved vitamin D status of adults who consumed more than one glass of juice per day. The research result shows that, consecutive 12 weeks ingestion of fruit juices fortified with 25 μg vitamin D₃/236.6 mL juice, has raised the serum 25(OH) D concentrations of adults by 150% (Biancuzzo et al. 2010). Fortification of bread has been practiced since 1930s. Single serving of rye and wheat bread fortified with 10μg of vitamin D₃/100g of bread has increased the serum 25(OH)D concentrations with negligible effect on parathyroid hormone (PTH). Nonetheless, increasing the amount of vitamin D₃ added in the fortification process to 125μg of vitamin D₃/serving and lengthening the intervention time to 1 year has increased serum 25(OH) D concentrations and reduced PTH. A 3 weeks bio-availability study done about effect of wild mushroom consumption has shown serum 25(OH) D concentrations increment which was comparable to taking 10μg vitamin D₂ supplements. However, compared to vitamin D₃ related researches the
studies showing the effectiveness vitamin D\textsubscript{2} in increasing serum 25 (OH) D concentrations are very limited and the studies are still under way (Biancuzzo et al. 2010).

2.4 Vitamin D fortification and the required accuracy in fortification process

People living in subtropical region of our world obtain inadequate amount of vitamin D caused from shortage of sunlight within the region. Therefore, people living within these area require vitamin D-fortified food products which helps them to lead a healthy life style (Ganesan, Brothersen, and McMahon 2011).

Food fortification process has been applied as a basic means of preventing micronutrients deficiency across the world. The effectiveness of the method is largely dependent on the skill and experience of the expertise involved within this field. Before considering food fortification method as a basic strategy of preventing micronutrients deficiency it is important to identify basic problems behind this scene. Inefficient food security program, narrow dietary choices among individuals, lack of basic nutrition knowhow and conditions of locally processed foods are mentioned as the basic causative factors for unhealthy nutrition style. Therefore, identification of the main reason should be done before relying on fortification process for combating nutrition problem. In order to successfully complete the fortification process it is highly recommended to consider the compatibility of food vehicle, fortificant and the process condition. This is because there are variable conditions which limits the efficiency of fortification process. For instance, multiple fortification of with in some food vehicles may result in considerably high cost of production and reduced bioavailability (PAPER 1995).

In food product fortification process delivering sufficient level of micronutrients to the consumers has stayed as the main challenge. Less stability of fortificants is also another problem associated with fortified food products. Working with proper dosage of fortifying ingredients is important for two basic reasons. Firstly, fortification process should sufficiently meet the recommended daily allowance (RDA) of micro nutrients for the targeted population. Secondly, since the amount of fortificant used is very low using extraordinary equipments that able to stream ingredients at a defined amount is highly essential. For example, the daily recommended level of Vitamin D in most European countries is 5 µg /day for adults and 10 µg for elder people between age of 60 to 65 years (Food and Authority 2006). Therefore, to deliver the right amount of micronutrients through fortified products (e.g. vitamin D) as per the daily recommended intake requirement, the fortification process should be done carefully and with high level of
accuracy. Sufficiency of micro nutrients and associated fortificant losses should be carefully controlled in order to reach targeted population. Apart from meeting the required amount of micronutrients in fortified foods keeping the original organoleptic property of the food and maintaining the stability of micronutrients is very important (Ganesan et al. 2011). For example, although most research works reported stability of vitamin D in milk and dairy products during processing and storage, most of fortified milk products available on the market are not inconsistent with their label claim. only 29% of vitamin D fortified milks available on the market had exact concentrations of vitamin D that were within 80 to 120% of the label claim (Holick et al. 1992).

Food processing industries should focus on basic points of fortification process. Stability / resistance toward degradation over time, establishment of processes for uniform fortificant distribution in foods and beverages and reduction of losses during fortification should take the highest priority in the process. Equipments which have capacity of protecting vitamin D degradation caused by UV light and refrigeration are recently available on the market. These equipments are able to maintain even distribution and colloidal stability of vitamin D in food matrix (Nutraceuticals World, 2010).

2.5 Stability of vitamin D in fortified foods

Fat-soluble vitamins and essential polyunsaturated fatty acids (PUFA) are nutrients that are sensitive to heat treatment and oxidation. Therefore, these type of vitamins can be partially lost during heat processing (Hrncirik 2010).

The stability of vitamin D widely studied in milk and dairy products and the result of most studies imply that it has shown stability under different processing and storage conditions (Hanson and Metzger 2010; Renken, S.A., Warthesen 1993). However, heating the pure/dry form of vitamin D in the presence of air at 150 °C resulted in total loss of vitamin D (Chen et al. 1965). Likewise, vitamin D has been described as a vitamin susceptible to oxidation with poor retention property in extruded food storage (Riaz, Asif, and Ali 2009). The studies related with vitamin D fortification in foods other than milk are very limited (Wagner et al. 2008). Thus further studies related to vitamin D stability are required.
2.5.1 Stability of vitamin D in milk and milk products

Stability of vitamin D in low-fat strawberry yogurt, in HTST treated processed milk and in UHT treated-processed chocolate milk was studied. In addition impact of increasing vitamin D concentration on sensory property and effect of storage condition on vitamin D stability within these milk products were also studied. Within this study the content of vitamin D added was increased from 2.5 µg /serving to 6.29µg/serving and a cold–water spreadable vitamin D₃ concentrate is used. The storage times were 21, 60 and 42 days for HTST-processed 2% fat milk, for UHT processed 2% fat chocolate milk and for Low-fat strawberry yogurt respectively. To get representative measurement, vitamin D was measured before startup and during the whole process. The sensory analysis was also conducted on day 14 for HTST-processed 2% fat milk, on day 40 for UHT for processed 2% fat chocolate milk and on day 28 for Low-fat strawberry yogurt. Increasing the vitamin D fortification level to 6.29µg/serving didn’t affect the sensory perception of the consumers and there was no observed vitamin D loss in all these milk products. In addition, vitamin D retention measurement after the storage days has shown that vitamin D was stable enough within these experimental regions (Hanson and Metzger 2010).

Stability of vitamin D₃ and flavor change of fortified Cheddar cheese were tested by adding 5 and 10 µg /serving emulsion form of vitamin D₃ in cheese milk. Stability of vitamin D₃ was 9 months and the flavor and taste of the cheese were equivalently liked by consumers comparative to none fortified cheese. In addition, the flavor of the cheese was unchanged for 9 consecutive months of storage (Ganesan et al. 2011).

Effect of vitamin D addition methods in fortification process was studied in Cheddar cheese production. To investigate this 10µg/ L of vitamin D was added in three different forms. As commercial water-soluble emulsion form of vitamin D (Vitex D), homogenized crystalline liposoluble vitamin D in a portion of cream for cheese milk standardization and water-soluble vitamin D entrapped in multilamellar liposomes (Prolipo-DuoTM) were used. Comparing vitamin D content of trial (fortified cheese) and control cheese, methods of vitamin D addition didn’t significantly alter the protein, fat, moisture and salt content in the trial cheese. Vitamin D best recovered when it was entrained in liposomes (61.5±5.4%) unlike in portion of cream and Vitex D which only recovered (40.5±2.2%) and Vitex D (42.7±1.7%) respectively. Furthermore method of vitamin D addition didn’t have an impact on the stability of the vitamin D for consecutive 3-5 months of ripening. However, following months of ripening liposome-
encapsulated vitamin D has shown poor stability in the experimental cheese samples. Vitamin D recovery in cheese indicates that 39, 57 and 59 % of vitamin D added to cheese milk either lost in whey or destroyed in cheese making of cheese with liposomes, Vitex D or vitamin D in cream, respectively. The highest vitamin D recovery in cheese making process was observed when vitamin D is encapsulated with liposome. This is related with improved protection of vitamin D in liposome encapsulation. In contrary, the poorest recovery of vitamin D in cheese was detected if vitamin is added to milk during homogenization which indicates this method did not give additional protection compared with Vitex D. The acidification caused from lactic acid bacteria fermentation and/or oxygen might have caused instability of vitamin D in cheese making process. Ripening of cheeses in the absence of air (vacuum packaging) and low temperature storage in the absence of light promoted stability of vitamin D in cheeses (Banville, Vuillemard, and Lacroix 2000).

Vitamin D₃ concentrations in milk after pasteurization process (at 72 °C for 16 s) was not affected in fortified cheese milks (Wagner et al. 2008). Likewise, vitamin D in natural or fortified foods has shown resistance to pasteurization temperature and sterilization temperature of 62.8 °C for 30 min and 115.6 °C for 15 min respectively (Krauss, Erb, and Washburn 1933).

Vitamin D₃ has shown resistance to destruction caused by heat in industrially processed fortified Cheddar and low –fat cheeses. Processing cheeses at 232 °C for 5 min and ripening it between 3-8 °C for over 1 year didn’t affect the stability of vitamin D. 91% and 55% of vitamin D₃ is recovered in fortified Cheddar and low-fat cheeses respectively. The remaining amount of vitamin D₃ is entrapped in the whey protein. Vitamin D₃ is also homogenously dispersed all over the cheese pieces. In addition after the fortification process the final output amount, chemical property and flavor of the Cheddar cheese is not altered (Wagner et al. 2008).

"(Leskova 2006)" described vitamin D₂ vulnerability to temperature, oxygen, light, and moisture. Stability of vitamin D₂ in milk processing, packaging and storage conditions were investigated. Three different heat treatments were compared for their impact on vitamin D₂ stability. These were pasteurization at 63°C/30 min, boiling and sterilization at 121 °C/15 min at 15 psi. The vitamin D₂ losses caused as result of heat treatments were insignificant. To analyze effect of storage condition on the stability of the vitamin D₂ milk samples were stored in plastic and glass bottles under refrigeration temperature for consecutive 7 days. Vitamin D₂ measurement after the
refrigeration storage has shown insignificant loss of vitamin D₂ in both packaging types. However, within the same storage condition the polyethylene bag has shown significant loss of vitamin D₂ from 14.9µg to 13.7 µg which is related with vitamin D₂ sorption in polyethylene pouches. To investigate effect of packaging under the influence of light milk samples were stored under the influences of three different light intensities (1485, 2970 and 4455 lux). The result revealed that glass packaging was effective enough to maintain the vitamin D₂ stability unlike a polyethylene pouch which caused significant loss of vitamin D₂ under the same storage condition and similar storage days (Kaushik, Sachdeva, and Arora 2014).

Research done on vitamin D₃ fortification practice in cheese manufacturing has shown 25 to 30% of vitamin D₃ loses when the cheese milk heated at 232°C for 5 minutes. On the same research work effect of storage condition on vitamin D₃ loss is studied after storing the cheese for more than 9 months between 4 - 6°C and 21 - 29°C. The finding of the work confirmed that there was no observable loss of vitamin D during these storage days (Upreti, Mistry, and Warthesen 2002).

Vitamin D₃ Stability test in cheddar cheese processing has shown 90% of vitamin D₃ retention (Ganesan et al. 2011). However, in another research work only 40 to 50% of Vitamin D₃ retained in cheddar cheese after the cheese making process (Banville et al. 2000). The observed difference of vitamin D retention between these two researches assumed to be related with forms of vitamin D₃ used in both fortification process (Ganesan et al. 2011).

"according to (King and Min 2002)" exposing milk to light during processing and storage promotes its oxidization to inactive form 5, 6-epoxide by riboflavin-photosensitized singlet oxygen.

The preparation of vitamin D₂ depicts that it has lower bioactivity that makes it sensitive to lose. In particular exposing the crystalline vitamin D₂ powder to varying temperatures, humidity and in different storage vessels fasten it’s lose. In addition poor stability and less purity in vitamin D₂ crystalline powder also promote greater risk toward toxicity compared to the associated vitamin D₃ metabolites (Houghton and Vieth 2006).

Uneven distribution of vitamin D in fortified food was one of the important issues in fortification process. Usually it is very essential to spread the fortificants throughout the food matrix/surface. Taking large amount of cheese mass with improper distribution of vitamin D₃ would create favorable conditions for toxicity (Wagner et al. 2008).
2.5.2 Vitamin D stability in none milk products

Vitamin D₃ stability in fortified margarine, egg and bread was studied in different household level cooking methods. Heating eggs and margarine product in oven for 40 min has shown 39-45% retention of vitamin D compounds. In contrary, frying method better retained the vitamin D₃ 82–84% within the same food. Likewise, boiling eggs also has shown comparative retention of vitamin D₃ 86–88% like frying method. Retention of vitamin D₃ and D₂ has been reported to be better in wheat bread compared to rye bread. After adding the same amount of vitamin D₃ initially in both bread types 69 and 85% is retained in rye and wheat bread respectively. Similarly 73% and 89% of vitamin D₂ is retained in rye and wheat bread respectively. Comparing retention of vitamin D in rye bread and wheat bread which are baked at identical temperature and time (at 200°C for 30 min) rye bread has shown poor retention. Analyzing the final vitamin D measurement in both bread types’ authors concluded that bread temperature cannot be the reason for lower retention of vitamin D in rye bread. This is because the final rye bread temperature after baking was significantly lower compared to the wheat bread (Jakobsen and Knuthsen 2014). The lower pH record in rye bread might caused the lower retention due to the acidic isomerization of vitamin D to isotachysterol (Jin et al. 2004). The observed retention level of vitamin D in egg (≈ 40%) after oven cooking indicates that the content of vitamin D in eggs might bind to protein or esters (non-specific storage form of vitamin D) that couldn’t defend the vitamin D from heat induced destruction. Even though cooking method plays main role in vitamin D loss, the loss is also dependent on type of the food and method of cooking process (Jakobsen and Knuthsen 2014).

Vitamin D₃ stability study together with 12 weeks of bioavailability investigation is carried out on juices fortified with 25 μg /240 mL of vitamin D₃. The investigation revealed that vitamin D₃ was stable at 4°C of storage temperature for consecutive 30 days and it was effective enough to improve examinees vitamin D status (Tangpricha et.al, 2003).

Vitamins D₂ and D₃ also exists in white to yellowish water insoluble crystalline powders. They are soluble in 95% ethanol, acetone, fats, and oils. They are also speedily soluble in chloroform and ether. Vitamin D₂ and D₃ in solution form exhibit reversible thermal isomerization creating equilibrium fusion containing their equivalent pre-vitamins. Stability of vitamin D in fats and oils is similar to the stability of the fat itself. If vitamin D is not confined within food matrix it is vulnerable to oxygen and light induced destruction. Exposure of vitamin D containing thin films
to heated air and/or diffusion of vitamin D containing alcoholic solution into aqueous phase in the presence of oxygen provoke its destruction. At alkaline condition vitamin D is stable. However, in acidic environment (even at mild acidity) it isomerise to 5,6-trans and isotachysterol which don’t have any potential of curing/preventing rickets (poor antirachitic activity) (George F.M. Ball 2005).

Inclusion of vitamin D in fats which have oxidization property promotes its destruction. According to (Bender 1978) methods of food processing, cooking and storage conditions generally do not affect the activity of vitamin D in foods. Vitamin D has shown resistance to smoking of fish, and sterilization of milk, and spray drying of eggs.

Bread is a common and principal diet across the world. For this reason it has drawn attention as fortification vehicle. Stability and addition level of Cholecalciferol (Vitamin D₃) stability, its dispersion property in bread and its bioavailability from fortified wheat and rye bread has been previously studied. In the research rye and wheat bread is fortified with 12mg/100 g of Cholecalciferol and its stability after bread baking is investigated. The Cholecalciferol stability measurement shows that 79 to 109% of cholecalciferol was recovered after the baking process which confirms its stability in bread baking process. In addition to these the study also confirmed that Cholecalciferol is evenly distributed throughout the dough matrix and in the final bread texture (Natri et al. 2006).

Even though literatures related with vitamin D₂ fortification process and its stability in food production are quite limited some literatures mentioned vitamin D₂ crystalline powder has shown its lower bioactivity property and poor stability. If vitamin D₂ crystalline powder is exposed to unstable temperature, humidity or to different storage containers it promotes the instability of vitamin D₂ (Houghton and Vieth 2006).

However, source of vitamin D₂ that was used under this research work was obtained from yeast *Saccharomyces cerevisiae*. The yeast is treated with UV light to photochemically convert the endogenous ergosterol component in the yeast to final vitamin D₂ alternatively called ergocalciferol (Stevenson 2012). The stability of vitamin D₂ sourced from the yeast *Saccharomyces cerevisiae* in bread baking process was not well studied.
2.6 Bread baking process

2.6.1 Bread recipes

Wheat flour constituting 50 to 60 % of (formula weight) bread recipes categorized as a major ingredient while other raw materials such as leavening agents (yeast or different bacterial strains, chemical leavenings), dairy materials, egg, and starch are grouped under minor ingredients. They are usually added 5 to 10% (formula weight). Micro ingredients are added up to 5% (usually to nearly 0.1%) and function as oxidation enhancer and fortification ingredients. They improve the baking activity and nutritional quality of baked product (Gorton 2009).

Salt is one of characterizing ingredient mainly used as a flavor enhancer in bread baking process and addition of salt (NaCl) between 1.5–2% maintains better bread volume (Biotechnology 2013; S P Cauvain 2003). In bread baking process sugar is used for substrate fermentation (G.Rubernthaler et. al 1963). Inclusion of oil mixture and solid fat in bread formula plays important role in gas retention. Effect of adding palm oil/Palm Sterin based shortening in white bread recipes has shown to improve the bread quality such as improved loaf volume, attractive texture and golden brown crust color (Artan 2010). Water will have impact in overall dough rheological property and formation of a continuous viscoelastic gluten network (Mastromatteo et al. 2013). The Farinograph, empirical mixing machine is used to determine water absorption property of wheat flour, optimum time for proper dough development and for the measurement of dough stability (Létang, Piau, and Verdier 1999). The amount of water absorption on the Farinograph graph is represented by the volume of water added in order to the dough reach 500 Brabender unit after the mixing process (Biotechnology 2013).

2.6.2 The leavening agent yeast

In bread baking process, the yeast (Saccharomyces cerevisae) is used as a leavening agent to promote generation of gases which will create proofing of the dough and oven spring. Yeast is the solely leavening agent used in bread baking unlike other chemical leavening agents used in many other bakery products. Yeast basically converts sugars formed from enzymatic hydrolysis of wheat starch or added sugars) to carbon dioxide gas and alcohol. The created alcohol will later be evaporated during the oven baking processes. The most common yeast types are fresh compressed yeast, yeast cake or crumbled yeast, liquid cream yeast, or active dry yeast (Hui 2006). Apart servings as a leavening agent yeast also serves as nutritional ingredient in different
foods. Nutritional yeasts is wealthy in containing significant nutrients such as the B vitamins, chromium, sixteen amino acids, more than fourteen minerals, and seventeen different vitamins (except vitamins A, C and E). In addition yeast is also prosperous in phosphorus, in protein and it is high energy food (Bruno 2009).

"(Panel 2014) " described baker yeast from”Lallemand SAS” efficiently enriched vitamin D_2 content of bread. This nutritional yeast is produced by treating the baker’s yeast (Saccharomyces cerevisiae) under UV irradiation process at 254 nm. The irradiation process transformed the ergosterol in the yeast to vitamin D_2 (ergocalciferol). The yeast also maintained its natural gassing power after UV treatment and it can be used in yeast-leavened bread, rolls, and fine pastry. Even though UV treatment promote baker’s yeast gene mutation, low number of viable mutant yeast and the final intended application of the novel yeast would not allow mutants to prevail in the final fortified product. This vitamin D enriched novel yeast can serve as naturally available food option for vegan diet.

2.7 Overview of bread baking process

Dough make up process comprises sequential steps of weighing intended ingredients, mixing of ingredients, fermentation process, dough dividing, molding and panning of proofed dough pieces for final baking process (Figure 3). During straight dough make up process all ingredients are mixed together and the dough is rested for short time after mixing. Mixing unit operation is the first unit operation that mixes desired level of major ingredients like wheat flour, yeast, salt and other required micro ingredients. In addition to these, the mixing process promotes incorporation of air bubbles and development of gluten network (structure).

When gluten structure fully and well develops it promotes further expansion of bread structure which allows full incorporation of gases liberated from the fermentation process. The strength of mixing or the mixing intensity required for optimum dough development depends on different factors. Such as, on mixing speed, mixer arrangements and wheat flour properties. Maintaining dough temperature between 30 to 32°C is very crucial requirement to achieve desired chemical and enzymatic reaction in the dough. However, higher temperature rise caused by high mixing intensity should be controlled. This is because, it weakness the dough structure and forms sticky dough complex (Hui and Corke 2006).

Development of optimum dough mass is achieved by maintaining the dough mixing intensity above its critical mixing speed and by controlling the amount of energy input (Ktenioudaki,
Butler, and Gallagher 2010). However, the relation between optimum dough development with respect to dough aeration and dough rheology is much unknown (Chin and Campbell 2005).

After completion of the mixing process dough resting is important for further rheological modifications and improvement of the dough pieces (Hui and Corke 2006; S P Cauvain 2003). Following the fermentation process the dough is divided into pieces and rounded before forwarding to the intermediate proving stage. Dough dividing and first molding stages are the consecutive steps that give desired weight and uniform shape to the dough respectively. The intermediate proving (for 4 - 10 minutes) further promotes dough relaxation before the final molding process. After intermediate proving, low pressure is applied on the dough pieces which

Fig.3: summarized explanation of commercial bread Baking process (Britannica 2014)
give its final shape. Following this the final proving between 27 °C to 40°C and at relative humidity of 85% for 41 to 60 minutes promote the yeast activity for further dough pieces expansion (Hui and Corke 2006; Karen Siffring & B.L.Bruinsma 1993).

Oven baking is the final stage of bread making process just before cooling and storage (S P Cauvain 2003). For most bread types the baking temperature and time combination varies from 200 to 240°C for 10 to 30 min respectively (Das, Raychaudhuri, and Chakraborty 2012). Oven spring, coagulation of protein, gelatinization of starches, crust development and crust browning are some of the main phenomenon happening during the oven baking process (Hui and Corke 2006). During the oven baking process closely monitoring the temperature and time combination is very important (Hui 2006).

### 2.8 Major bread quality indicators

Loaf volume is one of the main bread quality indicators which indicated the level of gases produced from the fermentation process and it indicates the amount of gases retained within the bread structure after the whole baking process. Apart from this loaf volume can also indicate the quality of the flour protein and the processing condition effectiveness toward developing well structured gluten network. It can also tell the stability of the whole baking process (Hui and Corke 2006).

Calculated value of the specific volumes also indicates the efficacy of dough fermentation process and level of gluten network plasticity caused from prolonged fermentation time. Prolonged fermentation time cause poor gluten plasticity which contributes to low loaf volume. Apart from these, specific loaves volume also tells how much the dough kneading process was optimum. Extensive dough kneading process result poor loaves volume (Jan Gliński, Józef Horabik 2011s).

Oven spring is another quality indicator which indicates bread height rise during oven baking stage. It compares the height difference between the standard proving stage and after baking process. Monitoring the optimum level of oven spring is very important. Less control over oven spring stage cause easy disassembling of crust structure from the top surface of the bread. The level of damaged starch in the flour is another important factor affecting the yeast activity and crust color formation. If the amount of damaged starch in the flour is lower, α-Amylase enzyme will only have access to liberate sugars for the yeast activity. This implies that low or negligible amount of sugars will be left for Millard reaction which is the solely source for crust color...
formation. However, if there are high level of damaged starch or extra added sugar as milk powder or as other sugar recipes promote high $\alpha$-Amylase level which drives intense crust color formation (Hui and Corke 2006).

Gas bubbles formed from the mixing process have major role of forming good bread texture. If the gas bubbles are not kept intact during the subsequent baking processes, the possibility of forming light gray crumb color and firmer texture is very higher. Hole structure formation on the crumb surface is considered as one of the quality indicator which tells the dough handling effectiveness toward maintaining the whole dough structure after curling process. It also shows the level of dough weakness caused in relation to added recipes and with processing conditions (Hui and Corke 2006).
3 EXPERIMENTAL RESEARCHES

3.1 Aims
The previous studies associated with vitamin D has been focusing on the bio-availability of vitamin D₃ and its stability. Furthermore the finding of most studies depicts that vitamin D₂ is less bio-available compared to vitamin D₃ (cholicalciferol). “Houghton & Vieth on 2006” presented a conclusion stating vitamin D₂ is not efficient to raise serum 25 (OH) D levels compared to vitamin D₃. Moreover they presented a negative connotation stating ergocalciferol should not be considered as appropriate nutrient for supplementation or as food fortificant. The stability studies of vitamin D₂ have been quite limited. In addition, food processing conditions that affect stability and nature of vitamin D are not well established.
Therefore, this research had the following basic objectives;

1. To investigate the stability of vitamin D₂ in white fortified wheat bread and to analyze vitamin D loss associated with the fortification process.
2. To study impact of process conditions on the stability of vitamin D₂. With the hypothesis of vitamin D susceptibility to oxygen and temperature, effect of mixing condition and baking temperature was especially examined.
3. To study impact of storage conditions on vitamin D₂ stability in the bread was investigated.
4. To study impact of vitamin D₂ containing bio-enriched yeast on bread quality

3.2 Materials and methods

3.2.1 Ingredients used for the experiment
The major ingredient wheat flour was Emännän Puolikarkea Vehnäjauho from Myllyn Paras Oy. The flour extraction rate, moisture content, ash content and protein content of the flour were 77%, 11.55%, 0.65% and 12.5% respectively. The bread formula designed for this experiment was based on flour weight (Healea et al. 2006) and the final targeted dough mass was nearly 2.5kg. The leavening agent used for the baking process was a yeast cream obtained from LALLEMAND Inc. The relative percentage and the amount of recipes added are listed on table 1.
Table 1: Recipes for the experimental bread

<table>
<thead>
<tr>
<th>List of Recipes</th>
<th>Per flour weight (%)</th>
<th>weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat flour</td>
<td>100%</td>
<td>1470</td>
</tr>
<tr>
<td>Water</td>
<td>59.33%</td>
<td>872.2</td>
</tr>
<tr>
<td>Yeast cream</td>
<td>4.8%</td>
<td>42.35</td>
</tr>
<tr>
<td>Salt</td>
<td>29.4%</td>
<td>29.4</td>
</tr>
<tr>
<td>Sugar</td>
<td>1.2%</td>
<td>17.64</td>
</tr>
<tr>
<td>Rape seed oil</td>
<td>5%</td>
<td>73.5</td>
</tr>
<tr>
<td>Vitamin D2 yeast</td>
<td>0.05%</td>
<td>1.3645</td>
</tr>
<tr>
<td>Total</td>
<td>199.73</td>
<td>2505.14</td>
</tr>
</tbody>
</table>

The rest of ingredients used for the baking process were obtained from commercially available products.

The amount of recipes shown above (in table 1) was calculated based on the formula given below (Healea et al. 2006)

\[
\text{(amount of total flour)} = \frac{\text{intended dough weight}}{\text{total percentages} / \text{total of flour percent (100)}} \quad \ldots \quad (1)
\]

3.2.2 Fortification ingredient

Ingredient used as a fortification agent was novel yeast obtained from LALLEMAND Inc. This novel yeast is enriched with Vitamin D2 after necessary UVB irradiation. Even though this yeast has gassing power it is not used as the primary baker's yeast. Rather it is only used for vitamin D enrichment purpose. The yeast is enriched with 432.5 µg of vitamin D/g of yeast. After performing necessary calculation 1.3645g of novel yeast was added in to the targeted dough mass (2.5kg) with the intention of providing 25 µg of vitamin D₂ /90 g of bread. The weighed amount of yeast was dissolved in luke warm water and then mixed with the dough.
3.3 Preliminary experiments

In order to precede with the baking process the wheat flour moisture content, water absorption and mixing behavior during dough formation was analyzed as preliminary experiment. Detail of the preliminary experiments performed is described as the following.

3.3.1 Moisture content determination of wheat flour

Determining the moisture content in wheat flour is important analysis for understanding the shelf life instability caused from enzymatic and microbial activity. Apart from these knowing the level of moisture content in the flour also helps to control the dough consistency obtained after the mixing process.

Oven drying method was used to determine the moisture content in the wheat flour (Matttila P, Piironen V 2001). The analysis compares the moisture difference before and after oven drying. Firstly, two aluminum crucibles with opened lid were placed in an oven for 1h at 130 °C. After 1 hour of oven drying the crucibles were kept in desiccators for 60 minutes. Following this, the empty crucibles were weighed with closed lids. 5g of wheat flour sample was placed in tarred crucibles and combined weight of sample and crucibles was registered. Then the samples were dried at 130°C for 6 hours by letting the lids stay opened. Closing back the lids the crucibles were kept in desiccators for 60 minutes for cooling. After sufficient cooling the dried crucibles were weighed and the values were inserted in the following formula (2) for further calculations. The formula indicated below was adopted from (Norimi et al. 2012) and customized for this research purpose.

\[
\text{Moisture Content (\%) } = \frac{M_{\text{dry}} - M_{\text{wet}}}{M_{\text{wet}}} \times 100
\]  

(2)

Where,

- \( M_{\text{wet}} \) is the weight of the crucible which contain wheat flour sample before drying
- \( M_{\text{dry}} \) is the weight of crucible which contain wheat flour sample after drying

After analyzing three sample replications the moisture content in the wheat flour was determined as 11.5%.
3.3.2 Water absorption and mixing behavior determination

The Farinograph is used for determining quality and behavior of the wheat flour in the baking process. This helps to get consistent dough characteristics. This method is applied based on method 54-21 of (AACC 1983) and was performed on 300 g of flour at 14% moisture base. The Farinograph determines the water absorption behavior of the wheat flour and mixing behavior of the dough. It also measures dough development time (min), dough stability (min) and degree of dough softness. The mixing behavior is recorded throughout the mixing process. For the test calculated amount of wheat flour (291.7g) was added to the mixing bowl. Following this, water from the burette was continuously added to the wheat flour containing 11.55% of moisture content and mixed continuously. When the dough consistency reached at 500FU addition of water was terminated. The value of 500 FU (Farinograph units alternatively called as Brabender units (BU)) shows the arrival of the average dough firmness. To get representative water absorption value and dough characteristics, three sample replications were done. Finally, based on the recorded data and interpretation of the Farinograph curve, the actual dough for the whole baking process was prepared with high energy mixing machine.

3.4 The bread baking process

Using formula (1) indicated above the bread recipes was designed and the following amounts of ingredients were measured according to the table 1. Baking protocols for varying mixing and baking schemes are presented on figure 4.

The amount of water added corresponded to the farinograph absorption value of 500 BU. High energy mixing device, Diosna SP160 spiral mixer was used for mixing the dough. All measured ingredients were poured to this machine to achieve 2.5kg of target dough mass. The first baking protocol was standard baking process. It had mixing time for 2+4 minutes. The second and third baking protocols had 2+2 minutes (short mixing time) and 2+6 (long mixing time) respectively.

After performing all mixing protocols, dough samples were taken for vitamin D$_2$ analysis. Following the mixing process the dough was allowed for 18 minutes of intermediate resting. Then the dough was sheeted, molded by hand and cut in 300g pieces. Each dough pieces was placed in baking pan having dimension of (15 cm X 6.5 cm X 9 cm) and proved for 55 minutes at 35°C and RH of 75%. Proving parameters of all baking arrangements were similar (35°C and 75% of RH for 55 minutes). Optimally proved dough pieces were baked in the stone oven.
Fig. 4: Baking parameters of experimental protocols

To investigate the effect of baking temperature and time combination on the stability of vitamin D2, three different baking conditions were utilized as shown on Figure 4.

### 3.4.1 Specific Volume of Bread

The AACC method 10-05.01 (2000) is the procedure used to obtain the specific volume of bread, through the division of volume (cc) by weight (g). This method has been implemented to
measure bread volume (Visvanathan R & Ranasalva N 2014). Recorded weight value of displaced rapeseeds immediately after removal from oven conveys the loaf volume. The process involves, placing of loaves in a container of a certain volume in which rapeseeds were run until the container was filled. The loaf volume was obtained from the volume of seeds displaced. Finally using the following formula (5) specific volume of breads (SVB) was calculated.

\[
S. \ V. \ B = \frac{\text{Loaf volume (cc)}}{\text{Loaf weight (g)}}
\]

3.4.2 **Image based crumb texture analysis**

Crumb textures of loaves obtained from all experimental designs were analyzed by capturing their crumb image. After oven baking and 1 hour of cooling process the 300g loaves were sliced into 6 parts and pictures were taken by *Sony Cyber-shot digital camera* which has 14.1 megapixels resolution capacity. After taking clear picture of the slices the images were analyzed using *ImageJ* application software (Abràmoff et al. 2004). Images analyses were performed by comparing the contrast difference existing between the pore structure and other parts of the crumb surface. Firstly, the image taken was changed to gray scale and the pixel sizes were calibrated to known length of measurements. Following resizing of the rectangular pictures of the slices; the pore structure distribution, radial pore size evaluation and total pore distribution throughout the crumb surface area were analyzed using ImageJ software.

3.4.3 **Vitamin D2 content determination in dough and bread samples**

*(Analytical method)*

Vitamin D$_2$ content in the dough and bread samples were measured to see how much vitamin D$_2$ was retained after the mixing process, in baking process and after storage days. To analyze these initially (before starting the mixing process) 0.278µg/g (11.1IU/g) of vitamin D$_2$ was added as ingredient through the novel yeast. Vitamin D$_2$ concentration after proving stage was also checked to see the change in vitamin D$_2$ concentration because of yeast fermentation. To investigate this, standard baking procedure (SBP) was followed and the vitamin D$_2$ change was measured. As mentioned above the whole bread baking process was designed to have 3 basic mixing and baking temperature arrangements. Vitamin D2 quantification was done after performing sampling from all experimental arrangements. The first sampling was done from the
standard mixing time (2+4) minutes. The second and last samples from the mixing arrangements were taken from 2+2 minutes and 2+6 minutes mixing conditions respectively. In addition, samples from each baking arrangements were also taken for vitamin D₂ analysis. Samples were taken from; standard baking (220°C, 20 minutes), low temperature baking (175 °C, 35 minutes) and high temperature baking process (245 °C, 15 minutes). The level of vitamin D₂ retention between controlled atmospheric storage of (23.4 °C and 35% RH) and freeze storage at (-18°C) were also measured. The samples were analyzed based on analytical method of vitamin D₂ determination at National Food Institute in Denmark as described below. The vitamin D₂ retention level associated with the protocols was statistically interpreted using JMP® Pro 11.0.0 software (SAS Institute Inc. 2013, USA).

3.4.3.1 Method for vitamin D₂ determination

Vitamin D₂ determination was run by couple HPLC technique (Waters, Milford, MA, USA). Semi-preparative HPLC was equipped with 600 controller and pump, a refrigerated 717Plus Auto sampler and a 2487 absorbance identifier. The system was equipped with a silica column (Luna, Si 60, 3 mm, 150×4.6 mm, Phenomenex, Torrance, CA, USA). The system for the analytical HPLC was equipped with a 515 HPLC pump, a 717Plus Autosampler, a 2996 Photodiode Array Detector (DAD) and a 2487 Absorbance Detector in combination with two VYDAC201TP54 (5µm, 250×4.6 ID, 5µm, 250×4.6 ID, Phenomenex, Torrance, CA, USA). Waters Empower 3 (Waters, Milford, MA, USA) was used for interpretation and processing of chromatograms and data.

Analytical method of vitamin D₂ determination earlier has been followed for determination of vitamin D in meat (Jakobsen et al. 2004) and for vitamin D₂ in the mushrooms (Kristensen, Rosenqvist, and Jakobsen 2012). Before starting vitamin D₂ determination, the bread and dough samples were homogenized in a food processor (Tecator 1094, Homogenizer, Foss Tecator, Höganäs, Sweden) for 1 min. Following this 10 g of homogenized bread and dough samples were taken for analysis and vitamin D₃ was added as an internal standard. The samples were extracted by alkaline saponification, liquid-liquid extracted by petroleum ether:dietylether (50:50) followed by clean-up by silica solid phase. The eluent for the preparative HPLC system was 2-propanol: methyltert-butyl-ether: cyclohexan: n-heptan (0.7:2:48.65:48.65). The flow rate used was 1.2 ml min. The retention time for vitamin D₂ was approximately 9 min; sample
collection was done between 0.7 min gap before and after the extraction of vitamin D₂. The retention time for ergosterol was approximately 14 min, which is a baseline for separation of vitamin D₂. For separation the analytical system was run with acetonitrile:methanol (80:20) as mobile phase, whereas detection was performed by photodiode array detector (220-320 nm) and quantification at 265 nm. Retention time was 20.3 and 22.8 for vitamin D₂ and vitamin D₃, respectively.
4 RESULTS

4.1 Dough mixing behavior and water absorption property
The water absorption behavior of the wheat flour was evaluated from the Farinograph curve. The wheat flour had 59.5% of water absorption. As indicated on fig. 2 the flour had development time of 2.4 minutes and stability for 6.13 minutes.

![Farinograph curve showing dough mixing behavior](image)

During the whole mixing process the appearance of the curves has shown a wide broad band width and slightly falling curves. These suggested that the dough was strong enough to withstand high stress applied from mechanical mixer.

4.2 Bread quality

4.2.1 Specific volume of breads
The mean specific volume measurement from the rape seed measurement is shown in table 5. The tables also contain breads mean weight and density with their standard deviations. Based on the statistical analysis (one way ANOVA) there was no significant difference in breads weight, density and specific volume were found between all baking protocols (P> 0.05). Fig. 3 shows the overall loaf volume and structure obtained from the SBP (standard baking processes) protocol.
Fig.3: Image of loaf obtained from standard baking arrangements. The line underneath bread represents 2.54cm. Details of the analysis indicated in Table 5.

Table 5: The following data represents mean values of weight, volume, density and specific volume (cm$^3$ g$^{-1}$) of three sample loaves (n=3) ± SD from each baking protocols.

<table>
<thead>
<tr>
<th>Bread samples taken from</th>
<th>Weight (g)</th>
<th>Volume (cm$^3$)</th>
<th>Density($\rho$)</th>
<th>Specific volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard baking process (SBP)</td>
<td>270±4,36</td>
<td>655±25,24</td>
<td>0,41±0,05</td>
<td>2,43±0,07</td>
</tr>
<tr>
<td>2+2 min mixing process (LEM)</td>
<td>273±5,29</td>
<td>654±30,19</td>
<td>0,42±0,05</td>
<td>2,39±0,08</td>
</tr>
<tr>
<td>2+6 min mixing process (HEM)</td>
<td>268,67±1,15</td>
<td>654,5±22,29</td>
<td>0,41±0,05</td>
<td>2,44±0,08</td>
</tr>
<tr>
<td>Low baking temperature (LBT)</td>
<td>270±10,83</td>
<td>656,7±32,66</td>
<td>0,41±0,10</td>
<td>2,43±0,16</td>
</tr>
<tr>
<td>High baking temperature (HBT)</td>
<td>279,33±1,53</td>
<td>658±11,14</td>
<td>0,42±0,03</td>
<td>2,36±0,04</td>
</tr>
</tbody>
</table>

*Values are the mean ± SD. There were no statistically significant differences between all baking protocols (P > 0.05).
Fig. 4: Scanned images of breads from all baking arrangements. These images are used for the **Image J** analysis. The line underneath bread represents 2.54cm. Details of the analysis indicated in Table 6. SBP=standard baking process, LEM=Low energy mixing, HEM=High energy mixing, LBT=Low baking temperature; HBT=High baking temperature.
4.2.2 Result from Image based crumb texture and porous structure

The following image (fig. 5) shows the analyzed breads texture from each baking protocols.

Fig. 5: Explanation of how the software ImageJ applies contrast difference in the scanned image to analyze the corner of pores and determines the regions representing voids before measuring their areas. SBP=standard baking process, LEM=Low energy mixing, HEM=High energy mixing, LBT=Low baking temperature; HBT=High baking temperature.
Following the adjustment of the threshold of the pictures, average pore size and percentage of pored distribution across the surface of the slices were calculated and results of the analysis from the Image J software is indicated on fig. 5 and in table 6. Median pore diameter distribution from each baking arrangements are also indicated on the histograms (fig.6).

**Fig. 6:** Slices porous structure distributions from all baking arrangements
The result calculated in table 6 shows the average pore size associated with each baking protocol. It is important to note that these measurements are restricted to rectangular bread samples having approximate dimension of 70mm width and height up to 90mm. Analyzed texture were taken from areas that didn’t have large pores and taken away from the edge.

Table 6: Characterization of porous structure distribution on bread slices baked under different baking protocols. Values are presented as mean ± standard error of the mean (n=3).

<table>
<thead>
<tr>
<th></th>
<th>Average pore size (xc) (µm)</th>
<th>Pore distributions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard baking process</td>
<td>29.09 ±4.09 (^{a})</td>
<td>174.97±5.03 (^{a})</td>
</tr>
<tr>
<td>(220°C for 20 minutes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low energy mixing</td>
<td>32.87 ± 3.13 (^{a})</td>
<td>175.96±1.18 (^{a})</td>
</tr>
<tr>
<td>(2+2 minutes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High energy mixing</td>
<td>60.75±2.55 (^{b})</td>
<td>165.57±11.83 (^{a})</td>
</tr>
<tr>
<td>(2+6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low baking temperature</td>
<td>58.35±0.35 (^{b})</td>
<td>167.93±1,73 (^{a})</td>
</tr>
<tr>
<td>(175 °C, 35 minutes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High baking temperature</td>
<td>58.51±4.91 (^{b})</td>
<td>169.53±5.53 (^{a})</td>
</tr>
<tr>
<td>(245 °C, 15 Minutes)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a,b}\) Mean within columns with different superscript are significantly different (p<0.0001\(*\)) from each other.
Depending on the one way ANOVA statistical analysis some of the baking protocols have shown significant difference of mean pore size distributions (P < 0.001***). Performing Tukey-Kramer mean comparison technique the mean porosity differences between slices of high energy mixing (HEM), LBT (low baking temperature) and HBT (high baking temperature) were insignificant. However, porosity of slices from low energy mixing (LEM) and standard baking process (SBP) were significantly different from the rest of protocols. There were no significant difference of average pore size between slices of LEM and SBP. The mean comparison process revealed that the highest pore sizes are recorded from the HEM baking protocol and the smaller pore diameter from SBP. This result also can be observed from fig.6 and in table 6.

Considering the relative distributions of porous structure over the crumb surface it has shown insignificant difference between baking protocols (P > 0.0001). Based on one way ANOVA statistical analysis the mean values of pore distribution from each baking protocol were compared and the highest distribution recorded was from LEM (175.96%). Even though pore distribution difference was insignificant the list value is recorded from HEM (165.57%). From the raw data of pore diameter distributions related with fig.6, the pores of breads baked in HBT process have shown the higher frequency of small diameter pores (between 0-20 µm) compared to other baking arrangements. Comparable frequencies of pores having size of between 0-20 µm were observed from SBP and LBT baking process. However, breads baked with HEM process have shown the lower number of small pores (< 400) which had pore diameter of between 0-20 µm.

4.3 Vitamin D2 content in dough and bread samples

Variations in mixing time did not change content of vitamin D and the vitamin D2 content difference between different mixing intensities (LEM, HEM and SBP-Mixing) were insignificant (P > 0.05).

The statistical analysis of vitamin D2 retention between different baking and temperature combinations again have shown insignificant differences between designed protocols (P > 0.05).
Fig. 7: vitamin D$_2$ concentration after mixing and baking process based on different mixing types and baking conditions. Superscripts with letter ‘a’ show the standard deviations.

The mean value of vitamin D$_2$ concentrations after each mixing and baking protocols are shown on fig. 7 (see above).

Fig. 8: vitamin D$_2$ concentration change in SBP protocol
Overall, calculating the difference between initial concentration of added vitamin D\textsubscript{2} and vitamin D\textsubscript{2} concentration in final breads of SBP there was an increment by 7.03% (fig.8).

Fig. 9 below shows the level of vitamin D\textsubscript{2} retention between controlled atmospheric storage of (23.4 °C and 35% RH) and freeze storage at (-18°C). The statistical analysis of vitamin D\textsubscript{2} retention between these storage protocols has shown insignificant difference between them (P > 0.05). It is important to note that these measurements are restricted to very limited number of samples and to the scope of this study.

Fig. 9: vitamin D\textsubscript{2} concentration change in two different storage conditions. Superscripts with symbol ‘a’ show the standard deviations
5 DISCUSSIONS

5.1 Bread quality and vitamin D₂ stability

5.1.1 Bread quality and impact of baking protocols on quality attributes

Specific volume of bread has shown a correlation with method of dough mixing and the level of dough improver addition. Mixing process also had major influence on specific volume of wheat bread (Milan Vukić et al. 2014). However, in current study mixing protocols did not have impact on the specific volume of bread. The energy input of high mixing intensity promoted the stretching of gluten matrix and induced incorporation of air bubbles. This later positively affected the specific volume of breads (Milan Vukić et al. 2014). According to their study the mixing conditions considered were fast mixing (80rpm, 300 sec) and intensive process (1400 rpm for 100 sec) which were different from the mixing protocols of the current study. In addition, their study focused to compare effect of mixing conditions on specific volume of breads in the presence of oxidative dough improvers.

In addition, different baking protocols did not influence the specific volume (P > 0, 05). When the baking parameter extended from short time-low baking temperature to long time - high baking temperature the breads specific volume between the baking protocols was nearly the same (P > 0, 05). Studies which show effect of temperature and time combination on specific volume of bread from 100% wheat flour are very few (AH 1990; Das et al. 2012; M 2005). According to "(Das et al. 2012)" loaf volume of coriander fortified bread was significantly affected with different baking time and temperature. Increasing temperature and baking time has increased breads loaf volume. In contrary, the current study has shown insignificant difference of bread specific volume between the baking protocols. Low specific volumes were obtained from all protocols compared to specific volume of common quality wheat bread (4, 5-5 ml/g). Apart baking parameters the amount of wheat flour protein and its quality, proving temperature and time combinations can also influence the loaf volume (Ragaee S and Abdel-Aal ESM 2006). The observed low loaf volume within my experiment could be associated with technical failures of the proving cabinet (unstable temperature and RH control) that might influence the yeast activity.
Even though specific volume of breads were similar between baking protocols, differences in crumb surface pore size were observed between (P < 0.0001***). Mixing process regulates the level of air inclusion within the dough matrix which later affects the amount of gas cell distributions in final crumb surface and the subsequent bread making process promotes enlargement of the gas cells (Baker J. C. and Mize M. D 1942). Consequently, the observed differences in crumb surface structure of mixing protocols are associated with the amount of air incorporated in the dough matrix. According to “(Cauvain and Young 2007)” the ultimate bread texture is entirely dependent on the expansion of gas cells which is created in the initial phase of mixing process. Furthermore the number, size and even distributions of gas cells are dependent on the mixing procedure, intensity applied energy and government of atmospheric condition in the mixer head space. All breads had similar pore distributions with minor differences of average pore diameter. Breads crumb structures which were obtained from SBP-mixing and LEM protocols have shown smaller pore diameter (fine crumb texture) which were significantly different from other baking protocols. The lowest pore size (diameter) was recorded from SBP even though pore size difference from LEM protocol was insignificant. In contrary, the highest pore diameter was recorded from HEM which was significantly different from mixing conditions of SBP and LEM protocols. This phenomenon could be related with the level of air incorporated (occlusion of air) in HEM condition. HEM might occlude the majority of the atmospheric air existing in the head space of the mixing device which arose from its mixing intensity and longer mixing duration (2+6 minutes).

In current experimental breads there were no signifcant difference of porous structure distributions among all baking and mixing protocols (P > 0.0001). Comparing the mean value of porous structure distributions it was observed that the highest distributions were recorded from SBP whereas the minimum distributions were viewed from HEM protocol. The average pore size was different in varying the baking protocols.

The slices pore diameter which obtained from SBP (220°C for 20 minutes) was the smallest compared to the pore diameter of LBT (175 °C, 35 minutes) and HBT (245 °C, 15 minutes) protocols. As it is shown in table 6 the slices’ pore diameter of SBP is significantly different from the slices’ pore diameter of LBT and HBT processes (P < 0.0001***). However, the difference between pore’s diameter of LBT and HBT protocols were insignificant.
Comparing the average pore diameter of baking protocols’ the highest pore diameter were viewed from HBT protocol. Depending on the raw data of fig.6 the slices obtained from HBT protocol have shown high frequency of small diameter pores (Ø 0-20 µm). In SBP and LBT protocols the frequency and number of pores having diameter of Ø 0-20 µm were nearly the same. However, bread slices from HEM protocol have shown low number (n < 400) of small pore diameters (Ø 0-20 µm).

5.2 Effect of mixing and baking parameters on the stability of vitamin D$_2$

Changing the mixing parameters from low energy- short time to high energy-long time mixing didn’t affect the stability of vitamin D$_2$ in dough and bread samples. Most of the researches conducted in relation to vitamin D$_2$ stability were mostly associated with effect of heat treatment and storage conditions. " (Leskova on 2006) " described vitamin D$_2$ vulnerability to temperature, oxygen, light, and moisture. However, the result obtained from the current study revealed that vitamin D$_2$ obtained from bio-enriched yeast was resistant toward dough aeration of different mixing intensities. In relation to the initial hypothesis of this study the level of dough aeration associated with different mixing condition didn’t influence the stability of vitamin D$_2$. The vitamin D$_2$ concentration differences between different mixing intensities (LEM, HEM and SBP-Mixing) were also insignificant (Fig.7).

The investigation of vitamin D$_2$ stability at varies baking protocols has shown that it was resistant toward loses associated with different baking time and temperature combinations. The vitamin D$_2$ concentration difference between the baking protocols was also insignificant (See Fig.7). This result was consistent with previous studies which have shown vitamin D$_3$ resistivity to loses caused by pasteurization and sterilization of milk, fortified Cheddar cheese and low-fat cheese productions (Krauss et al. 1933; Wagner et al. 2008). In research work of "(Upreti et al. 2002)" significant lose of vitamin D$_3$ (25-30%) was observed in fortified cheese production. Vitamin D$_3$ was affected when the cheese milk was heated at 232°C for 5 minutes. In contrary, in the current research work the vitamin D$_2$ used in bread samples (vitamin D$_2$ originated from bio-enriched yeast) was resistant to lose when the baking temperature-time combination varied from LBT (175 °C, 35 minutes) to HBT (245 °C, 15 minutes). 90% of vitamin D$_3$ was retained in cheddar cheese processing (Ganesan et al. 2011). Whereas, in another study only 40 to 50% of vitamin D$_3$ was retained cheddar cheese processing (Banville et al. 2000). The conclusion given toward
this phenomenon was associated with forms of vitamin D3 used for the fortification process that affected the level of retention. The observed level of vitamin D2 retention within the current study was solely based on vitamin D2 originating from bio-enriched yeast.

"(Jakobsen & Knuthsen, on 2014) "have compared the level of vitamin D2 retention between rye and wheat bread. The level of vitamin D2 retention between these bread types was based on one baking condition (at 200 °C for 30 min). However, with in the current study variable baking protocols were considered which shows the level of vitamin D2 when the intensity of baking temperature-time combinations were increased from lower to higher intensities. As indicated on Fig.7 and depending on the statistical analysis the vitamin D2 was resistant to lose at varies baking protocols.

Vitamin D2 concentration change between baking unit operation of SBP protocol is shown in fig.8. Looking the statistical analysis of SBP the vitamin D2 concentration has shown increment by 7.03% compared to the startup concentration. The percentage of increment was calculated based on vitamin D2 mean concentration difference between the final bread and initial added amount. The increment observed could be related with production of vitamin D2 as result of yeast exposure to light sources during measurement and mixing unit operations. Since, the baking process was not fully protected from light exposure the yeast might keep producing vitamin D2 during the subsequent baking unit operations.

5.3 Stability of vitamin D2 in different storage conditions
Different studies have shown stability of vitamin D at varies storage conditions (Banville et al. 2000; Ganesan et al. 2011; Hanson and Metzger 2010; Leskova 2006). However, most of the studies focused on stability of vitamin D in milk and milk products storage. Beyond these the method of storage was limited to one type of storage condition. Effect of two different packaging types (plastic and glass bottles) on the stability of vitamin D2 in milk samples was compared. The milk samples were stored under refrigeration temperature for consecutive 7 days. The result shows vitamin D2 was stable in both packaging types (Leskova on 2006). However, in the current study stability of vitamin D2 in bread samples (from SBP) were stored at controlled atmospheric storage (at 23.4 °C and 35% RH) and freeze storage (at -18°C) for retention measurement. The vitamin D2 was also stable in both storage types and the vitamin D2
concentration differences between bread samples of both storage protocols were insignificant as shown on Fig.9.

6 CONCLUSIONS

This study aimed to investigate stability of vitamin D$_2$ originating from bio-enriched yeast during the baking process and assessed its impact on bread quality. The vitamin D$_2$ retention measurement was done based on different mixing and baking protocols. The assessment is conducted to determine the level vitamin D$_2$ retention after each unit operation. The vitamin D$_2$ content within the dough and bread samples were measured following analytical method which run by couple of HPLC technique. Image J application software was used to analyze texture of the experimental breads during this study. Finally, the results obtained from this study statistically analyzed using JMP® Pro 11.0.0 software (SAS Institute Inc. 2013, USA).

The outcome of the baking experiment associated with impact of vitamin D$_2$ containing bio-enriched yeast on the quality of bread resulted low specific volume of bread compared to the common wheat bread specific volume However, different mixing and baking protocols resulted very similar specific volumes indicating that processing variables had minor role in volume development. However, in relation to the observed low loaf volume of protocols it is important to control the efficiency of proving unit operation. SBP (with 2+4 min mixing and 220°C, 20 min baking parameter) and LEM-baking process (with 2+2min mixing and 175 °C, 35 min baking parameter) yielded bread with evenly distributed fine porous structure. Therefore, Applied baking protocols could be considered as adequate method in the fortification of vitamin D$_2$ originating from bio-enriched yeast.

Vitamin D$_2$ retention measurement has shown that there was no detected lose of vitamin D$_2$ due to different mixing and baking protocols. It can be concluded that vitamin D$_2$ stability was not affected by changing the intensity and length of mixing time.

The vitamin D$_2$ concentration difference between the baking protocols was insignificant indicating that baking protocols didn’t have impact on the stability of vitamin D$_2$. In addition, the designed storage conditions: controlled atmospheric storage (23.4 °C and 35% RH) and freeze storage (-18 °C) fully retained vitamin D$_2$ content in bread samples and there was no significant
deference vitamin D₂ retention between these storage conditions after 10 consecutive storage days.

It was observed that with SBP protocol there was an increment of vitamin D₂ concentration with 7.03% during the whole baking process. In order to clearly understand how the vitamin D₂ increment was detected it is important to analyze impact of other unit operation such as proving stage, which was not part of this study. Since analytical method of vitamin D₂ measurement is very expensive and time taking technique, the samples taken during this study were limited to small numbers. Therefore, the conclusion given above only restricted predictive power for other experimental set ups.

In conclusion, depending on the scope of this study vitamin D₂ stability (typically the vitamin D₂ obtained from the UV treated yeast) was stable enough throughout the whole bread baking process and its presence in bread fortification process didn’t affect the quality of bread. Future work should aim to widen this study work and it would be important to take more samples for vitamin D₂ measurements. This will help to get more representative measurements for creating valid conclusions. Furthermore more works are needed to assess the level of vitamin D₂ retention under different storage and packaging conditions. There is evidence that suggest light exposure affected stability of vitamin D₂ in milk processing. Therefore studying effect of light on vitamin D₂ stability during bread baking process and under different storage conditions will be important. A recent study found that type of milk packaging also determined the level of vitamin D₂ retention in milk products. Therefore it is also essential to study stability of vitamin D₂ under different bread packaging types. Studying stability of vitamin D₂ (obtained from UV treated yeast) in different type of breads and baking methods will also be essential.
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