FACULTY OF SCIENCE
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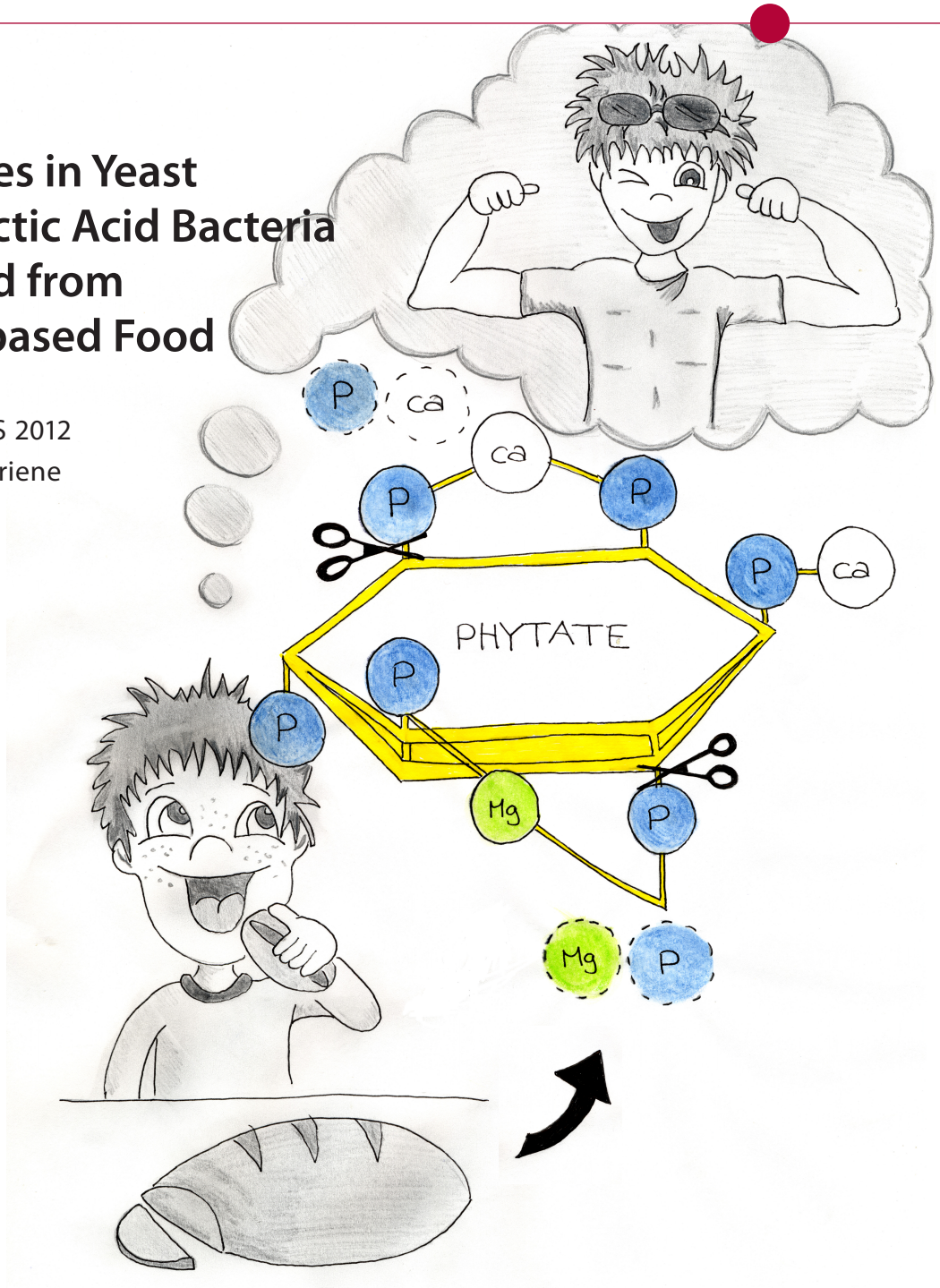
Phytases in Yeast and Lactic Acid Bacteria Isolated from Grain-based Food

PHD THESIS 2012

Lina Nuobariene



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Phytases in yeast and lactic acid bacteria isolated from grain-based food

Quality and Technology ▪ Department of Food Science

Faculty of Science ▪ University of Copenhagen

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- To my Mother and Father

- Mamai ir Tėveliui

The doctor of the future will give no medicine,
But will interest his patients in the care of human frame, in diet,
And in the cause and prevention of disease

– Thomas Alva Edison

Preface

This PhD thesis has been accomplished to fulfil the requirements for obtaining a PhD degree at the Quality and Technology Section, Department of Food Science, Faculty of Life Science, University of Copenhagen. This PhD project was financed by a scholarship from faculty of LIFE Science, University of Copenhagen therefore grateful acknowledge goes to them for the funding source that made my PhD work possible.

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I am thankful to all my colleagues at Q&T for contributing to a pleasant, welcoming and professionally fruitful working environment. Special thanks go to Marta Jolanta Popielarz and my officemate Solveig Warnecke. Marta, thank you for your time we have spent together. We have shared many ups and downs in our lives and you always been there for me no matter what occasion. Solveig, I am grateful for your attentive listening and for guiding me along the Danish society.

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* * * * *

Mama ir Tėti, šį *darbą skiriu Jums. Jūs patys nuostabiausi. Silvija ir Aivarai, Jūs esat mano šviesa. Ačiū sesytėm Virginijai ir Vilandai už Jūsų palaikymą ir buvimą šalia kai man to labiausiai reikėdavo. Aš Jus visus labai myliu.*

Lina Nuobariene

Copenhagen, 2012 July

Abstract

Nowadays, consumption of whole-grain breads is gaining popularity across the world due to increasing awareness of its nutritional benefits. Whole grains contain wide range of nutrients, such as dietary fibre, vitamins and minerals. Despite that whole grains contain 20-70% of the daily requirements of the minerals, their absorption in human gut are very low due to antinutritional phytic acid salt (phytate), and this may lead to mineral deficiencies. To increase the bioavailability of minerals, enzymatic degradation of phytate and its dephosphorylated isomers is needed.

The main aim of this thesis was therefore to isolate and identify phytase active strains of yeasts and lactic acid bacteria to be used in bread making. The use of selected combination of yeast and lactic acid bacteria with high phytase activity could find use as starter cultures to improve mineral bioavailability in whole-grain bread fermented in short time as alternative to sourdough bread and/or yeast fermented bread. All experiments were carried out at conditions optimal for leavening of bread (30°C and pH 5.5).

Another aim of the study was to isolate and identify phytase active yeasts and lactic acid bacteria from commercial sourdoughs. In addition, comparison between sourdoughs from different bakeries and countries on yeast strain distribution and their phytase activities was done.

Further, physical parameters, such as pH and temperature have profound influence on growth of yeast cells and production of phytase from them. Thus the third aim of this study was to purify and characterize the phytase enzyme isolated from *Saccharomyces cerevisiae* L1.12 – the yeast strain which show the highest extracellular phytase activity among all tested strains.

To investigate whether the yeast and lactic acid bacteria strains produce phytases, selective defined minimal media, containing phytic acid dipotassium salt as the only phosphate source, were developed. Consequently, our results show that the specific extracellular phytase activities of all tested yeast strains were at least 20-fold higher than the intracellular phytase activities. However, tested lactic acid bacteria have been shown to produce intra- and extra-cellular phytases with the clear tendency to be species dependent. The highest specific extracellular activities were observed in

Saccharomyces cerevisiae L1.12 (10.6U/10^{10} CFU), *S. cerevisiae* L6.06 (8.2 U/10^{10} CFU), *Lactobacillus frumenti* 2.1 (13 U/10^{10} CFU), and *L. frumenti* 2.5 (16 U/10^{10} CFU). The high phytase activities of the strains mentioned above, observed under conditions optimal for bread dough leavening, suggest that these strains may be a particularly interesting source of phytase for the production of whole-grain bread with high content of bioavailable minerals.

Furthermore, in this study, *Saccharomyces pastorianus*, *Candida humilis*, and *Lactobacillus frumenti* have for the first time been recognized as phytase positive strains.

Comparison of sourdoughs from different bakeries and countries for yeast strain distribution and their phytase activities revealed that yeast isolated from Lithuanian sourdoughs have rather higher phytase activity than the yeast isolated from Danish one.

Implemented Concanavalin A chromatography for purification of extracellular phytase produced by *S. cerevisiae* L1.12 resulted in 8.1-fold purification with a yield of 3.6% to a maximum specific activity of 42.9 U/mg. The optimum pH and temperature were found to be 4.0 and 35°C, respectively. In addition, more than 80% of maximum purified phytase activity was determined between pH 3.5-5.5. These results suggest that phytase, produced by generally recognized as safe (GRAS) yeast *S. cerevisiae* L1.12, with its desirable activity profile under dough leavening conditions, has potential for application in bread processing.

The results of these investigations are presented in two published papers in international peer reviewed journals, one submitted to an international journal and one manuscript under preparation.

List of Publications

PAPER I

Lina Nuobariene, Åse S. Hansen, Lene Jespersen and Nils Arneborg
Phytase-active yeasts from grain-based food and beer
Journal of Applied Microbiology **110** (2011) 1370-1380

PAPER II

Lina Nuobariene, Åse S. Hansen and Nils Arneborg
Isolation and identification of phytase-active yeasts from sourdoughs
LWT- Food Science and Technology **48** (2012) 190-196

PAPER III

Lina Nuobariene, Dalia Eidukonyte, Åse S. Hansen, Grazina Juodeikiene and Finn K. Vogensen
Isolation and identification of phytase-active lactic acid bacteria from sourdoughs
[Submitted to International Journal of Food Microbiology]

PAPER IV

Lina Nuobariene, Åse S. Hansen and Jens C. Sørensen
Purification and characterization of phytase produced by yeast Saccharomyces cerevisiae isolated from rye sourdough
[Manuscript under preparation]

List of Abbreviations

LAB	lactic acid bacteria
Rep-PCR	repetitive element sequence-based polymerase chain reaction
IP_6	phytic acid
IP_5	*myo*-inositol pentaphosphate
IP_4	*myo*-inositol tetraphosphate
IP_3	*myo*-inositol triphosphate
IP_2	*myo*-inositol diphosphate
IP_1	*myo*-inositol monophosphate
PHO pathway	phosphate signal transduction pathway

Table of Contents

Chapter 1

Introduction

Nowadays, consumption of whole-grain breads is gaining popularity across the world due to increasing awareness of its nutritional benefits. Over the past years, major epidemiological studies have consistently shown that consumption of whole-grain foods is protective against the development of chronic diseases. Moreover, whole grains have been found to help regulation of blood glucose and protect against type 2 diabetes (De Munter et al., 2007) as well as to reduce the risk of obesity (Van de Vijver et al., 2009; Good et al., 2008). The importance of whole-grain foods intake on blood glucose and weight regulation is considered as a result of the content of dietary fibres and magnesium in whole grains. Other studies concern the contribution of dietary fibres, folate and, to a certain extent, vitamin B_6 and magnesium from whole-grain foods as protective components against cardiovascular diseases and certain type of cancer (Slavin, 2004).

Besides dietary fibres, the whole-grain foods are also important sources of minerals. It was shown that 100 g of whole grains contain 20-70% of the daily requirements of the minerals, such as iron, zinc, potassium, phosphorus, magnesium, and manganese (Cordain, 1999). Despite that whole grains are relatively rich in minerals, their absorption in human gut are very low (Cheryan, 1980) due to antinutritional phytic acid salts (phytate), and this may lead to mineral deficiencies.

Iron deficiency is the most common nutrient deficiency, widespread both in western and developing countries (WHO, 2002). In Denmark, many teenage girls (15%) and pre-menopausal women (up to 40%) as well as pregnant women (75-80%) have low

or depleted iron store. Also many rapid growing teenage boys have low iron status (Fødevaredirektoratet, 2002). Diets based upon whole grain wheat (Hallberg, 1987) and other cereals (Hurrell, 2002) have been consistently shown to reduce iron absorption. Zinc represents the other nutritionally significant mineral that has been associated with phytate inhibition effect.

Phytic acid, also known as *myo*-inositol 1,2,3,4,5,6-hexakis phosphate, consists of a *myo*-inositol ring with six phosphate groups esterified to each carbon atom. It is the principal storage form of phosphorus in plant tissue, typically accounting for 60-90% of total seed phosphorus (Reddy, 2002). In addition, phytic acid in plant tissue mainly occurs as a mixed calcium-magnesium-potassium salt, so-called phytate (Ockenden et al., 2004; Graf, 1983). Due to its chemical structure, phytic acid is a very stable, high negatively charged molecule over a wide pH range. Thus, phytic acid acts as a strong chelator of cations and binds minerals, such as iron, zinc, calcium, magnesium (Raboy, 2003). Formed phytate complexes are insoluble at physiological pH, and, therefore, minerals and phosphate are unavailable for absorption in the human intestine (Lopez et al., 2002; Brune et al., 1992). To increase the bioavailability of minerals, enzymatic degradation of phytate and its dephosphorylated isomer is needed (Sandberg et al., 1999).

Phytate degrading enzymes - phytases - are enzymes naturally found in cereals (Eeckhout and Depaepe, 1994) and microorganism (Olstorpe et al., 2009; Lambrechts et al., 1992; Ullah and Gibson, 1987). Phytase catalyse the stepwise dephosphorylation of phytate to lower phosphoric esters of *myo*-inositol and phosphoric acid via penta- to monophosphates.

Cereals exhibit high phytase activities with values ranging from 900 to 2886 U/kg dry matter in wheat grain and from 4100 to 6100 U/kg dry matter in rye grain (Greiner and Egli, 2003; Eeckhout and Depaepe, 1994). However, the activities in wheat were considered insufficient to notably improve the mineral bioavailability in whole grain wheat bread (Türk and Sandberg, 1992; Harland and Harland, 1980). Several studies were carried out on phytase activity from baker's yeast (*Saccharomyces cerevisiae*) during leavening of bread dough (Andlid et al., 2004; Türk and Sandberg, 1992; Harland and Frolich, 1989; Harland and Harland, 1980). They showed that only marginal amount of phytate was degraded during 3 h fermentation. Studies on phytase activity from lactic acid bacteria identified few strains of *Lactobacillus* spp. to express intracellular activities only (Reale et al., 2004; De Angelis et al., 2003; Lopez et al., 2000).

From an industrial point of view, the extracellular phytase activity would be more important for bread making than the intracellular phytase activity, because cells of the

yeast and lactic acid bacteria should be intact in dough in order to ensure a good fermentation. In this case, the intracellular phytase will not have access to phytate in the dough. So far, no high phytase active yeasts and lactic acid bacteria were identified for bread industry in order to increase mineral bioavailability.

One of the aims of this study was therefore to identify strains of yeasts and lactic acid bacteria with high activity of phytase to be used in bread making to increase the content of bioavailable minerals in bread. All experiments were carried out at conditions optimal for leavening of bread (30°C and pH 5.0). Moreover, the use of selected combination of yeast and lactic acid bacteria with high phytase activity could find use as starter cultures to increase the mineral bioavailability in whole-meal bread fermented in short time as alternative to sourdough bread and/or yeast fermented bread. In addition, high phytase active yeast in combination with baker's yeast may also be used in yeast fermented bread.

Another aim of the study was to isolate and identify phytase active yeasts and lactic acid bacteria from commercial sourdoughs. In addition, comparison between sourdoughs from different bakeries and countries on yeast strains distribution and their phytase activities was done.

Physical parameters, such as pH and temperature have profound influence on growth of yeast cells and production of phytase from them. Therefore, the third aim of this study was to purify and characterize the phytase isolated from the most prominent yeast strain for phytase production.

The subsequent chapters of this thesis will be as follow:

Chapter 2 provides background information of the cereals, their anatomy and the nutritive value of whole grains.

Chapter 3 describes the microbiology of bread and main techniques of yeast and lactic acid bacteria identification (PAPER II and PAPER III).

Chapter 4 provides background information on the phytate structure, distribution in cereals and effect on human health.

Chapter 5 describes the factors which influence on phytase synthesis, as well as analytical methods for phytase identification. Moreover, results of phytase activities in yeast and lactic acid bacteria, and purified phytase characteristics are discussed. In addition, this chapter provides background information on the cereals and microbial phytases (PAPER I, PAPER II, PAPER III AND PAPER IV).

Chapter 2

Cereals and Whole-Grain Food; what is Beyond it?

During the Mesolithic era (10000-8000 years ago), in the face of increasing human population numbers, agriculture became the dominant way of life. Together with agriculture, cereal-based diets almost replaced the animal-based diet of hunter-gatherers, and became the dominant caloric and protein source in many cultures (Harlan, 1992). Moreover, Lorenz and Lee (1977) reported that consumption of cereal products comprise up to 80% of the total caloric intake in human population, and in at least half of the countries of the world, bread provides more than 50% of the total caloric intake.

In our days more than 90% of population consume cereal grains in its highly refined state, which may lead the development of the chronic diseases, such as obesity, type 2 diabetes and cardiovascular diseases. Over the last four decades, attention has turned to whole-grain food, recognizing that their fiber content, vitamins, minerals, phytoestrogens and antioxidants may protect against chronic diseases (Martin et al., 2011).

The nutritional value of wheat takes an important place among our daily food. Therefore, the main themes of this chapter revolve around the component parts of the wheat kernel and their chemical composition. Moreover, nutritional value of wheat and the definition of whole grain food will be pointed out.

2.1 Cereals classification

Cereals are the one-seed fruits (caryopsis) that belong to the grass family *Poaceae*, former known as *Gramineae* (Delcour and Hoseney, 2010; Belitz et al., 2009). The common cereals in bread-making, i.e. wheat and rye, belong to the genus *Triticum* and *Secale*, respectively (**Figure 2.1**). The *Triticum* genus includes different wheat species, such as einkorn, emmer wheat, durum wheat, spelt wheat and common wheat (bread wheat), whereas the *Secale* genus includes only one rye species – *Secale cereale* (cereal rye).

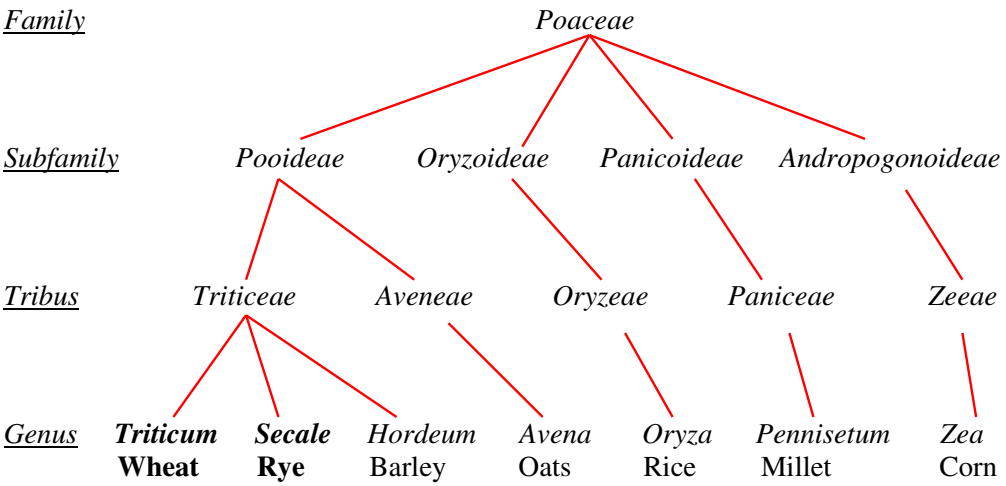

Figure 2.1 Cereals classification (Belitz et al., 2009)

The major cereals of the world are: maize, rice, wheat, barley, sorghum, millet, oats, and rye (**Table 2.1**). Maize is widely grown throughout the world and has the highest production of all the cereals with 844.2 million tonnes being produced in 2010 (FAOSTAT, 2012). Wheat is also widely grown around the world and has been the main ingredient for food, such as bread, of the major civilization in Europe and North Africa for more than 8000 years. Wheat is the most produced cereal in Europe, followed by maize and barley. Rye is the second cereal crop used for bread making in areas east of the French-German border and north of Hungary. Rye is widely cultivated in Eastern Europe (5.7 million tonnes in 2010), Scandinavian (0.48 million tonnes in 2010) and the Baltic countries (0.18 million tonnes in 2010).

Table 2.1 World cereal production in 2010 [adapted from FAOSTAT(2012)]

Cereals	Cereal production in different continents of the world (million tonnes)				
	World	EU	America	Africa	Asia
Maize	844.2	85.5	447.9	64.3	246.1
Rice	672.0	4.4	37.2	22.9	607.3
Wheat	650.9	201.1	112.7	22.0	292.4
Barley	123.5	73.5	16.0	6.3	20.0
Sorghum	55.9	0.7	22.5	20.9	9.9
Millet	29.3	0.3	0.3	15.3	13.3
Oats	19.6	12.0	5.1	0.2	1.0
Rye	12.3	10.6	0.5	0.05	1.1

2.2 Cereals anatomy

Wheat and rye as well as other cereals, in contrast to forage grasses form a relatively large fruit, called a caryopsis. Wheat grain is composed of a number of tissues with specific composition and structures, as illustrated in **Figure 2.2** (Kent and Evers, 1994).

All cereal caryopsis consist, in milling terms, of three major parts: bran, endosperm, and germ. Schematic representation of the principal locations of the nutritional and bioactive compounds among wheat grain tissues is given in **Table 2.2**, modified after Hemery et al. (2007).

The bran fraction consist of several distinct layers; pericarp (fruit coat), testa (seed coat), nucellar tissue and aleurone layer. Wheat bran covers about 14-15% of the kernel weight, whereas rye bran accounts for about 10-12% of the kernel weight (Posner, 2000; Lorenz, 2000).

The pericarp has a multilayer structure and consists of outer pericarp (epidermis and hypodermis) and inner pericarp (cross cells and tube cells). In all cereal grains the pericarp is dry in maturity where most of the cells are dead and empty. The outermost layer of the pericarp has a protection role for the endosperm and embryo. Moreover, the most outer tissues are rich in insoluble dietary fibres (**Table 2.2**), i.e. cellulose, lignin and complex xylans (Hemery et al., 2007; Hoseney, 1994). The innermost layer in many cereals is an incomplete layer and contains chloroplasts in the immature grain (Kent and Evers, 1994).

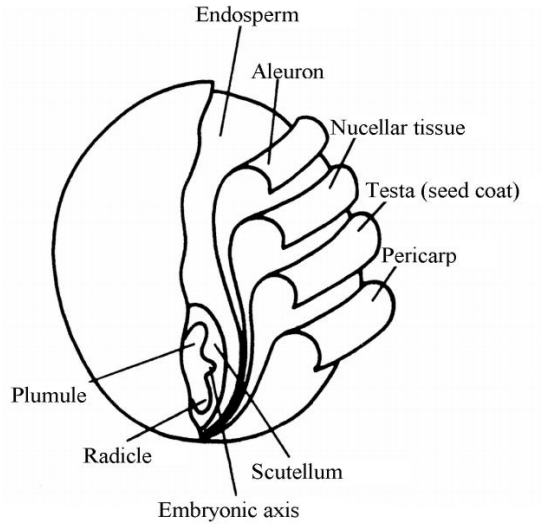

Figure 2.2 Wheat grain; the main common characteristics (Kent and Evers, 1994)

Testa and nucellus are the two next layers, so-called seed coat. The seed coat is a multifunctional organ that plays an important role in embryo nutrition during seed development and in protection against detrimental agents from the environment (Weber et al., 1996). Moreover, testa regulates the water uptake and contains pigments that for wheat grains gives either white if the content of pigment are low or red to brownish colour, whereas for rye grains it gives the characteristic blue-green colour (Hoseney, 1994).

The aleurone layer is located between the nucellar layer and starchy endosperm and constitutes about 5-8% of the wheat kernel (Posner, 2000). This tissue of mature cereals is physiologically active, in contrast to the outer layers, whose cells died during late cereals maturation after having encountered considerable development changes. The aleurone layer contains high levels of β-glucans compared with whole grain (Hemery et al., 2007) and appreciable amounts of ferulic acid, which is recognized for its antioxidant properties (Smith and Hartley, 1983). Moreover, this tissue is rich in proteins (32% on dry weight basis), and also contain lipids (9% on dry weight basis), B vitamins and enzymes. Additionally, important quantities of minerals, such as magnesium, phosphorus, selenium, iron, zinc and potassium are found in the aleurone layer (**Table 2.2**). In spite of its real mineral wealth, it is necessary to consider that the aleurone layer contains 84-88% of the total anti-

nutritional phytate, which has a strong binding affinity to important minerals, thus making them unavailable for absorption in the human intestine (Reddy, 2002). The phytate content in cereals and its interaction with minerals will be discussed more in details in chapter 4.

Table 2.2 Schematic representation of the location of nutritional compounds and phytic acid in wheat grain tissues [modified after Hemery et al. (2007)]

Nutritional compound	Bran fraction			Endosperm	Germ
	Pericarp	Testa	Aleurone		
Insoluble fibres	***	***	**	*	**
Soluble fibres	-	-	*	**	*
Proteins	-	-	**	**	*
Lipids	-	*	*	-	***
Starch	-	-	-	***	-
B vitamins					
Thiamine	-	-	*	-	***
Riboflavin	-	-	*	-	***
Niacin	-	-	***	-	*
Pantothenic acid	-	-	**	-	*
Pyridoxine	-	-	***	-	**
Biotin	-	-	***	-	*
Folate	-	-	***	-	**
Vitamin E	-	-	*	-	***
Minerals					
Magnesium	*	*	***	-	**
Phosphorus	*	*	***	-	*
Manganese	*	*	**	-	**
Zinc	*	*	**	-	*
Iron	*	*	**	-	*
Ferulic acid	*	*	***	*	**
Phytic acid	-	-	*******	-	*

*, **, ***; Indicate if a compound is present, concentrated, or highly concentrated in a tissue
-; indicates that a compound is not found or present in very low concentrations in a tissue

The endosperm is the largest tissue of the grain which represents 82-85% of wheat and *app.* 87% of rye grain weight (Kent and Evers, 1994). This tissue consists of

starch granules embedded in or surrounded by an amorphous protein matrix. The function of starchy endosperm is to store the nutrients in form of starch which, afterwards, could be mobilized to support growth of the embryonic axis at the onset of germination.

The germ, also known as the embryo represents 2.5-3.0% of the grain weight and consist of embryonic axis and scutellum (Fardet, 2010). The embryonic axis is separated from the endosperm by the scutellum, which is a source of lipids (13-27% on dry weight bases) and hydrolytic enzymes as well as rich in proteins (25-34% on dry weight bases). Moreover, scutellum functions as a transfer organ between the developing seedling and its nutrient supply (endosperm). The mineral level in germ is also rather high and varies between 4.5 and 5.5% on dry weight basis (Belitz et al., 2009; Cornell, 2003). However, among all these goods the wheat and rye germ also contain *app.* 12% of the total phytate (O'Dell et al., 1972).

2.3 Nutritional value of whole grains

Cereal grains play an important role in supplying not only the important macronutrients, such as proteins and carbohydrates, but also the micronutrients such as vitamins and minerals, which are responsible for regulation metabolic pathways, and their deficiencies lead to drastic health disparities (McGuire and Kathy, 2011). Macronutrients are responsible for everyday functions of the body, such as building and repairing tissue, generating energy and heat, regulating hormones, and maintaining normal brain and nerve function.

The chemical composition of the cereals varies widely and depends on the environmental conditions, soil, variety and fertilizer (Riahi and Ramaswamy, 2003). However, the macronutrients of rye are comparable to wheat in terms of protein, lipid, starch, and ash, whereas fiber content is higher in rye grain than in wheat grain (**Table 2.3**).

Table 2.3 Chemical composition of macronutrients in wheat and rye

	Protein	Lipid	Starch	Dietary fiber	Ash
Wheat	10-16	1-3	60-80	9-15	1-2
Rye	7-15	1-2	56-73	14-19	1

Results are expressed % on dry matter basis
Source; (Verwimp et al., 2004; Hansen et al., 2003; Hansen et al., 2002; Posner, 2000; Lorenz, 2000; Zhuge et al., 1991; Cummings, 1987)

For example, the endosperm, which is the main part of the grain that is milled to produce flour, mostly contains starch and has lower protein and lipid content than the germ and bran. Further, the endosperm has a very low content of nutritionally essential vitamins and minerals. In contrast to the endosperm, the grain bran is a source of B-vitamins and has a high content of dietary fibres, which have been reported to have positive health effects on cardiovascular diseases and lowering cholesterol content in the blood (Slavin et al., 1997; Hoseney, 1994). Moreover, the grain bran is rich in minerals (40-70% of the total mineral content in grain), especially calcium, potassium, phosphorus, iron and magnesium.

To benefit from the nutrition provided by whole grain and especially the grain bran, people should eat a diet based on wholemeal products such as high extraction wheat and rye flour (100%) breads, whole-grain bread and whole-grain breakfast cereals because the process that produce low extraction flour (75% and lower) removes the bran and a large portion of the highly nutritious ingredients is refined away (**Table 2.4**). Pederson et al. (Pedersen et al., 1989) observed that in refined flour (extraction rate 75% and lower) the concentration of calcium decreases more than two times, whereas the concentration of phosphorus, iron, zinc and copper decrease about three times in comparison to whole grain flour (**Table 2.4**). Technology and microbiology of bread will be discussed in chapter 3.

Table 2.4 Chemical composition of different extraction rates wheat flour [modified after Pedersen et al. (1989)]

	Extraction rates, % (dry basis)		
	100	75	66
Energy, kJ/g	18.5	18.3	18.4
Ash	1.8	0.6	0.5
Protein (Nx6.25)	14.2	13.5	12.7
Fat	2.7	1.4	1.1
Starch plus sugar	69.9	82.9	84.0
Crude fiber	2.4	0.3	0.2
Dietary fiber	12.1	2.8	2.8
Mineral			
Calcium, mg/g	0.44	0.25	0.23
Phosphorus, mg/g	3.8	1.3	1.2
Zinc, ppm	29	8	8
Copper, ppm	4.0	1.6	1.3
Iron, ppm	35	13	10
Vitamins			
Thiamine, μg/g	5.8	2.2	1.4
Riboflavin, μg/g	0.95	0.39	0.37
Vitamin B_6, μg/g	7.5	1.4	1.3
Folate, μg/g	0.57	0.11	0.06
Biotin, μg/g	116	46	25
Niacin, μg/g	25.2	5.2	3.4

Over the past years, major epidemiological studies have consistently shown that consumption of whole-grain products is protective against the development of chronic diseases. Whole grains have been found to help regulate blood glucose and protect against type 2 diabetes (De Munter et al., 2007) as well as to reduce the risk of obesity (Van de Vijver et al., 2009; Good et al., 2008). The importance of whole-grain intake on blood glucose and weight regulation is considered a result of the content of dietary fibres and magnesium in whole-grain. Other studies concern the contribution of dietary fibres, folate and, to a certain extent, vitamin B_6 and magnesium from whole-grain products protective against cardiovascular disease and certain type of cancer (Slavin, 2003).

Although epidemiological studies have shown that the consumption of two or three servings of whole-grain products per day is sufficient to get beneficial health effects (Lang and Jebb, 2003) the recommended consumption of whole-grain products differ from one country to another (**Table 2.5**).

Table 2.5 Recommendation for whole-grain products consumption

	Recommended Servings per day						Reference
	Children	Teens	Adults				
Age in years	2-13	14-18	19-50		51+		
Sex	Girls and Boys		Female	Male	Female	Male	
Australia[a]	5-9	5-11	4-9	6-12	4-7	4-9	(Australian Government, 2003)
Canada[b]	3-6	6-7	6-7	8	6	7	(Health Canada, 2007)
Denmark	55 g whole-grain[c]		75 g whole-grain[d]				(DTU, 2008)
Germany	Min 30 g of dietary fiber daily						(DGE, 2012)
Netherlands[a]	4-5	5-6	5-6		4-5		(Netherlands, 2012)

[a] one serving equal two slices of bread or one cup breakfast cereal flakes
[b] one serving equal one slice of bread (35 g), 45 g of bagel or 30 g of breakfast cereal flakes
[c] recommendation based on 1800 calories diet
[d] recommendation based on 2400 calories diet

Despite indications that whole grain diet beneficially influence on protective against the development of chronic diseases, whole grain cereals are also a source of anti-nutrients with adverse health effect. The main anti-nutrient is phytate, which are known to inhibit the absorption of essential minerals present in cereals (Kumar et al., 2010). The antinutritional effect of phytate will be discussed more in details in chapter 4.

Beside the phytate, cereal grains contain other antinutrients such as enzyme inhibitors which can inhibit digestion; tannins which irritate the digestive tract; and complex sugars which the body cannot break down.

2.4 The definition of whole-grain food

According to the European Food Information Council (EUFIC) 'Whole grains or foods made from them contain all the essential parts and naturally-occurring nutrients of the entire grain seed. If the grain has been processed (e.g. cracked, crushed, rolled, extruded, and/or cooked), the food product should deliver approximately the same rich balance of nutrients that are found in the original grain seed' (EUFIC, 2009). However, during grain milling operations the small losses of grain components and bioactive compounds appear, so the whole-grain flour rarely contain the same proportions of endosperm, bran and germ as the whole grain before milling. Therefore, the Whole Grain Task Force proposed the best adapted definition to Western countries technologies: *'Whole grains shall consist of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components – the starchy endosperm, germ and bran – are present in the same relative proportions as they exist in the intact caryopsis to the extent feasible by the best modern milling technology'* (Jones, 2008; AACC International, 1999).

Chapter 3

Microbiology of Bread

The history of sourdough is as old as the history of leavened bread itself. Way back in ancient days (as early as 3000 B.C.) humans first figured out how to promote the fermentation and leavening of grains to first be brewed into beverages and then, later to be backed into bread (Wood, 1996). For the more than six thousand years bread has been made in the same way using spontaneous dough fermentation, and the dough has been left to rise slowly for many hours before it is baked.

However, during the industrial revolution in the 19 century the straight dough breads have been introduced where the main sourdough fermentation step was reduced or even excluded (Decock and Cappelle, 2005). The use of sourdough as a leavening agent was widely replaced by industrially produced metabolically active baker's yeast (*Saccharomyces cerevisiae*). The use of straight dough process substantially reduced the time required for pre-proofing of sourdough.

Interest in sourdough bread production again gain popularity during recent decades due to increased consumer demand for bread with high nutritional value as well as good texture and aromatic taste. At present, the sourdough is employed worldwide in the production of wheat bread, cakes, and crackers (Ottogalli et al., 1996; Brummer and Lorenz, 1991).

In this chapter principles of sourdough and straight dough bread as well as microbiology of bread will be discussed.

3.1 Sourdough bread and straight dough bread

Sourdough

Sourdough is a mixture of flour and water that is spontaneously fermented with lactic acid bacteria (LAB) and sourdough yeasts, whose fermentation confers to the resulting bread characteristic such as palatability and high sensory quality (Corsetti and Settanni, 2007). Sourdough is extremely complex ecosystem, mainly represented by LAB and yeast. LAB to yeast ratio in sourdough is generally 100:1 and the levels of LAB and yeasts ranged from 10^7 to 10^9 CFU/g and from 10^5 to 10^7, respectively (Gobbetti, 1998). In sourdough fermentation yeasts mainly contribute to the dough leavening by producing CO_2 via the alcoholic fermentation of the sugars (Sugihara et al., 1971). Yeasts are also very important for the production of the metabolites, such as alcohols, esters and carbonyl compounds which contribute to the development of the bread flavour, whereas the LAB are mainly responsible for the acidification of sourdough and enhanced the formation of the yeast volatile compound (Damiani et al., 1996; Hansen and Hansen, 1994). Furthermore, the enzymatic activities of yeast and LAB as well as of cereals by enzymes such as phytases have an influence on the bread nutrition (Haefner et al., 2005). In addition, it was shown that yeast phytase activities are species or even strain-dependent (PAPER I) (Hellström et al., 2010), so a special interest arose to involve the desired microorganisms in to fermentation in order to increase bread nutrition.

The tradition of making wheat bread with addition of sourdough is widely used in Italy (Corsetti et al., 2001), Spain (Barber and Baguena, 1988), Greece (De Vuyst et al., 2002), (Faid et al., 1994), Iran (Azar et al., 1977). In Northern Europe, for example, Finland, Sweden, Denmark, Lithuania as well as in Germany, sourdough is employed especially in the rye bread production (Hansen, 2004; Rosenquist and Hansen, 2000).

Most sourdoughs used in both wheat and rye bread baking can be initiated by (i) spontaneous fermentation, (ii) adding a piece of mature sourdough, or (iii) adding a defined starter culture.

Spontaneous fermentation sourdough preparation is a multiple stage process that starts with a mixture of flour and water left for a specific period of time. Usually water and flour mixture are kept for 1-2 days at ambient temperature where spontaneous fermentation take place due to the naturally occurring microorganisms in the flour (Hansen, 2004; Linko et al., 1997).

Mature sourdough has traditionally been used by artisan bakeries (Minervini et al., 2012). This sourdough are characterized by continuous (daily) propagation (back slopping) of the dough at ambient temperature (20 to 30°C) where the fermented sourdough is used for bread production, but part of it is used as starter by initiating a new sourdough (Hansen, 2004). Conventionally, 2-5% of old sourdough is used as an inoculum for the new batch (Linko et al., 1997).

Starter cultures for sourdough fermentation are pure cultures of dried LAB, or a mixture of LAB and sourdough yeasts. To initiate fermentation the starter are mixed with flour and water, and kept for several hours for multiplication and fermentation of the microorganisms. Subsequently, fermented dough is used as a sourdough.

Straight dough

In straight dough method one-stage process is used where all ingredients are mixed at once to form the final dough which afterwards is fermented to maturity without or with degassing by punching during the fermentation step. The fully fermented dough is divided into desired weights pieces. The dough pieces are than rounded, given a relaxation period, shaped, and after proofing are baked (Kulp and Ponte, 2000) (**Figure 3.1**).

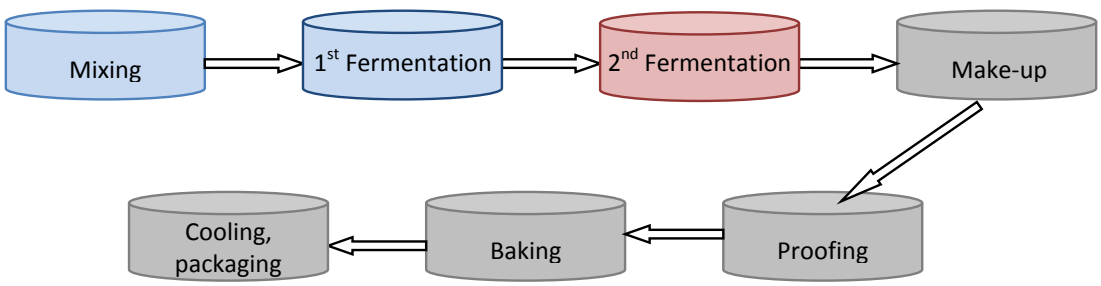

Figure 3.1 The principles of straight dough method

3.2 Yeasts in sourdough (PAPER II)

Microbiological studies have shown that more than 20 yeast species have been isolated and identified from different sourdoughs (De Vuyst and Neysens, 2005). The most representative yeast species in both rye and wheat sourdough belong to the genera *Saccharomyces* and *Candida* (**Table 3.1**). *Saccharomyces cerevisiae*, *Candida krusei* and *Candida humilis* (synonym *Candida milleri*) are the most frequently encountered yeast species, followed by *Kazachstania exigua* (formerly named as *Saccharomyces exiguus*, anamorph *Candida* (*Torulopsis*) *holmii*) and *Pichia kudriavzevii* (formerly named as *Issatchenkia orientalis*, anamorph *C. krusei*). Less frequently detected yeast species in sourdough ecosystem are, *Hansenula anomala* (synonym *Pichia anomala*), *Pichia saitoi*, *Pichia membranaefaciens*, *Debaryomyces hansenii* and *Saccharomyces uvarum* (Succi et al., 2003; Meroth et al., 2003a; Foschino et al., 1999; Gobbetti et al., 1994a).

The extensive variability in the number and type of yeast species found in sourdoughs are influenced by several factors, including leavening temperature, pH, yeast propagation cycles, the flour type, dough hydration, environment and location (Gobbetti, 1998; Rossi, 1996). In generally, the yeasts present in sourdough are acid-tolerant to the organic acid produced by LAB (Mäntynen et al., 1999).

Saccharomyces cerevisiae was found to be the dominant species in five out of seven tested Lithuanian rye sourdoughs, followed by *P. kudriavzevii* which was isolated from the other two sourdoughs out of seven (PAPER II). Valmorri et al. (2010) reported that *S. cerevisiae* accounted for 85% of all isolates found in 20 wheat sourdoughs samples collected from different artisan bakeries in Italy. Other dominant species were *C. milleri* (11%), *C. krusei* (2.5%), and *Torulaspora delbrueckii* (1%). *Saccharomyces cerevisiae* was also the dominating yeast in homemade rye sourdoughs from Finland (Salovaara and Savolainen, 1984).

Candida humilis was found to be the predominant species in Danish rye as well as in wheat sourdoughs, whereas *K. exigua* and *P. kudriavzevii* dominated only in rye sourdough (PAPER II). Gullo et al. (2003) and Garofalo et al. (2008) found *C. humilis* in sourdough from Italy for the production of durum wheat bran bread and Panettone (sweet Italian bread), respectively.

Table 3.1 Yeasts isolated from wheat and rye sourdoughs from different countries

Species	Country		References
	Wheat sourdough	Rye sourdough	
C. humilis	Italy, Denmark	Denmark	(PAPER II) (Garofalo et al., 2008; Gullo et al., 2003)
C. holmii	Italy	Germany	(Meroth et al., 2003b; Foschino et al., 1999)
C. krusei	Italy	Germany	(Valmorri et al., 2010; Meroth et al., 2003b; Gobbetti et al., 1994a; Spicher et al., 1979)
C. milleri	Italy, Morocco	Finland	(Valmorri et al., 2010; Vernocchi et al., 2004; Mäntynen et al., 1999; Boraam et al., 1993)
C. stellata	Italy		(Zorzanello and Sugihara, 1982)
D. hansenii	Greece	Germany	(Meroth et al., 2003a; Paramithiotis et al., 2000)
H. anomala	Italy		(Gobbetti et al., 1994a)
I. orientalis	Portugal		(Almeida and Pais, 1996)
[a]*K. exigua*		Denmark	(PAPER II)
M. pulcherrima	Italy		(Palomba et al., 2011)
[b]*P. kudriavzevii*		Denmark, Lithuania	(PAPER II)
[c]*P. occidentalis*		Denmark	(PAPER II)
P. membranaefaciens	Greece Portugal		(Paramithiotis et al., 2000; Almeida and Pais, 1996)
P. saitoi		Germany	(Spicher et al., 1979)
S. cerevisiae	Italy, China, Greece, Morocco Portugal	Denmark, Finland, Germany, Lithuania	(PAPER II) (Palomba et al., 2011; Zhang et al., 2011; Vernocchi et al., 2004; Meroth et al., 2003a; Corsetti et al., 2001; Paramithiotis et al., 2000; Rosenquist and Hansen, 2000; Rocha and Malcata, 1999; Gobbetti et al., 1994a; Boraam et al., 1993; Strohmar and Diekmann, 1992; Salovaara and Savolainen, 1984; Spicher et al., 1979)
S. exiguus	Italy		(Gobbetti et al., 1994b; Zorzanello and Sugihara, 1982; Sugihara et al., 1971)
S. uvarum		Germany	(Meroth et al., 2003a)
Tr. delbrueckii	Italy Portugal		(Valmorri et al., 2010; Almeida and Pais, 1996)
T. holmii		Germany, Finland	(Salovaara and Savolainen, 1984; Spicher et al., 1979)
Y. lipolytica	Greece		(Paramithiotis et al., 2000)

C., *Candida*; *D.*, *Debaryomyces*; *H.*, *Hansenula*, *I.*, *Issatchenkia*; *M.*, *Metschnikowia*; *P.*, *Pichia*; *S., Saccharomyces*; *Tr.*, *Torulaspora T., Torulopsis*; *Y. Yarrowia*

[a]*Kazachstania exigua* (formerly named as *Saccharomyces exiguus*, anamorph *Candida holmii*)

[b]*Pichia kudriavzevii* (formerly named as *Issatchenkia orientalis*, anamorph *Candida krusei*)

[c]*Pichia occidentalis* (formerly named as *Issatchenkia occidentalis*)

3.3 Lactic acid bacteria in sourdough (PAPER III)

Microbiological studies have shown that the most relevant bacteria isolated from both rye and wheat sourdoughs mostly belong to the genus *Lactobacillus* (for references see **Table 3.2**). Several studies reported the presence also of non-*lactobacillus* species, such as *Leuconostoc*, *Pediococcus*, and *Weissella* which can dominate during the early fermentation process (Paramithiotis et al., 2005; Corsetti et al., 2001).

Lactobacilli isolated from sourdough, dependently on their carbohydrate fermentation type may consist of obligately heterofermentative, facultatively heterofermentative or obligately homofermentative species (**Table 3.2**). Facultatively heterofermentative and obligately homofermentative lactic acid bacteria (LAB) are able almost completely to ferment hexoses to lactic acid by Embden-Meyerhof-Parnas (EMP) pathway, whereas obligately heterofermentative species ferment hexoses by phosphogluconate pathway producing lactate, ethanol or acetic acid and carbon dioxide. Pentoses are fermented by heterofermentative LAB, whereas homofermentative LAB lack phosphoketolase and are not able to ferment pentoses (Kandler and Weiss, 1986).

The variation in the composition of microorganisms isolated from sourdough depends on the fermentation processes and conditions, such as flour type and extraction rate, fermentation temperature and time, water content in dough. On the basis of the technology applied, three types of sourdough can be distinguished; type I, type II, and type III (Chavan and Chavan, 2011; Hansen, 2004; Vogel et al., 1999; Kandler and Weiss, 1986). Type I is so-called traditional sourdough based on spontaneous fermentation and continuous refreshments (every 24-48 h) at ambient temperature (20-30°C) to keep the microorganisms metabolically active. Type II sourdough is based on large scale one-step fermentation in an industrialized environment at higher temperatures (40-50°C), higher hydrations (easily pump able in an industrial bakery) and longer fermentation times (from 2 up to 5 days). Type III sourdough is based on dough fermentation initiated by adding defined starter culture (commercially available).

Table 3.2 Lactic acid bacteria isolated from wheat and rye sourdoughs from different countries

Species	Country		References
	Wheat sourdough	Rye sourdough	
Obligately heterofermentative			
L. acidifarinae	Belgium		(Vancanneyt et al., 2005)
L. brevis	Greece Italy Morocco Portugal	Germany Sweden	(Vancanneyt et al., 2005; De Vuyst et al., 2002; Rocha and Malcata, 1999; Vogel et al., 1994; Boraam et al., 1993; Okada et al., 1992; Spicher and Lonner, 1985)
L. buchneiri	Morocco		(Boraam et al., 1993)
L. fermentum	Lithuania	Germany Sweden Lithuania	(PAPER III) (Vogel et al., 1994; Spicher and Lonner, 1985)
L. fructivorans		Germany	(Vogel et al., 1994)
L. frumenti		Germany Lithuania	(PAPER III) (Müller et al., 2000)
L. hilgardi	Italy		(Okada et al., 1992)
L. namurensis	Belgium	Belgium	(Scheirlinck et al., 2007a)
L. panis		Denmark Germany	(Rosenquist and Hansen, 2000; Wiese et al., 1996)
L. pontis	Belgium	Germany	(Scheirlinck et al., 2007a; Vogel et al., 1994)
L. reuteri	California	Lithuania	(PAPER III) (Okada et al., 1992)
L. rossiae	Italy		(Valmorri et al., 2006; Corsetti et al., 2005)
L. sanfranciscensis	Belgium Greece Italy	Germany	(Scheirlinck et al., 2007a; Valmorri et al., 2006; Corsetti et al., 2005; De Vuyst et al., 2002; Vogel et al., 1994; Okada et al., 1992; Strohmar and Diekmann, 1992)
L. viridescens		Sweden	(Spicher and Lonner, 1985)
L. zymae	Belgium Greece		(Vancanneyt et al., 2005)
Facultatively heterofermentative			
L. alimentarius	Italy		(Valmorri et al., 2006)
L. casei subsp. *casei*	Morocco	Switzerland Sweden	(Boraam et al., 1993; Okada et al., 1992) (Spicher and Lonner, 1985)
L. casei subsp. *rhamnosus*			
L. hammesi	France		(Valcheva et al., 2005)
L. nantensis	France		(Valcheva et al., 2005)
L. paralimentarius	Italy Belgium, Greece	Japan	(Scheirlinck et al., 2007b; Valmorri et al., 2006; De Vuyst et al., 2002; Cai et al., 1999)
L. plantarum	Italy Belgium Morocco	Sweden	(Scheirlinck et al., 2007b; Valmorri et al., 2006; Boraam et al., 1993; Spicher and Lonner, 1985)
Obligately homofermentative			
L. amylovorus		Denmark	(Rosenquist and Hansen, 2000)
L. acidophilus		Denmark Sweden	(Rosenquist and Hansen, 2000; Spicher and Lonner, 1985)

L. curvatus	California Portugal	Switzerland	(Rocha and Malcata, 1999; Okada et al., 1992)
L. delbrueckii subsp. *delbrueckii*		Sweden	(Spicher and Lonner, 1985)
L. farciminis		Sweden	(Spicher and Lonner, 1985)
L. mindensis		Germany	(Ehrmann et al., 2003)
P. pentosaceus	Greece	Lithuania	(PAPER III) (Gul et al., 2005; De Vuyst et al., 2002; Paramithiotis et al., 2000)

L., Lactobacillus; *P., Pediococcus*

Obligately heterofermentative *Lactobacillus sanfranciscensis* are the predominant LAB in type I sourdoughs (Siragusa et al., 2009; Kitahara et al., 2005; Corsetti et al., 2001; Gobbetti, 1998). Also other obligately heterofermentative LAB species, such as *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus rossiae*; facultatively heterofermentative *Lactobacillus alimentarius*, *Lactobacillus paralimentarius* and *Lactobacillus plantarum*; and obligately homofermentative *Lactobacillus farciminis*, *Pediococcus pentosaceus* and *Weissella* spp. have been found in spontaneous traditional and homemade sourdoughs that are characterized by low incubation temperatures and continuous back-slopping (PAPER III) (Siragusa et al., 2009; Corsetti et al., 2001; Gobbetti, 1998).

The dominant strains in industrial processes of type II sourdoughs with an extended fermentation period and higher temperature are mainly heat- and acid- tolerant obligately heterofermentative LAB, such as *Lactobacillus pontis*, *Lactobacillus panis*, *Lactobacillus reuteri*, *Lactobacillus frumenti* and *L. fermentum* (PAPER III) (Vogel et al., 1999; Wiese et al., 1996). However, homofermentative *Lactobacillus amylovorus* have also been isolated from this type of sourdough (Vogel et al., 1999).

Commercially available dried sourdough starters commonly consist of mixture of different LAB to assure good acidification and aromatization. *Lactobacillus plantarum*, *Lb. brevis* and *Pediococcus pentosaceus* have been identified from these starters (Lönner et al., 1986).

3.4 Identification of yeast and lactic acid bacteria (PAPER II and PAPER III)

For many years yeasts and bacteria have traditionally been classified on the basis of their morphology and physiological-biochemical properties (Barnett et al., 2000). However, classical methods are time and material consuming. They require technical expertise and can yield ambiguous results because the reliability and distinctive capacity of closely related isolates are low (Verweij et al., 1999).

Alternative to traditional methods, molecular methods for fungal and bacterial identification have been developed. At the end of 20 century genome comparison was shown to be a valuable tool in yeast classification (Price et al., 1978). Moreover, invention of polymerase chain reaction (PCR) technology by Kary Mullis (Mullis, 1990) contributed to the progress in a molecular biology and revolutionized the identification of yeast and bacteria.

3.4.1 Repetitive element sequence-based polymerase chain reaction (rep-PCR)

Repetitive element sequence based-polymerase chain reaction (rep-PCR) was shown to be the rapid and accurate method for sourdough yeast (PAPER II) (Andrade et al., 2006) and LAB (PAPER III) (Van der Meulen et al., 2007) identification.

The rep-PCR method was developed based upon the observation that each bacterial and fungal genome contains numerous conserved non-coding repetitive elements. These repetitive elements are distributed more or less randomly over the genome with intervening sequences of variable length which are specific for each fungal and bacterial strain (Versalovic et al., 1991). Therefore, enzymatic amplification using specific primers designed to amplify intervening DNA between two adjacent repetitive elements results in variable sized amplicons. Subsequently, size-based analysis of these amplicons provides a 'molecular fingerprint' (**Figure 3.2**) that can be used to group bacteria and yeast at the species level as well as to discriminate them between two closely related species (Hierro et al., 2004; Morakile et al., 2002; Lopes et al., 1998).

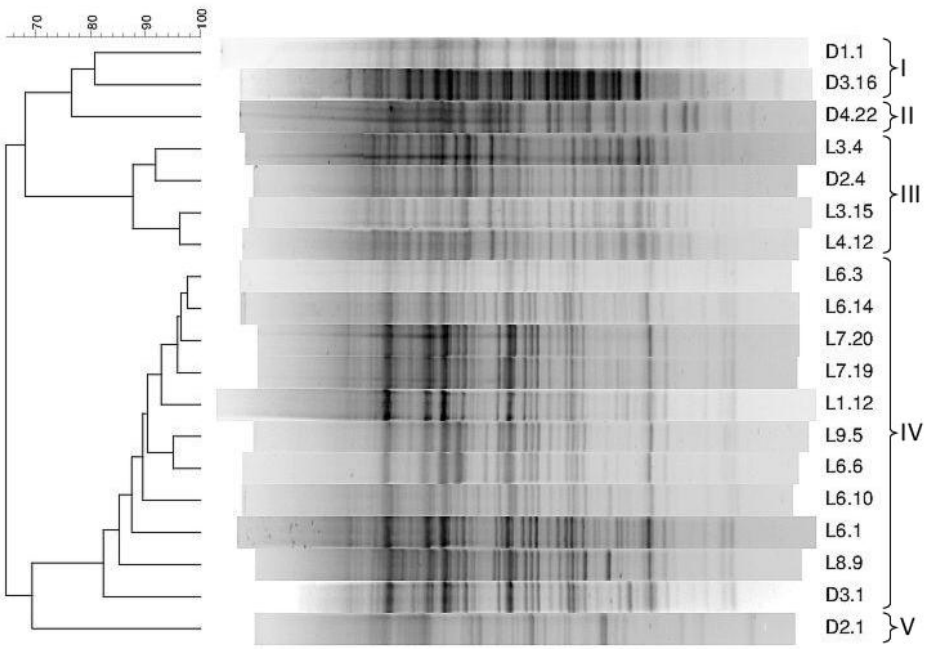

Figure 3.2 rep-PCR-based dendrogram and gel image representing the fingerprint patterns of isolates, belonging to the following species; I – *Candida humilis*, II – *Pichia occidentalis*, III – *Pichia kudriavzevii*, IV – *Saccharomyces cerevisiae*, V – *Kazachstania exigua* (PAPER II)

rep-PCR principles

Typically, PCR consist of an approximately 30 repeated cycles. Each of the cycles includes 3 steps; (1) template denaturation, (2) primer annealing, and (3) primer extension (**Figure 3.3**).

(1) *Template denaturation*. The first step in each cycle requires a high temperature (94-95°C) to denature the DNA molecule which carries a target sequence. During denaturation two complimentary DNA strands are separated.

(2) *Primer annealing*. The second step in each cycle requires lowering the temperature (40-45°C) to allow annealing of the primers to the DNA. It is very important to choose the right temperature which depends on the melting temperature of the primer. If the temperature is too low the primers may bind non-specifically while if the temperature is too high the primers cannot anneal efficiently.

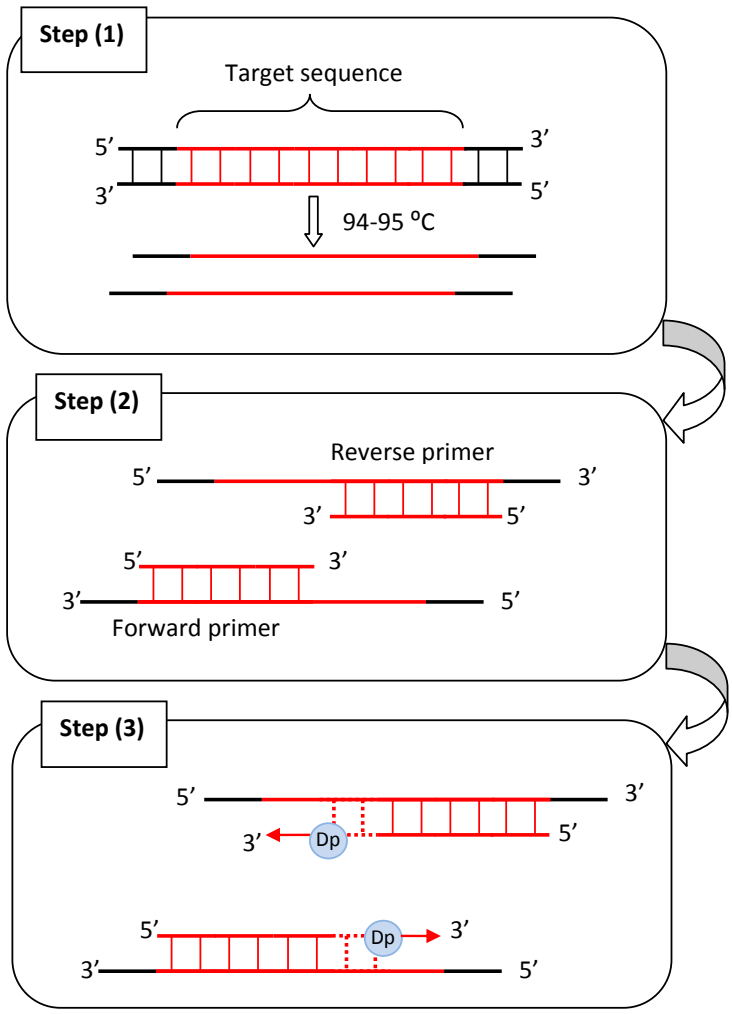

Figure 3.3 Outline of PCR technique; step (1), template denaturation; step (2), primer annealing; step (3), primer extension.

Primers used for rep-PCR fingerprinting are designed to have a sequence which is the reverse complemented of a target DNA. Thus, short polynuceotides primers, such as $(GTG)_5$, REP1R-Dt, and REP2R-Dt have been found to be a promising genotypic tool for rapid and reliable speciation and typing of yeasts and lactobacilli (Nielsen et al., 2007; Versalovic et al., 1991).

(3) *Primer extension*. The third step in each cycle requires extension of the annealed primers from the 3'-ends of both DNA strands by DNA polymerase. To withstand the

repeated exposure to high temperature, a thermostable DNA polymerase is used, which attach additional complementary nucleotides to the developing DNA strand.

Prior to the first cycle, during an initialisation step, the rapid increase of temperature is used to completely denature DNA. After the last cycle the final elongation step is used to ensure that any remaining single-stranded DNA is completely copied.

3.4.2 Ribosomal RNA (rRNA) sequencing

Nowadays, ribosomal RNA (rRNA) sequencing is the most commonly used method to identify sourdough-related microorganisms (Meroth et al., 2003a; Vogel et al., 1994). For bacteria and yeast many gene targets have been recognized as useful tools for identification. The main requirement for selected gene target is to have a conserved segment that is common to all yeast (bacteria) and that is flanked by variable regions. During cycle sequencing, variable regions generate unique nucleotide base fragments (sequence), so-called 'signature' for different species. Subsequently, the obtained sequences are compared with reference sequences that are deposited in GenBank (http://www.ncbi.nlm.nih.gov). Nucleotide sequences are generally reported in terms of 'percent identity' between a pair of sequences and most taxonomists accept a percent identity of ≥97% and ≥99% to classify microorganism to genus and species, respectively. In this study all sequenced yeast and LAB isolates showed 99.8-100% similarities to sequences in GenBank (PAPER II and PAPER III).

Many studies for yeast identification have employed the sequencing of the D1/D2 domain of the 26S rRNA gene. The D1 and D2 are located approximately in the first 650 bases of the larger-subunit 26S rRNA gene. These domains are highly variable and divergent among closely related yeast species and therefore are ideal for identification purpose (PAPER II) (Kurtzman and Robnett, 1998).

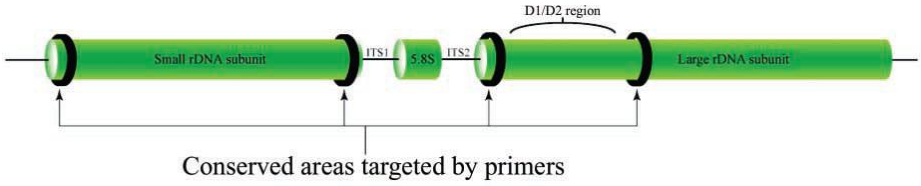

Figure 3.4 Small- and large-subunit rRNA genes; circles represent conserved regions that serve as gene targets for RNA sequencing of yeast. Figure adapted from Petti (2007).

The gene target that is mostly used for bacterial identification is 16S rRNA (**Figure 3.5**). This gene was selected as a candidate molecule for a number of reason; (1) its present in almost all bacteria; (2) its sequence is sufficiently conserved and has not changed over time; (3) the 16S rRNA gene is large enough (approximately 1500 bases) for informatics purpose (Patel, 2001).

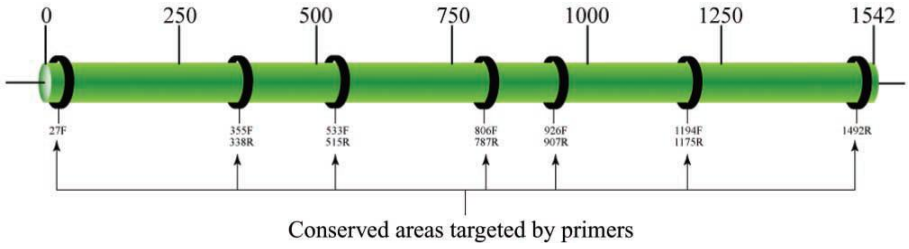

Figure 3.5 16S gene; circles represent conserved regions that serve as gene targets for DNA sequencing of bacteria. Figure adapted from Petti (2007).

Chapter 4

Phytates in Cereals

Phytate has for the first been described by Hartig (1855) as small, non-starch round particles isolated from various plants seeds. In addition, phytate has been shown to be a principal storage form of phosphorus in plant tissue, typically accounting for 60-90% of total seed phosphorus (Reddy, 2002). Although phytate has an important role in plants, in the human nutrition it is considered to be the most important antinutritional compound that reduces the bioavailability of essential mineral (Lopez et al., 2002; Brune et al., 1992). In this chapter structure, occurrence and content as well as the importance of phytate will be discussed.

4.1 Structure of phytic acid

Phytic acid also known as *myo*-inositol 1,2,3,4,5,6-hexakis or phytate as phytic acid salt consist of a *myo*-inositol ring with six phosphate groups esterified to each carbon atom (**Figure 4.1**). Two pH-dependent conformations of phytic acid have been described in the literature: the low-pH possesses one axial and five equatorial phosphates (1a/5e), whereas the high-pH form has and inverted 5a/1e structure (Costello et al., 1976). The fact that phytic acid can have different stereochemistry conformations over a wide pH range makes this compound quite reactive during the processing and consumption of food.

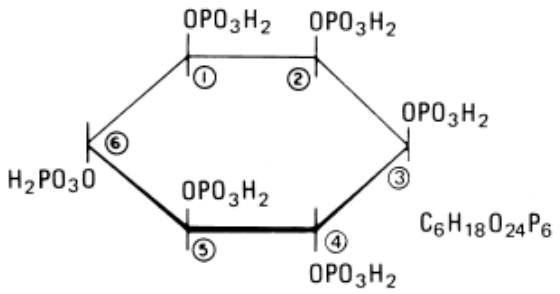

Figure 4.1 Structure of phytic acid

Many different terms for phytic acid are used in the literature. In the following text free *myo*-inositol 1,2,3,4,5,6-hexakis will be termed phytic acid or abbreviated IP_6, while phytic acid salts will be termed phytate. The following abbreviations will be used for the degradation products of phytic acid/phytate: IP_5 for *myo*-inositol penta-, IP_4 for *myo*-inositol tetra-, IP_3 for *myo*-inositol tri, IP_2 for *myo*-inositol di-, and IP_1 for *myo*-inositol mono-phosphate. IP_1, IP_2, IP_3, IP_4 and IP_5 will also be referred to as lower inositol phosphates.

4.2 Biological function and occurrence of phytic acid/phytate in cereals

Phytic acid is widely distributed in most cereal grains, legumes, nuts, and oilseeds (Reddy, 2002). In both cereals and legumes, phytic acid accumulates rapidly during seeds ripening, reaching a maximum level at maturity (Bohn et al., 2008; Raboy and Dickinson, 1987). In mature seeds phytic acid mainly occurs as a mixed calcium-magnesium-potassium salt (Ockenden et al., 2004; Graf, 1983). A few important physiological roles have been proposed for the accumulation of phytate in seeds. It is widely accepted that phytate serves a major store of phosphorus, typically accounting for 60-90% of total seed phosphorus, and *myo*-inositol for growing seeds (Reddy et al., 1989). Further, phytate have been reported to be protective against oxidative stress during the seed life span (Ali et al., 2010).

In whole wheat grains the content of phytate varies from 0.32% to 1.55% of the kernel weight, whereas a slightly higher content of phytate was observed in whole rye grains (0.54-1.87%) (**Table 4.1**).

Table 4.1 Phytate content in wheat and rye cereals

Cereal	Number of varieties	Phytate content, %	Reference
Wheat			
	3	1.16-1.36	(Averill and King, 1926)
	38	0.66-1.35	(Lolas et al., 1976)
		0.32	(O'Dell et al., 1972)
		1.15	(De Boland et al., 1975)
		0.9	(Khokhar et al., 1994)
	2	0.82-0.96	(Morris and Ellis, 1981)
	11	0.39-1.35	(Frossard et al., 2000)
		0.49-0.93	(Kasim and Edwards, 1998)
Rye			
	3	1.04-1.87	(Averill and King, 1926)
	11	0.54-1.46	(Frossard et al., 2000)
	16	0.81	(Fretzdorff and Weipert, 1986)

The distribution of phytate varies in different cereals. In the case of wheat, rye, rice, nearly 85% of the phytate is found in the pericarp and aleurone layers, while the major proportion of phytate in corn exist in the germ (Fretzdorff and Weipert, 1986; Cheryan, 1980; O'Dell et al., 1972). The phytate distribution between wheat aleurone layer, germ, and endosperm have been shown to be approximately 86%, 12%, and 2% , respectively (O'Dell et al., 1972). In evaluating 38 wheat varieties, Lolas et al. (1976) found a phytate range of 0.66-1.35% dry weight in whole kernels whereas the bran portion had phytate levels ranging from 4.59 to 5.52% dry weight. Anjum et al. (2002) corroborate these earlier findings by measuring phytate content in different milling fractions of spring wheat. He found that phytate content in wheat bran was at least three times higher than in whole-wheat flour and about 10-12 times higher than in straight-grade flour.

4.3 Formation of phytic acid-mineral complex

The unique chemical structure of phytic acid molecule contains 12 dissociable protons with pK_a values from 1.5 to 9.5. Six protons have been shown to be strongly ionized with pK_a of *app.* 1.5, two – weakly ionized with pK_a of *app.* 6-7, and four – very weakly - with pK_a of *app.* 9.5 (Evans et al., 1982; Costello et al., 1976). Thus, phytic acid has a strong chelating potential at broad pH region. Phytic acid easily chelate positively charged nutritionally important dvivalent minerals such as calcium (**Figure 4.2A**), magnesium, zinc, cooper, iron and potassium, as well as proteins (**Figure 4.2B**) and starch (**Figure 4.2C**), and forms very stable insoluble complexes. Such complex formation was well documented to obstruct mineral absorption in human and animal intestine (Joung et al., 2007; Davidsson et al., 1995; Hurrell et al., 1992; Sandström, 1987). The stability of phytic acid-mineral complexes as well as factors affecting their solubility will be discussed further in sub-chapter 4.5.

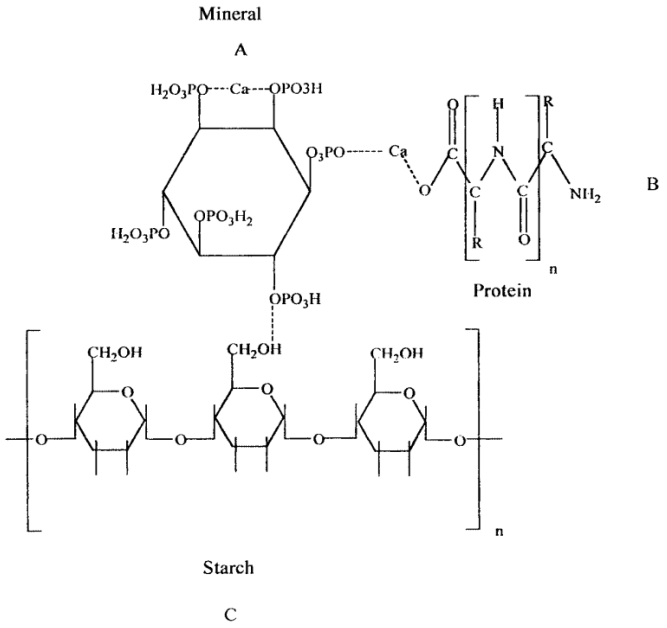

Figure 4.2 Interaction of phytic acid with minerals (**A**), proteins (**B**), and starch (**C**), according to Rickard and Thompson (1997)

4.4 Antinutritive effects of phytate and micronutrients deficiencies

During the past 30 years many studies have focused on phytate as antinutritional factor in the human's diet (Sandberg et al., 1999; Wise, 1995; Sandström and Sandberg, 1992; Brune et al., 1992). As previously described, the antinutritional aspect of phytate occurs due to efficient chelation of nutritionally important dvivalent minerals and proteins at intestinal pH values, forming precipitated complexes that are not available for intestinal uptake (Cheryan, 1980). Phytate has been shown to decrease the human absorption of important minerals such as iron (Hallberg et al., 1987), zinc and calcium (Fredlund et al., 2006; Sandström and Sandberg, 1992; Lönnerdal et al., 1989). Moreover, it was well documented that the diets high in cereals and legumes are also high in phytate; therefore these diets are low in bioavailable minerals and may lead to mineral deficiencies (Harinarayan et al., 2007; Hallberg et al., 1987; Forbes et al., 1984).

4.4.1 Phytate and Fe^{2+} bioavailability

According to the World Health Report (WHO, 2002) iron deficiency is the most common nutritional deficiency, widespread in both developing and developed countries. Its deficiency leads mainly to anemia fatigue and cognitive impairment as well as reducing growth and physical strength. Over two billion people are affected by iron deficiency because of the high iron demands of infant growth and pregnancy (Stoltzfus and Dreyfuss, 1998). In Denmark, many teenage girls (15%) and pre-menopausal women (up to 40%) as well as pregnant women (75-80%) have low or depleted iron store. Also many teenage boys have low iron status (Fødevaredirektoratet, 2002).

A number of studies have shown that phytate content in food is strongly related to iron absorption in human intestine (Brune et al., 1992; Hallberg et al., 1989). In a study of Hallberg (1987) the marked inhibitory effect of bran on iron absorption was observed. He showed that 5-10 mg of phytate added to a wheat roll containing 3 mg iron inhibited iron absorption by 50%. Moreover, Sandberg et al. (1989) concluded that addition of isolated inositol hexa- and penta-phosphates to a white-wheat roll under simulated physiological conditions significantly reduced the iron absorption. In agreement with these findings Mendoza et al. (1998) also observed that the iron absorption from tortillas made with genetically modified low phytate maize (368 mg phytate per 100 g) was about 50% higher than from tortillas made with native maize (847 mg phytate per 100 g).

4.4.2 Phytate and Zn^{2+} bioavailability

Zinc represents the other nutritionally significant mineral that has been associated with phytate binding. Zinc is essential for the function of many enzymes and thus is involved in a large number of metabolic processes, including RNA and DNA synthesis. It was shown that persons suffering from zinc deficiency had depressed immunity, impaired taste and smell, onset of night blindness and impairment of memory (Shankar and Prasad, 1998; Walsh et al., 1994).

Numerous studies have implicated phytate as a causative factor in poor zinc absorption from cereal-based foods. In 1973, Reinhold and co-authors established a general inverse relationship of phytate-rich bread and zinc bioavailability from these diet (Reinhold et al., 1974). This finding was also supported by studies on bread made from different extraction rate wheat flour (Sandström et al., 1980). Studies on zinc bioavailability from different foods showed that zinc absorption from phytate-rich soy is much lower than from milk and milk-based foods (Sandström et al., 1983). Several subsequent single meal studies also clearly showed the negative correlation between the presence of phytate in food matrix and zinc absorption (Sandström and Sandberg, 1992; Kivistö et al., 1989; Nävert et al., 1985).

4.4.3 Phytate and Ca^{2+}, Cu^{2+}, Mn^{2+} and Mg^{2+} bioavailability

Studies on humans have also showed an inhibitory effect of phytate on calcium absorption as well as on other minerals (Rimbach et al., 1995; Lönnerdal et al., 1989; Reinhold et al., 1973). Lönnerdal (1989) found that phytate had strong inhibitory effect on calcium and zinc in sucking rats. Moreover, the negative effect appeared to be more pronounced for absorption of zinc than for calcium. Studies done by Nickel et al. (1997) on rat model failed to observe any negative effect of phytate on calcium absorption.

An inhibition effect of phytate on mineral absorption was also reported for magnesium (Bohn et al., 2004; Reinhold et al., 1976) and manganese (Davidsson et al., 1995), However, phytate does not appear to influence cooper absorption in human intestine (Turnlund et al., 1985).

4.4.4 The effect of phytate on protein digestibility

Phytate is known to form strong complexes with positively charged proteins at both acidic and alkaline pH (Cheryan, 1980). This interaction may affect changes in protein structure that can obstruct enzymatic activity, protein solubility and protein digestibility. *In vitro* studies have shown that the extent of phytate-protein interactions depend on various factors, such as pH, the source of protein and dietary levels of magnesium and calcium (Kemme et al., 1999; Cheryan, 1980).

It was also observed that phytates inhibit the digestive enzymes such as α-amylase (Knuckles and Betschart, 1987; Deshpande and Cheryan, 1984), lipase (Knuckles, 1988) or proteases such as trypsin and chymotrypsin (Deshpande and Damodaran, 1989; Knuckles, 1988; Singh and Krikorian, 1982).

4.5 The solubility and stability of phytic acid-mineral complex

Studies have shown that the solubility of phytic acid-mineral complexes and mineral availability depends on factors such as pH of the solution, the molar ratios of minerals to phytic acid, and the phosphate groups bond to the inositol molecule (Siener et al., 2001; Frossard et al., 2000; Persson et al., 1998; Cheryan, 1980; Jackman and Black, 1951). Further, the interaction of phytate with single minerals and their binding strength differs for different minerals but generally increases with increasing atomic mass (Persson et al., 1998; Vohra et al., 1965). Vohra et al. (1965) found that the order of the ability of the mineral cations to form complexes with phytic acid is: $Cu^{2+}>Zn^{2+}>Ni^{2+}>Co^{2+}>Mn^{2+}>Ca^{2+}$. Similar binding order was also shown by Cheryan (1980) and Persson et al. (1998). They reported that phytic acid with Zn^{2+} and Cu^{2+} forming the most stable complexes followed by Mn^{2+}, Ca^{2+} and Fe^{2+} in decreasing order of stability.

4.5.1 pH and the solubility of phytic acid-mineral complex

The effects of pH on phytic acid-mineral complex have been intensively studied over the pH range of 2.0 to 12.0 (Champagne and Phillippy, 1989; Kaufman and Kleinberg, 1971). Most phytates tend to be more soluble at lower pH compared to higher pH value (Torre et al., 1991). The solubility of Ca-, Zn- or Cu-phytate increases at pH values lower than 4-5, whereas Mg-phytate is soluble at pH up to 7.5

(Brown et al., 1961). In contrast, Fe-phytate is insoluble at pH values in the range of 1.0 to 3.5 and solubility increases at pH above 4. Moreover, it was shown that with decrease in the number of phosphate groups presented in the inositol molecule, the pH required to precipitate the salts of the various inositol phosphate increased asymptotically (Champagne and Phillippy, 1989).

4.5.2 The molar ratio of minerals to phytic acid

The molar ratio of minerals to phytic acid is an important determinant of mineral bioavailability from human diets.

Hurrell et al. (1992) measured iron absorption in adult human fed with soy protein. He found that decreasing the molar ratios of IP_6:Fe from 5.17 to 2.16, did not improve iron absorption, which remainded almost at the same level (10-24%). Decreasing the molar ratios to 0.47, the iron absorption increased approximately two-fold, while decreasing the molar ratios to 0.17 and below the iron absorption increased approximately three- four-fold. Summarizing these results the authors concluded that the IP_6:Fe molar ratios should be below 1 and preferably 0.4 to predict increase in iron bioavailability (Hurrell, 2004). Siegenberg et al. (1991) varied phytate levels in iron-fortified wheat bread rolls mead from wheat flour in combination with phytate-containing and phytate-free maize bran. They reported that iron absorption was very similar when the phytate content was increased from 10 to 14 mg per meal. However, when the phytate content was increased to 22 mg per meal, the iron absorption was significantly inhibited.

A daily IP_6:Zn molar ratios below 10 are said to have little effect on the bioavailability of zinc, whereas ratios above 20 have been associated with biochemical and/or clinical evidence of zinc deficiency (Oberleas and Harland, 1981). However, Bosscher et al. (2001) found that IP_6:Zn molar ratios a little below 8 markedly decrease zinc availability from the green beans, whereas Bindra et al. (1986) reported that molar ratios above 17 was critical for zinc absorption in Punjabi diets. Moreover, calcium has been reported to exacerbate the inhibitory effect of phytate on zinc absorption (Fordyce et al., 1987; Morris and Ellis, 1980). *In vitro* studies have shown that human who have a [Ca][IP_6]:[Zn] ratio >200 may have increased risk of impaired Zn bioavailability (Bosscher et al., 2001; Ellis et al., 1987).

4.5.3 Myo-inositol derivatives and the solubility of their salts

Lönnerdal et al. (1989) have found that *myo*-inositol hexa- (IP_6) and penta-phosphates (IP_5) inhibit zinc and calcium absorption in sucking rats, while no effect was observed for the lower *myo*-inositol phosphates such as tetra- (IP_4) and tri-phosphate (IP_3). In agreement with this findings Sandström & Sandberg (1992) have shown that IP_6 and IP_5 inhibit zinc absorption in humans, whereas the lower inositol phosphates had no significant effect. Added IP_4 to white bread also had no effect on zinc absorption in humans, whereas IP_5 and IP_6 depressed zinc absorption (Sandström and Sandberg, 1992). Addition of IP_5 and IP_6 to a white-wheat roll under simulated physiological conditions had a strong negative effect on iron solubility, but no effect was found for IP_4 and IP_3 (Sandberg et al., 1989). However, studies of iron absorption in a human from different bread meals showed a strong negative correlation between the sum of IP_6-IP_3 and iron absorption, suggesting that IP_4 and IP_3 also inhibit iron absorption (Sandberg et al., 1999; Brune et al., 1992).

Persson et al. (1998) studied the interaction of different inositol phosphates with minerals at pH 5-7 and observed that the higher inositol phosphates (IP_6 and IP_5) bound more metal ions than lower inositol phosphates (**Table 4.2**). Moreover, it was shown, that the mineral binding capacities as calculated per phosphate group was similar for IP_6, IP_5, IP_4 and IP_3, but the binding strength was lower for the IP_4 and IP_3 (Persson et al., 1998). It was in agreement with Kaufman and Kleinberg (1971) findings, who claimed that cleavage of phosphate groups from inositol phosphate results in a weaker complexes, followed by increase in solubility. In addition, Sandberg et al. (1999) reported that in order to improve iron absorption from cereal-based foods, degradation of phytates and its dephosphorylated isomers IP_5, IP_4 and IP_3 is needed.

Table 4.2 Number of metal ions bound per inositol molecule at pH ~ 5-7 (Persson et al., 1998)

IP_i	$n_{Cu^{2+}}$	$n_{Zn^{2+}}$	$n_{Cd^{2+}}$
IP_6	5.8	4.9	5.3
IP_5	5.7	4.8	5.1
IP_4	3.3	3.0	3.3
IP_3	3.1	3.0	2.4

4.6 Therapeutic potential of phytate

Despite of the negative impact of phytate on food nutrition value which was discussed above, some novel positive metabolic effects of phytate or some of its degradation products have been recognized.

Dietary phytate has been reported to prevent kidney stone formation (Grases et al., 2000) and against dental caries (McClure, 1963). Numerous studies in the medical literature have shown phytate to inhibit the formation of free radicals (-$OH^{\bullet}$), thus playing role as antioxidant and anticarcinogenic (Graf and Eaton, 1990; Graf et al., 1984). Other advantages of phytate capacity to bind cations are referred to as improving the glucose response (glycemic index) (Yoon et al., 1983) as well as decreasing plasma cholesterol and triglycerides (Katayama, 1995). Moreover, it was shown that phytate is responsible for detoxification of heavy metals such as cadmium and lead (Rimbach and Pallauf, 1997).

Chapter 5

Phytases; Enzymatic Degradation of Phytate

Phytases (*myo*-inositol hexakis phosphohydrolase) are a specific class of phosphohydrolases capable to catalyse the stepwise dephosphorylation of phytate to lower phosphoric esters of *myo*-inositol and phosphoric acid via penta- to monophosphates. This enzymatic activity produces available phosphate and non-chelated minerals for human absorption (Konietzny and Greiner, 2002). The aim of this chapter is to sketch an overall picture and call attention to the general characteristics of the phytases and to provide an overview of currently known phytase sources. Moreover, the phytase activities in yeasts isolated form grain-based food (PAPER I) and sourdoughs (PAPER II) as well as phytase activities in lactic acid bacteria isolated from sourdoughs (PAPER III) will be discussed.

5.1 Classification of phytases

Phytases have been categorized on three bases: depending on the carbon position of the *myo*-inositol ring from which the hydrolysis of phytate is initialled, their catalytic mechanism and pH of activity.

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) in consultation with the International Union of Pure and Applied Chemistry (IUPAC-IUBMB) Joint

Commission on Biochemical Nomenclature (JCBN) phytases are classified into three subgroups based on the carbon in the *myo*-inositol ring of phytate at which dephosphorylation is initiated; i.e. 3-phytase (EC 3.1.3.8), 5-phytase (EC 3.1.3.72) and 4-phytase, frequently called 6-phytase when name based on 1L-numbering system and not on 1D-numbering (EC 3.1.3.26). In the following text 4(D)/6(L)-phytase will be termed 4-phytase.

Confusion in numbering the Haworth projection of the six carbons of *myo*-inositol ring is mitigated by use the form of a Agranoff's turtle (**Figure 5.1**), in which the axial hydroxyl is its head (Agranoff, 1978).

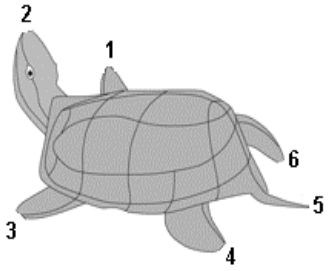

Figure 5.1 Agranoff's turtle (Agranoff, 1978)

According to the current recommendation of the Nomenclature Committee of the International Union of Biochemistry the D-configuration numbering should be used (NC-IUB, 1989), where the *myo*-inositol ring are numbered in a counter-clockwise manner with the turtle's right paw as C_1 (**Figure 5.2A**). However, the old nomenclature, based on L-configuration, where the *myo*-inositol ring are numbered in a clockwise manner with the turtle's right paw as C_3 (**Figure 5.2B**) is still frequently used.

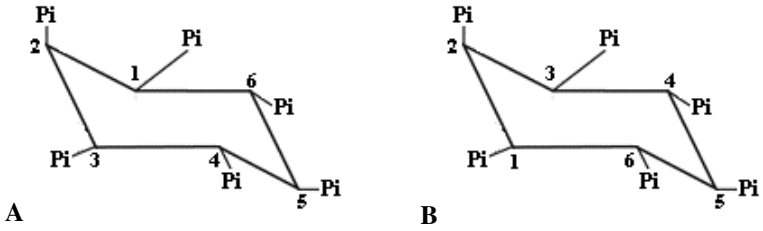

Figure 5.2 Numbering of *myo*-inositol. **A**: D-configuration numbering; **B**: L-configuration numbering

According to their catalytic mechanism phytases can be referred to histidine acid phosphatases (HAPs), β-Propeller phytases (BPPs), cysteine phosphatases (CPs) and purple acid phosphatases (PAPs) (Mullaney and Ullah, 2007).

Depending on their pH optima for catalytic activity, phytases have been divided into acid and alkaline phytases. Most bacterial, fungal, yeast and plant phytases belong to the HAPs with maximal phytate-degrading activity under the acidic conditions (Greiner and Konietzny, 2006). In contrast, alkaline phytases from legume seeds (Scott, 1991), lily pollen (Barrientos et al., 1994) and *Bacillus* spp. (Tran et al., 2011) have an optimal activity under neutral and alkaline conditions and belong to BPPs. BPPs differ from HAPs in the specificity of hydrolysis of phosphate esters on the *myo*-inositol ring (first attack the 5-position). They have high substrate specificity for phytic acid as well as biochemical properties such as calcium ion requirement for enzymatic catalysis and are not inhibited by fluoride at concentrations that completely inhibit HAPs activity (Oh et al., 2004; Scott, 1991).

5.2 Phytate degradation pathways

Most phytases of microbial, especially of fungal origin are considered to be 3-phytases (Irving and Cosgrove, 1972), whereas those of plant origin are 4-phytases (Tomlinson and Ballou, 1962). The 3-phytase (*myo*-inositol-hexakisphosphate 3-phosphohydrolase) first attacks the 3-position phosphate of the phytate on the D-configuration, while the 4-phytase (*myo*-inositol-hexakisphosphate 4-phosphohydrolase) first attacks the 4-position phosphate of the phytate on the D-configuration. These phytases have been designated on the basis of the removal of C_3 or C_4 from phytic acid molecule, so the first yielded *myo*-inositol pentaphosphates are 1D-*myo*-Ins(1,2,4,5,6)P_5 and 1D-*myo*-Ins(1,2,3,5,6)P_5, respectively. Yielded different

lower inositol phosphates dependently on cleaved phosphate position may again become a substrate for further hydrolysis.

Studies on phytate degradation by the yeast phytases indicated that phytate is dephosphorylated by sequential removal of phosphate groups via 1D-*myo*-Ins(1,2,4,5,6)P_5, 1D-*myo*-Ins(1,2,5,6)P_4 and 1D-*myo*-Ins(1,2,6)P_3 to finally IP_2 which can be either 1D-*myo*-Ins(1,2)P_2 or 1D-*myo*-Ins(2,6)P_2 with the end product 1D-*myo*-Ins(2)P_1 (**Figure 5.3**) (Adelt et al., 2003; Greiner et al., 2001; Türk et al., 2000). However, Quan et al. (2003) discovered that the phytate degradation pathway by *Candida krusei* phytase was significantly different in comparison with that of previous reported yeast phytases. In their case the phosphate had been initially hydrolysed at the D-3 and/or D-4 positions of 1D-*myo*-Ins(1,2,3,4,5,6), resulting in the formation of 1D-*myo*-Ins(1,2,4,5,6)P_5 and/or 1D-*myo*-Ins(1,2,3,5,6)P_5, respectively.

Studies of phytate dephosphorylation by the spelt phytase D21, the rye phytase, the barley phytases P1 and P2, and the oat phytase (Greiner and Alminger, 2001) as well as by wheat phytase (Nakano et al., 2000) indicated that the phytate degradation pathway, with the exception of IP_5 isomer was identical with those generated by the yeast (**Figure 5.3**). However, phytate degradation pathway disused above have been shown to be not a general for all microbial and cereal phytases. The wheat phytase F2 (Lim and Tate, 1973) and mung bean phytase (Maiti et al., 1974) generated 1D-*myo*-Ins(2)P intermediates shown in **Figure 5.3**.

Independent of their bacterial or plant origin, acid phytases studied so far in respect to phytate degradation release five of six phosphate groups of phytate, and the final degradation product was identified as 1D-*myo*-Ins(2)P (Greiner et al., 2002; Nakano et al., 2000; Wyss et al., 1999). These results indicate that phytases have a pronounced stereospecificity and a strong preference for equatorial phosphate groups, whereas they are virtually unable to cleave the axial phosphate group at C_2 position. Dephosphorylation of IP_2 was shown to occur only in the presence of high enzyme concentration during prolonged incubation (Greiner, 2007).

The alkaline phytase isolated from pollen (Hara et al., 1985) has been considered to be 5-phytase (*myo*-inositol-hexakisphosphate 5-phosphohydrolase). It first attacks the 5-position phosphate of the phytate to yield the symmetrical 1D-*myo*-Ins(1,2,3,4,6)P_5. Additionally, alkaline phytases have been shown to stop degradation at 1D-*myo*-Ins(1,2,3)P_3 (Barrientos et al., 1994).

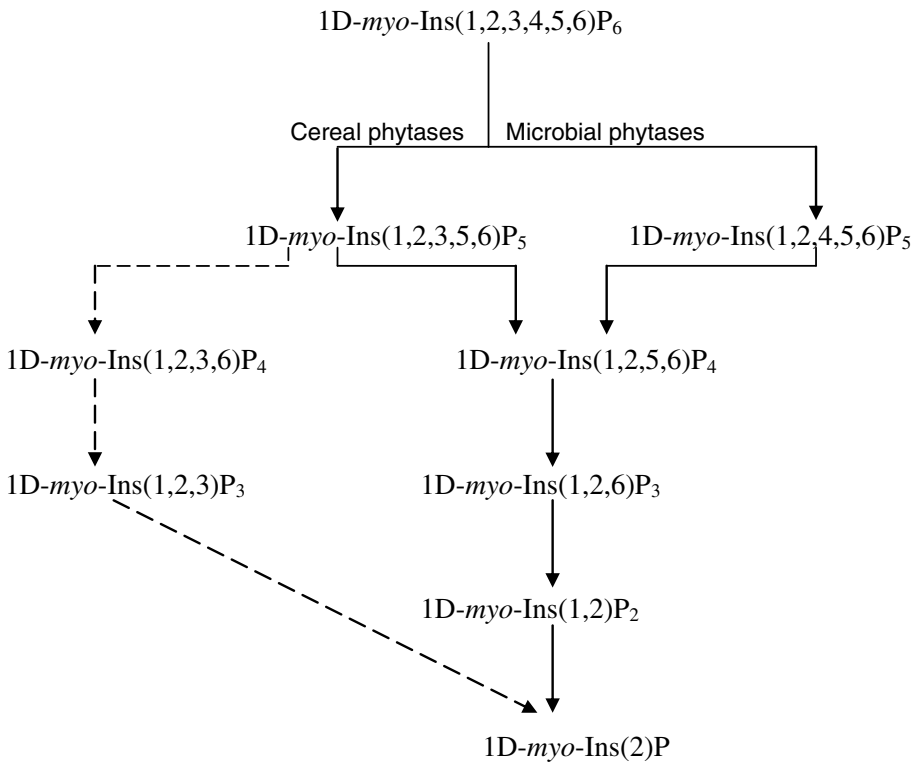

Figure 5.3 Pathways of phytate degradation by: acid phytases from cereals and yeast (solid line); wheat phytase F2 and mung bean phytase with optimum pH between 7.0 and 7.5 (dashed line)

5.3 Cereal phytases

Phytases have been widely reported in a numerous plant species including wheat, rye and barley (for references see **Table 5.**1) as well as in, maize (Laboure et al., 1993), rice bran (Eeckhout and Depaepe, 1994), soybean (Hamada, 1996) and other cereals, legumes, oilseeds and pollen of higher plants.

In the following text one unit of the cereal phytase will be defined as the amount of phytase that liberates 1 μmol/mL inorganic phosphate per minute from phytate under certain conditions mentioned in **Table 5.1**.

The phytase activity varies substantially among different plant species. Viveros et al. (2000) tested a total of 183 samples including cereals, cereal by-products, legumes and oilseed for phytase activity and found that the highest phytase activity occurred in

rye and wheat bran with values of 7339 U/kg and 4624 U/kg, respectively. From all tested cereals, rye had the highest phytase activity with value of 5147 U/kg, followed by wheat and barley with values of 1637 U/kg and 1016 U/kg, respectively (**Table 5.1**). The lowest phytase activities were observed in oat, corn, millet and legume seeds with values ranging from 32 U/kg to 258 U/kg. Steiner et al. (2007) confirmed these findings by analysing a total of 113 samples of whole cereals, wheat and rye bran, and legumes for phytase activity. In their study the highest phytase activities also occurred in wheat and rye bran (9241-9945 U/kg dry matter), intermediate in cereals such as wheat, barley, rye, and triticale (2323-6016 U/kg dry matter), and lowest in legume seeds and oats (262-496 U/kg dry matter). Their observations regarding cereals are in agreement with those of Viveros et al. (2000) by reporting the highest phytase activity in rye grain (**Table 5.1**).

Other studies also confirmed that activities in rye are in general higher than in other cereals and ranging from 2144 U/kg (Marounek et al., 2011) to 5200 U/kg (Delia et al., 2011). Phytase activity in barley vary from 88 U/kg (Liu et al., 1998) to 2323 U/kg (Steiner et al., 2007). For legume seeds reported phytase activity values vary between 69 U/kg in bean (Marounek et al., 2011) and 324 U/kg in lupins (Steiner et al., 2007).

In general, literature data shows a considerable variation in phytase activities among the same plant species. For example, Cossa et al. (2000) reported wheat phytase activity values ranging from 520 to 1390 U/kg, similar to those reported by Eeckhout and Depaepe (1994) , whereas Steiner et al. (2007) reported at least two times higher phytase activity in wheat (2886 U/kg) (**Table 5.1**). This variation might be due to a several reasons. At first, the analytical procedure used for the determination of phytase activity (direct incubation or extraction method) may have an impact on the value obtained. Second, it could be questioned, if data are representative due to the low number of observations in most of experiments (**Table 5.1**). Third, differences in phytase assay conditions between experiments may influence the results. Much of this variation can be also explained through cultivar differences, weather conditions and possibly through grain storage time and conditions (Barrier-Guillot et al., 1996).

Table 5.1 Phytase activities in wheat, rye and barley

Phytase source	Phytase activity, U/kg	Number of cultivars	Analytical procedure [a]	Phytase assay conditions			References
				pH	T, °C	Phytate conc., mM	
Wheat							
	2886	18	DI [a]	5.0	37	0.10	(Steiner et al., 2007)
	520-1390		EX [b]				(Cossa et al., 2000)
	670		EX	5.0	40	2.00	(Azeke et al., 2011)
	1156	5	EX	5.5	37	5.00	(Delia et al., 2011)
	1193	13	DI	5.5	37	1.86	(Eeckhout and Depaepe, 1994)
	503	37	EX	5.5	37	5.10	(Selle et al., 2003)
	1637	30	DI	5.5	37	1.86	(Viveros et al., 2000)
	735		DI	5.0	37	5.00	(Brinch-Pedersen et al., 2003)
Rye							
	6016	13	DI	5.0	37	0.10	(Steiner et al., 2007)
	5384	2	EX	5.5	37	5.00	(Delia et al., 2011)
	5130	2	DI	5.5	37	1.86	(Eeckhout and Depaepe, 1994)
	5147	6	DI	5.5	37	1.86	(Viveros et al., 2000)
Barley							
	2323	15	DI	5.0	37	0.10	(Steiner et al., 2007)
	755	3	EX	5.0	37	0.10	(Delia et al., 2011)
	582	9	DI	5.5	37	1.86	(Eeckhout and Depaepe, 1994)
	348	6	EX	5.5	37	5.10	(Selle et al., 2003)
	1016	21	DI	5.5	37	1.86	(Viveros et al., 2000)

[a] DI, direct incubation;
[b] EX: extraction procedure

5.3.1 Localisation of phytases in cereals

In cereals, such as wheat, rye, and barley phytase is mainly associated with the aleurone layer. Peers (1953) examined the distribution of phytase in various anatomical fractions of the wheat grain and suggested that phytase was more dispersed throughout wheat grain than its substrate, phytate. Further, he found that the phytase with the highest activity is located in aleurone layers. Singh and Sedeh (1979) confirmed these findings by showing comparatively higher phytase activity in soft wheat bran (containing epidermis, testa and aleurone layers) than in the endosperm flour. In rye grains as well as in wheat grains the activity of phytase is highest in the outer layers of the bran (epidermis, testa and nucellar tissue) followed by the aleurone cells. Fretzdorff and Weipert (1986) observed that the activity of phytase in the bran fractions is 2-6 times higher than the activity in the grains with removed outermost layer of the pericarp. In addition, Brinch-Pedersen et al. (2000) observed that recombinant phytase primarily is synthesised in one or more tissues of the pericarp. At the mid-stage of grain development phytase synthesis was observed in seed coat and aleurone layer while at late-stage the primary site for phytase synthesis have been indicated to be the endosperm (Brinch-Pedersen et al., 2000).

5.3.2 Characteristics of cereal phytases

So far characterized cereal phytases belong to the acidic phytases and are most active at weak acidic to neutral pH (**Table 5.2**). The pH optimum of the wheat phytases varies between pH 5.15 in wholemeal (Peers, 1953) and 5.6 - 7.2 in bran (Lim and Tate, 1973). The triticale and rye grains exhibit the pH optimum of 5.4 (Singh and Sedeh, 1979) and 6, respectively (Nielsen et al., 2008; Greiner et al., 1998). In germinated barley two different phytases were observed with the optimal phytate hydrolysis at pH 5 and 6 (Sung et al., 2005; Greiner et al., 2000). Phytase P2 was identified as a constitutive enzyme, whereas phytase P1 was induced during barley germination (Greiner et al., 2000).

The temperature optima reported for wheat, triticale, rye and barley phytases range from 45 to 55°C (for references see **Table 5.2**).

The purified phytases have been characterized as monomeric proteins with molecular masses ranging from 47 kDa for wheat bran to 67 kDa for barley. The K_m values for phytate as substrate were calculated to be from 22 µM to 830 µM for wheat phytase, and from 0.3 µM to 72 µM for rye and barley phytases. Relatively low K_m values have been reported for the phytase from barley (P1: 72 µM) and wheat bran (F_1: 22

μM), whereas relatively high K_m value have been reported for the phytase from whole wheat extract (830 μM) indicating that wheat bran and barley phytases have 35-10 times higher affinity to phytate than phytase isolated from whole wheat (**Table 5.2**).

Table 5.2 Characteristics of cereal phytases; optimum pH and temperature, Michaelis-Menten constant K_m, molecular weight MW

Phytase source	pH	Temp., °C	K_m, μM	MW, kDa	Reference
Wheat					
Wholemeal	5.15	55	300	nv	(Peers, 1953)
Bran	6.0	45	830	nv	(Bohn et al., 2007)
Bran F_1	5.6	nv[a]	22	47	(Lim and Tate, 1973)
Bran F_2	7.2	nv	200	nv	
Rye					
Wholemeal	6.0	45-55	nv	nv	(Nielsen et al., 2008)
Wholemeal	6.0	45	300	67	(Greiner et al., 1998)
Barley					
Wholemeal P1	5	45	72	67	(Greiner et al., 2000)
Wholemeal P2	6	55	190	67	
Wholemeal P1	6	55	nv	96	(Sung et al., 2005)
Wholemeal P2	5	50	nv	66	

[a]nv; no value were reported

5.4 Analysis of phytase activity in microorganisms (PAPER I, PAPER II, and PAPER III)

Suitable enzymes assays are necessary fundamentals for screening large numbers of microbial isolates such as those that can be found in complex system like the sourdough. Although effective for characterizing individual isolates, standard solution assays for measuring phytase activity are unsatisfactory for the rapid screening of large numbers of microbial isolates. A more suitable method to investigate whether the microorganisms produce phytase is the use of different microbial growth-based tests. Frequently, growth-based tests on solid medium have been used which relay on the presence of a translucent zone around colonies as the result of the disappearance

of precipitated calcium or sodium phytate indicating the microorganisms ability to produce phytase (Lambrechts et al., 1992; Howson and Davis, 1983). However, it was then shown that the size of the clear zone around colonies could not be quantitatively correlated with the amount of synthesised phytase (Fredrikson et al., 2002; Nakamura et al., 2000; Lambrechts et al., 1992). Lambrechts et al. (1992) stated that *Pichia anomala* did not hydrolyse phytate as no clearing zone was detected, whereas Olstorpe et al. (2009) found growth of *P. anomala* in the agar assay, supplemented with the high purity phytic acid dipotassium salt. Thus it must be that *P. anomala* has high intraspecies variation and not all strains belonging to this species possess phytase activity.

In this PhD study, to check the ability of yeasts to synthesize phytase, yeast strains were cultivated on Defined Minimal Medium (Delft) plates, supplemented with phytic acid dipotassium salt as the only phosphorus source (Defft+Phy). As control, yeast strains were cultivated on phosphate-containing (Delft+P, positive control) and phosphate-free (Delft-P, negative control) (PAPER I and PAPER II). To check the ability of lactic acid bacteria to synthesize phytase Chemically Defined Medium (CDM) was used (PAPER III). A positive control was done in order to validate that all essential nutrients to ensure good yeast and microbial growth are added. A negative control was done in order to validate that due to phosphate starvation yeast and lactic acid bacteria stop their growth.

However, due to the fact that some microorganisms may grow on solid phytate medium but not in liquid medium and *vice versa* (Tseng et al., 2000), growth test in liquid medium in parallel to the test on solid medium is mandatory. Hence, a liquid culture assay in microtiter plates was used, which yielded more useful data than growth test on solid medium (PAPER I, PAPER II and PAPER III) (Olstorpe et al., 2009). The advantage of this method is that yeast and microbial growth can be followed over time and appreciable differences between the experimental and control growth as well as differences in the duration of lag phase and the maximum specific growth rate ration between isolates can be detected (PAPER I).

Following the isolation of microorganisms that have the ability to degrade phytate in /on media, it is necessary to confirm the phytase activity. Most of the assays used to quantify phytase activity developed so far are based upon the colorimetric quantification of the orthophosphate released by the hydrolysis of phytate. To quantify phytase activity, phosphate released from phytate has to be linear with time. Typically, microbial cells or extracellular phytase extract are incubated with phytate under defined conditions (temperature, pH, reaction time) and phosphate released during enzymatic activity is determined. Two major approaches are used in quantification of the released phosphate including the onetime point method (Quan et

al., 2002; Engelen et al., 2001) and the multiple time point linearity analysis method (PAPER I, PAPER II, PAPERIII) (Olstorpe et al., 2009). The onetime point method measures phytase activity over one period of time, for example 60 min of incubation (Engelen et al., 2001), by assuming a constant velocity of inorganic phosphate hydrolysis under a given assay conditions. However, Olstorpe et al. (2009) observed that the rate of phosphate release decreased in most yeast phytases assays after 5-30 min of incubation, and hence, one time point method may provide incomplete information about phytase activity. We therefore measured the concentration of phosphate released from phytate every 15 min during the 45 min of phytate hydrolysis. Subsequently, the phytase activity was calculated from the steepest slope of the activity curve (**Figure 5.3**).

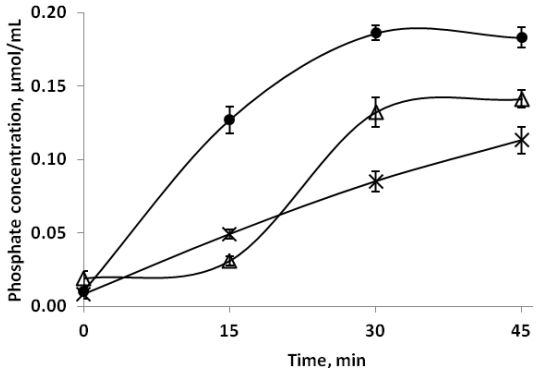

Figure 5.3 The change of phosphate concentration during time from phytate as result of phytase activity; (•) *Candida krusei* P2, (Δ) *Saccharomyces cerevisiae* KVL015, (×) *Candida krusei* K132 (Based on results in PAPER I)

In the following text one unit of the yeast or microbial phytase will be defined as the amount of phytase that liberates 1 µmol/mL inorganic phosphate per minute from phytate under certain conditions.

Moreover, in PAPER II and PAPER III we used a microtiter plate method to assess the phytase activity instead of the single cuvette method as described in PAPER I. The microtiter plate method was found to be more rapid, and that the results are comparable with those of the single cuvette method. Moreover, the microtiter plates

allow the measurement of many samples in parallel with sample volume down to 200-225 μL which decrease the cost of each assay.

5.5 Yeast phytases

Phytases have been isolated from fungi, yeast and bacteria. Most microbial phytases as well as cereals phytases belong to the histidine acidic phosphatases and exhibit considerable variations in biochemical and kinetics properties.

Phytases produced by yeast may be extracellular or intracellular including cell-bound phytases. From an industrial point of view, the extracellular phytase activity would be more important for bread making than the intracellular phytase activity, because the yeast cells should be intact in the dough in order to ensure good fermentation, in this case, the intracellular phytase will not have access to phytate in the dough.

Several studies have shown the yeast phytase to be extracellular with activities ranging from 14 to 566 mU/mL. The first report on extracellular phytase from baker's yeast (*S. cerevisiae*) was in 1984 (Nayini and Markakis, 1984). Later, Sano et al (1999) screened about 1200 yeast strains for their ability to hydrolyse phytate and among all these, strains belonging to *Arxula adeninivorans* exhibited the highest phytase activities at 70ºC (362-566 mU/mL). Nakamura et al. (2000) evaluated extracellular phytase levels in several hundred yeast species. Strains, mainly belonging to *Candida spp.* and *Pichia spp.* had the highest levels of phytase in phosphate-depleted medium (17-342 mU/mL). In our study, to the best of our knowledge we showed for the first time that S*accharomyces pastorianus* KVL isolated from lager beer (PAPER I) and *Candida humilis* isolated from sourdough (PAPER II) exhibit high extracellular phytase activities.

Strains of *Schwanniomyces castellii* (Lambrechts et al., 1992), *Pichia anomala* (Vohra and Satyanarayana, 2001), *Candida krusei* (Quan et al., 2001) were reported to produce intracellular (cell-bound) phytases.

Several studies were carried out on phytase activity from baker's yeast (*S. cerevisiae*) during leavening of bread dough (Andlid et al., 2004; Türk et al., 2000; Türk et al., 1996; Harland and Frolich, 1989; Harland and Harland, 1980). Hellström et al. (2010) isolated nine yeast species from Tanzania togwa and showed that *Issatchenkia orientalis* (anamorph *Candida krusei*) and *Hanseniaspora guilliermondii* completely degraded all phytate in YPD medium, supplemented with 5 mmol/L Phy, within 24 h of fermentation at 30°C and pH 6.5. This is in agreement with the results obtained by

Türk et al. (2000) from fermentation with baker's yeast in synthetic medium supplemented with 0.88 mg/mL Phy at 30°C and pH 5.3 (conditions relevant for leavening of bread dough). However, in their previous work, Türk et al. (1996) reported that the phytase activity from baker's yeasts during dough fermentation is too low to significantly influence the iron absorption from bread.

Our results confirmed previous findings showing that phytase activity may be detected in the extracellular as well as in the intracellular medium of all tested isolates. However, the specific extracellular phytase activity was found to be at least 20-fold higher than the specific intracellular activity (PAPER I and PAPER II). Moreover, phytase activities for yeast isolated from sourdough (PAPER II) were at least five times higher than the phytase activities of yeast isolated from beer (PAPER I). The reason for this difference may be that the yeasts isolated from sourdough are adapted to grow in phytate rich medium and thus produce more phytase.

5.5.1 Regulation of yeast phytase synthesis

In yeast as well as in bacteria, phytase is an inducible enzyme and its expression is subjected to a complex regulation. Various physical and nutritional parameters are known to affect the production of phytase. The most important physical parameters are pH and temperature. Nutritional parameters include carbon and phosphorus sources, and occurrence of metal cations.

5.5.1.1 Effect of extracellular phosphorus on phytase synthesis

Phosphorus is needed for many important reactions in the yeast cells. It is an essential component for the synthesis of nucleic acid and phospholipids, as well as is involved in energy transfer reactions in the form of ATP and ADP. All these reactions require millimolar concentrations of phosphate whereas most of environmental concentrations are substantially lower (Wykoff and O'Shea, 2001). Therefore, under phosphate-starved conditions, production and secretion of the phosphatases are activated in yeast to ensure a sufficient phosphate supply to the cells. In *S. cerevisiae*, the phosphate signal transduction (PHO) pathway was shown to regulate the expression of a set of phosphate-responsive genes, including PHO5 and Pho84 that are involved in the scavenging and specific uptake of phosphate from extracellular sources. Gene PHO5 is known to encode repressible acid phosphatase (rAPs) whose transcription is repressed when yeast is grown in high concentration of inorganic phosphate (Yoshida et al., 1989). Gene Pho84 was shown to encode a high-affinity phosphate transporter that acts in response to low phosphate level (Bunya et al., 1991).

In a phosphate-rich medium inorganic phosphate is transported into the yeast cell by a low-affinity phosphate transport system. Subsequently, the transcriptional activator Pho4 is phosphorylated in the nucleus by the cyclin-dependent kinase (CDK) complex Pho80-Pho85 (Kaffman et al., 1994). Phosphorylated Pho4 binds with the export receptor Msn5 and then is rapidly exported from the nucleus to the cytoplasm, and transcription of genes PHO5 and Pho84 is turned off. In contrast, when yeasts are starving for phosphate, the CDK inhibitor Pho81 inactivates the Pho80-Pho85 complex preventing Pho4 phosphorylation. Hence, unphosphorylated Pho4 was shown to be concentrated in the nucleus and transcription of phosphate-responsive genes are activated (Kaffman et al., 1998).

The PHO pathway in context of IP_6 has been studied in *S. cerevisiae* (Andlid et al., 2004). Authors reported that the high levels of extracellular inorganic phosphate repress the biosynthesis of phytase, whereas absence of inorganic phosphate activated phosphate-responsive genes. However, a small amount of phosphate (~0.004 g/100 mL) in the growth medium was shown to stimulate phytase synthesis in *Aspergillus niger* (Soni and Khire, 2007).

5.5.1.2 Effect of carbon source on phytase synthesis

The effect of carbon sources on the phytase production by yeast has been investigated by several researches. Galactose was found to be a better carbon source than glucose for phytase synthesis in *Arxula adeninivorans* (Sano et al., 1999), and in *S. cerevisiae* isolated from mash of wine (In et al., 2008). However, Vohra and Satyanarayana (2001) noted that galactose was a less effective carbon source than glucose for phytase production by *Pichia anomala*. Phytase production was also reported to be induced in the presence of glucose as the carbon source in *Candida krusei* isolated from soil (Quan et al., 2001).

5.5.1.3 Effect of metal cations on phytase synthesis

Metal cations have been shown to modulate phytase activity. Moreover, the requirement for metal cations for phytase activity differs depending upon the sources. The extracellular phytase from *Schwanniomyces castellii* was strongly inhibited by 5 mM Zn^{2+} and Cu^{2+} while the presence of 5 mM Mg^{2+} and Ca^{2+} resulted only in slight inhibition (Segueilha et al., 1992). These findings were partially confirmed by Quan et al. (2002) who reported completely inhibition of phytase activity by Zn^{2+} and almost 70% inhibition by 5 mM Mg^{2+}. In et al. (2008) showed that low concentrations of Mg^{2+} was a week activator for phytase synthesised by *S. cerevisiae*. Addition of 5 mM Fe^{2+} caused approximately 50% inhibition of phytase activity in *S. castellii* (Segueilha et al., 1992). In contrast, the phytase activity in *Candida krusei* (Quan et

al., 2002) was slightly inhibited by addition of 1 mM Fe^{2+}, however increased Fe^{2+} concentration up to 5 mM resulted in the recovery of phytase activity. Vohra and Satyanarayana (2001) reported that 0.15 mM of Fe^{2+} slightly activated phytase production by *P. anomala*.

5.5.2 Purification and characterization of yeast phytases (PAPER IV)

Up to now there have been only few reports on the yeast extracellular phytase purification and enzymatic properties. During the last two decades, extracellular phytases from yeast, including the wild strain of *Schwanniomyces castellii* (Segueilha et al., 1992), marine yeast *Kodamaea ohmeri* (Li et al., 2008), and *S. cerevisiae*, isolated from the mash of traditional Korean wine (In et al., 2009) have been purified to homogeneity (**Table 5.3**).

A combination of various methods has been usually employed for purifying phytases. A typical ammonium sulphate fractionation or acetone precipitation followed by gel filtration, ion-exchange chromatography, affinity chromatography or hydrophobic interaction techniques are employed.

In this study ultrafiltration and Concanavalin A (Con A) affinity chromatography techniques have been used to purify phytase isolated from rye sourdough yeast *S. cerevisiae* L1.12 (PAPER IV). The phytase was purified 8.1, with a yield of 3.7% to a maximum specific activity of 42.9 U/mg proteins. To the best of our knowledge, Con A affinity chromatography has never been used for phytase purification. However, Guimarães et al. (2004) using ion-exchange chromatography followed by Con A affinity chromatography purified phytase from *Aspergillus caespitosus*.

So far characterized yeast phytases belong to the acidic phytases and are more active at lower pH in comparison to the optimum pH of cereals phytases. The pH and temperature optimums for examined S. *cerevisiae* L1.12 phytase were found to be 4.0 and 35°C (**Figure 5.4**) (PAPER IV) is in agreement with previous studies. Most of the yeast phytases are optimally active in the pH range between 3.6 and 4.6 (for references see **Table 5.3**), whereas the temperature optima are wider and range from 35 to 75°C (for references see **Table 5.3**). Nakamura et al. (2000) observed the pH optimum of 4.0-5.0 when assayed extracellular yeast phytases at temperature of 50-60°C, whereas pH optimum of 3.0-4.0 was observed when phytase was assayed at 37°C.

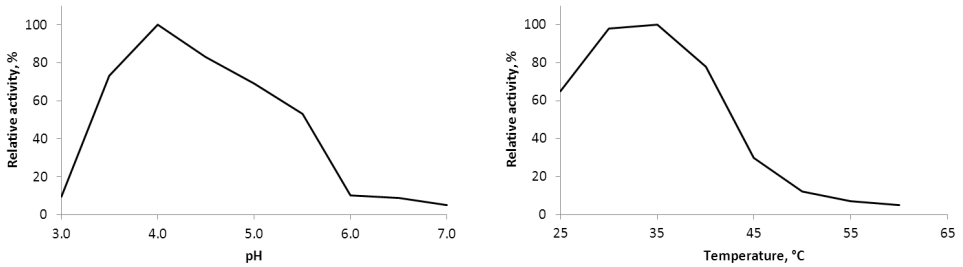

Figure 5.4 *Saccharomyces cerevisiae* L1.12 phytase activity as a function of pH and temperature (PAPER IV)

Purified yeast phytases have been found to be the high molecular weight proteins with glycosylation levels around 30% (In et al., 2009; Segueilha et al., 1992). Their molecular weight ranging from 64 kDa for *P. anomala* (Vohra and Satyanarayana, 2001) to 630 kDa for *S. cerevisiae* (In et al., 2009).

Table 5.3 Characteristics of yeast phytases; optimum pH and temperature, Michaelis-Menten constant K_m, molecular weight MW

Yeast	pH	Temp., °C	K_m, µM	MW, kDa	Phytase	Reference
Schwanniomyces castellii	4.4	77	38	490	E[b]	(Segueilha et al., 1992)
Pichia anomala	4.0	60	200	64	I[c]	(Vohra and Satyanarayana, 2001)
Pichia rhodanensis	4.5	70-75	250	nv[a]	E	(Nakamura et al., 2000)
Arxula adeninivorans	4.5	75	250	nv	E	(Nakamura et al., 2000; Sano et al., 1999)
Candida krusei	4.6	40	30	330	I	(Quan et al., 2002)
S. cerevisiae	4.0	35	421	nv	E	(PAPER IV)
S. cerevisiae	3.6	40	660	630	E	(In et al., 2009)

[a]nv; no value were reported
[b]E; extracellular phytase
[c]I; intracellular phytase

The K_m values for phytate as substrate were calculated to be from 30-38 μM for *C. krusei* and *S. castellii* phytases (Quan et al., 2002; Segueilha et al., 1992) to 600 μM for *S. cerevisiae*, isolated from the mash of traditional Korean wine, phytase.

5.6 Lactic acid bacteria phytases

So far, only few strains of LAB, i.e. *Lactobacillus amylovorus* and *L. plantarum* present in a wide variety of microbial systems of plant origin, showed consistent extracellular phytase activity with value of 125-146 U/mL (Sreeramulu et al., 1996). However, Reale and co-authors (2007) were unable to confirm these findings using the same *L. amylovorus* strain under identical growth conditions. Furthermore, few strains of LAB isolated from sourdough, i.e. *Lactobacillus plantarum, L.brevis, L. curvatus*, *L. sanfranciscensis, L. fermentum, L. plantarum* were shown to express intracellular (cytoplasmic and/or cell-wall bound) phytase activity in phytate rich medium (Reale et al., 2004; De Angelis et al., 2003; Lopez et al., 2000). However, our results demonstrated that the phytase activities of tested LAB strains isolated from sourdough are extra- and intracellular (PAPER III). In addition, the highest volumetric extracellular activities (56-60 $mU \cdot ml^{-1}$) were found among *L. frumenti* isolates which, to the best of our knowledge, has never been described as a phytase positive species (PAPER III). Observed extracellular phytase activities in *L. fermentum* and *Pediococcus pentosaceus* are consistent with Songre-Ouattara et al. (2008) report showing that *L. fermentum* isolated from fermented pearl-millet slurries produce extracellular phytase with value of 276 U/mL (one unit (U) was defined as the amount of enzyme required to liberate 1nmol of phosphate from sodium phytate per min under the assay conditions). Additionally, we found that *L. fermentum* also produces intracellular phytase. In contrast, Zamudio et al. (2001) found that *L. fermentum* and *P. pentosaceus* at pH 4.5 and 37°C exhibit very low extracellular phytase activity (less than 6.3 mU/mL) and do not produce intracellular phytase activity.

Up to now there have been only few reports on the lactic acid bacteria phytase purification and enzymatic properties. The purified phytases have been characterized as monomeric proteins with molecular masses around 50 kDa (De Angelis et al., 2003; Zamudio et al., 2001). The optimum pH for *L. sanfranciscensis* phytase has been observed to be 4.0 (De Angelis et al., 2003), whereas phytases from *L. amylovorus* and *Leuconostoc mesenteroides* were most active at pH 5.5 (Oh and In, 2009; Zamudio et al., 2001). The optimum temperature for the LAB phytase is mostly between 45°C and 65°C (De Angelis et al., 2003; Zamudio et al., 2001).

Chapter 6

Conclusions and Perspectives

This PhD project has focused on obtaining knowledge about the phytase activity synthesised by yeast and lactic acid bacteria isolated from grain-based foods and beer in order to select the high phytase-active ones. Selected yeast and lactic acid bacteria may be used as a starter to increase the mineral bioavailability in whole-grain bread fermented in short time as alternative to sourdough bread.

The highest extracellular phytase activities were observed in isolates of *S. cerevisiae* (L1.12 and L6.06) and *L. frumenti* (2.1 and 2.5) and therefore they may be the best candidates for improving mineral bioavailability in whole-grain bread (PAPER II and PAPER III).

The extracellular phytase activities were much higher than the intracellular phytase activities for all the tested yeast strains and isolates. However, tested lactic acid bacteria isolated from sourdough have been showed to be both intra- and extra-cellular phytases with the clear tendency to be species dependent. (PAPER I, PAPER II and PAPER III).

In addition, lager beer yeast *S. pastorianus*, yeast strain *C. humilis* isolated from Danish sourdough and lactic acid bacteria *L. frumenti* isolated from Lithuanian sourdough have, to the best of our knowledge, for the first time been described as phytase positive strains (PAPER I, PAPER II and PAPER III).

Yeasts isolated from sourdoughs produced five times higher extracellular phytase activities than yeasts isolated from kenkey or beer. These results lead us to

hypothesise that the yeasts isolated from sourdoughs are adapted to grow in phytate rich medium and thus produce more phytase (PAPER I and PAPER II).

Furthermore, an interesting pattern was observed regarding the phytase active yeasts isolated from sourdoughs. Yeast isolates with the highest extracellular phytase activities were rather isolated from Lithuanian sourdoughs than from Danish one (PAPER II). Sourdoughs yeast species composition and their phytase activities differ between countries and it may be that sourdough fermentation conditions (such as temperature) are additional factors which should be taking into consideration in further investigations.

Concanavalin A chromatography process was successfully implemented to purify phytase from *S. cerevisiae* isolate L1.12 which resulted in 8.1-fold purification with a yield of 3.6% to a maximum specific activity of 42.9 U/mg. The optimum pH and temperature were found to be 4.0 and 35°C, respectively (PAPER IV).

It must not be omitted to perform phytase activity assay on each isolate which shows even minimal growth in liquid medium supplemented with phytate. *Saccharomyces pastorianus* KVL008, one of the negative controls, which exhibited very slow growth in medium, unexpectedly showed the highest extracellular phytase activity among all the tested strains (PAPER I). Therefore further research should be carried out as at present we do not have a clear explanation for this phenomenon.

In conclusions, one of the most prominent *S. cerevisiae* isolates in combination with one of the most prominent *L. frumenti* isolates, mentioned above, appear to be well suited for inclusion in a starter culture for the whole-grain bread making. Due to the fact that the phytase activities were highly variable among different strains within the species, once more indicating the importance of selecting the right strain for developing a good starter culture. Moreover, future studies should concentrate on isolating additional beneficial strains from relevant food system. Functional culture of appropriate microorganisms should then be found, and interaction among the different strains as well as effect on mineral bioavailability in whole grain bread investigated.

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Paper 1

Phytase-active yeasts from grain-based food and beer

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ORIGINAL ARTICLE

Phytase-active yeasts from grain-based food and beer

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Abstract

Aims: To screen yeast strains isolated from grain-based food and beer for phytase activity to identify high phytase-active strains.
Methods and Results: The screening of phytase-positive strains was carried out at conditions optimal for leavening of bread dough (pH 5·5 and 30°C), in order to identify strains that could be used for the baking industry. Two growth-based tests were used for the initial testing of phytase-active strains. Tested strains belonged to six species: *Saccharomyces cerevisiae*, *Saccharomyces pastorianus*, *Saccharomyces bayanus*, *Kazachstania exigua* (former name *Saccharomyces exiguus*), *Candida krusei* (teleomorph *Issachenkia orientalis*) and *Arxula adeninivorans*. On the basis of initial testing results, 14 strains were selected for the further determination of extracellular and intracellular (cytoplasmic and/or cell-wall bound) phytase activities. The most prominent strains for extracellular phytase production were found to be *S. pastorianus* KVL008 (a lager beer strain), followed by *S. cerevisiae* KVL015 (an ale beer strain) and *C. krusei* P2 (isolated from sorghum beer). Intracellular phytase activities were relatively low in all tested strains.
Conclusions: Herein, for the first time, beer-related strains of *S. pastorianus* and *S. cerevisiae* are reported as phytase-positive strains.
Significance and Impact of the Study: The high level of extracellular phytase activity by the strains mentioned previously suggests them to be strains for the production of wholemeal bread with high content of bioavailable minerals.

Introduction

Increased consumption of wholegrain products is recommended because several epidemiological studies find that intake of wholegrain food is protective against cardiovascular disease, certain type of cancers, type 2 diabetes and obesity (Slavin 2003). Wholegrain bread also provides an important source of minerals in diet, such as zinc, iron, magnesium, potassium (Spiegel *et al.* 2009); however, they contain absorption inhibitors of minerals, such as phytic acid [IP6] (*myo*-inositol hexaphosphate), the main storage form for phosphorus in plants (Febles *et al.* 2002; Kumar *et al.* 2010).

Phytic acid is highly charged with six phosphate groups extending from the central *myo*-inositol ring and acts as a strong chelator of cations and binds minerals, such as Zn^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+} (Raboy 2003). These phytate complexes are insoluble at physiological pH, and, therefore, minerals and phosphate are unavailable for absorption in the human intestine (Brune *et al.* 1992; Iqbal *et al.* 1994; Lopez *et al.* 2002). The absorption of iron and zinc from a given food in the human's intestine depends on its amount of phytate. A higher amount of phytate leads to smaller amounts of bioavailable minerals (Navert *et al.* 1985; Brune *et al.* 1992; Hurrell *et al.* 1992). To increase the bioavailability of minerals, enzymatic degradation of phytate and its dephosphorylated isomer IP5 (inositol pentaphosphate) is needed (Sandberg *et al.* 1999).

Characterized phytases are enzymes that catalyse the stepwise dephosphorylation of phytate to lower phosphoric esters of *myo*-inositol and phosphoric acid via penta- to monophosphates. This enzymatic activity produces available phosphate and nonchelated minerals for human absorption (Konietzny and Greiner 2002).

Phytases are widespread in nature and present in plants, such as cereals and legumes (Eeckhout and DePaepe 1994; Viveros *et al.* 2000; Steiner *et al.* 2007), and in microorganisms, such as bacteria and fungi (Howson and Davis 1983; Ullah and Gibson 1987; Lambrechts *et al.* 1992; Berka *et al.* 1997, 1998; Nakamura *et al.* 2000; Olstorpe *et al.* 2009). Cereal phytase varies in activity depending on source. For wheat, reported phytase activities range from 900 to 2886 U kg^{-1} dry matter, for rye from 4100 to 6100 U kg^{-1} dry matter and for barley from 400 to 2323 U kg^{-1} dry matter (Eeckhout and DePaepe 1994; Greiner and Egli 2003; Steiner *et al.* 2007). However, this activity in wheat is not enough to improve mineral bioavailability sufficiently in wheat bread (Harland and Harland 1980; Harland and Frolich 1989; Turk and Sandberg 1992; Turk *et al.* 1996). On the other hand, phytate is fully degraded during commercial rye bread production (Nielsen *et al.* 2007). Mineral bioavailability in bread can be increased, using high phytase-active yeasts, in addition to cereal phytase. A few studies have shown phytase activities in a large number of yeast strains ranging from 14 to 566 mU ml^{-1} (Lambrechts *et al.* 1992; Sano *et al.* 1999; Nakamura *et al.* 2000; Olstorpe *et al.* 2009).

Several studies were carried out on phytase activity from baker's yeast (*Saccharomyces cerevisiae*) during leavening of bread dough (Harland and Harland 1980; Harland and Frolich 1989; Turk *et al.* 1996, 2000; Andlid *et al.* 2004). Phytase from baker's yeast was first extracted by Nayini and Markakis (1984) and they found an optimum pH to be 4·6 and optimum temperature of 45°C. On the other hand, In *et al.* (2009) found optimum pH for *S. cerevisiae* phytase to be 3·6 at 40°C and this is in accordance with Turk *et al.* (1996) who found a similar optimum pH of 3·5 at 30°C. Hellstrom *et al.* (2010) isolated nine yeast species from Tanzania togwa and showed that *Issatchenkia orientalis* (anamorph *Candida krusei*) and *Hanseniaspora guilliermondii* completely degraded all IP6 in YPD medium, supplemented with 5 mmol l^{-1} IP6, within 24 h of fermentation at 30°C and pH 6·5. During the first 3 h of fermentation, only marginal amount of IP6 was degraded. This is in agreement with the results obtained by Turk *et al.* (2000) from fermentations with baker's yeast in synthetic medium supplemented with 0·88 mg ml^{-1} IP6 at 30°C and pH 5·3 (conditions relevant for leavening of bread dough).

To our knowledge, there are no high phytase-active yeasts available for bread industry today, so the potential of identification of yeasts to be used for bread making with high content of bioavailable minerals is of outstanding importance. The objective of this study was therefore to screen different yeast strains isolated from grain-based food and beer for phytase activity under conditions optimal for leavening of bread dough (30°C and pH 5·5).

Materials and methods

Yeast strains

Yeast strains ($n = 39$) used in this study were isolated from grain-based food and beer, and their isolation source and country of origin are listed in Table 1. Yeast strains belong to the species *S. cerevisiae* ($n = 13$), *Saccharomyces pastorianus* ($n = 10$), *Saccharomyces bayanus* ($n = 1$), *Kazachstania exigua* (former name *S. exiguus*) ($n = 1$) and *C. krusei* (teleomorph *I. orientalis*) ($n = 12$). Additionally, two reference yeast strains (CBS7377 and CBS8335), belonging to *Arxula adeninivorans*, were used (Sano *et al.* 1999; Olstorpe *et al.* 2009).

Yeast strains were stored at −80°C in YPG medium (D-(+)-glucose, 10 g l^{-1}; bacto-yeast extract, 3 g l^{-1}; bacto-peptone, 5 g l^{-1}; pH 5·5) containing 20% glycerol.

Growth media and preparation of yeast inocula

Three different defined minimal media, modified from Albers *et al.* (1996), were used to investigate the ability of the strains to grow on media with phytate as the only phosphor source: Delft + P (positive control; phosphate-containing medium), Delft − P (negative control; phosphate-free medium) and Delft + Phy (phytate medium; phytic acid dipotassium salt as phosphate source) (Table 2).

Trace minerals and vitamins were prepared separately as 100-fold concentrated stock solution, filter sterilized (0·2-μm Minisart filters; Bie & Bernzten, Herlev, Denmark) and stored at 4°C. Potassium, ammonium and magnesium salts were prepared separately as 50-fold concentrated stock solution, autoclaved at 121°C for 15 min and stored at 4°C. Heat-sensitive phytic acid dipotassium salt (Bae *et al.* 1999; Fredrikson *et al.* 2002) was freshly prepared as 50-fold concentrated stock solution, filter sterilized (0·2-μm Minisart filters) and added after autoclaving and cooling to 45°C. To stabilize the pH during yeast cultivation, 50 mmol l^{-1} succinic acid/NaOH buffer, pH 5·5, was used (Turk *et al.* 2000; Andlid *et al.* 2004).

For solid medium, bacto-agar (20 g l^{-1}) (Becton Dickinson, Broendby, Denmark) was suspended in corresponding medium (Delft + P, Delft − P and Delft + Phy) and autoclaved at 121°C for 15 min. After cooling to 45°C, 100-fold concentrated solutions of vitamins and trace minerals and 50-fold phytic acid dipotassium salt solution was added.

Yeast inocula were prepared in two steps. First, few yeast colonies were resuspended in 10-ml sterile YPG medium and cultivated in a shaking water bath (170 rev min^{-1}) at 30°C overnight. Second, yeast culture was resuspended in 250-ml Erlenmeyer flasks, containing

Table 1 Yeast strains (n = 39) used in this study, their isolation source and country of origin

Species	Strains	Isolation source	Country of origin
Arxula adeninivorans	CBS7377	Garden soil	South Africa
	CBS8335	Soil	Italy
Candida krusei	K3	Kenkey*	Ghana
	K21	Kenkey	Ghana
	K131	Kenkey	Ghana
	K132	Kenkey	Ghana
	K168	Kenkey	Ghana
	K190	Kenkey	Ghana
	K191	Kenkey	Ghana
	K204	Kenkey	Ghana
	P1	Pito†	Ghana
	P2	Pito	Ghana
	P9	Pito	Ghana
	P11	Pito	Ghana
Kazachstania exigua	CBS7901	Sourdough	Italy
Saccharomyces bayanus	CBS380	Turbid beer	Denmark
Saccharomyces cerevisiae	ATCC26108	Laboratory strain	
	DGI342	Baker's yeast	Denmark
	CBS1171	Brewer's top yeast	the Netherlands
	CBS1234	Baker's yeast	the Netherlands
	CBS1236	Baker's yeast	France
	P3	Pito	Ghana
	P4	Pito	Ghana
	P5	Pito	Ghana
	P6	Pito	Ghana
	P10	Pito	Ghana
	KVL013	Ale beer	Denmark
	KVL014	Ale beer	Denmark
	KVL015	Ale beer	Denmark
Saccharomyces pastorianus	KVL001	Lager beer	Denmark
	KVL004	Lager beer	Denmark
	KVL005	Lager beer	Denmark
	KVL006	Lager beer	Denmark
	KVL007	Lager beer	Denmark
	KVL008	Lager beer	Denmark
	KVL009	Lager beer	Denmark
	KVL010	Lager beer	Denmark
	KVL016	Lager beer	Denmark
	KVL017	Lager beer	Denmark

*Kenkey, fermented maize dough from two local production sites in Accra, Ghana. Sampling was performed during the fermentation (Jespersen *et al.* 1994).

†Pito, top-fermented sorghum beer from Tamale, northern Ghana. Sampling was performed on the dried yeast used for inoculation.

50 ml of YPG medium, and cultivated in a shaking water bath (170 rev min^{-1}) at 30°C overnight. The inoculation level was set to OD_{600} = 0·1.

Subsequently, cells were harvested by centrifugation (Sorvall RT6000D; Buch & Holm, Herlev, Denmark) at 5000 ***g*** for 10 min, 4°C, washed three times with 20-ml sterile ultrapure water and diluted to an initial OD_{600} = 1. Prepared yeast inocula were used for growth test on agar plates, growth test on microtitre plates and phytase extraction.

Table 2 Modified defined minimal medium used in the experiment: Delft + P (positive control; phosphate-containing medium), Delft − P (negative control; phosphate-free medium) and Delft + Phy (phytate medium; phytic acid dipotassium salt as phosphate source)

	Medium composition, mg l^{-1}		
Component	Delft + P	Delft − P	Delft + Phy
D-Glucose	20 000	20 000	20 000
Phytic acid dipotassium salt	NI	NI	1850
KH_2PO_4	3510	NI	NI
KCL	NI	2000	1700
$(NH_4)_2SO_4$	7500	7500	7500
$MgSO_4{\cdot}7H_20$	740	740	740
Trace metals			
EDTA	30·00	30·00	30·00
$CaCl_2{\cdot}6H_2O$	8·30	8·30	8·30
$ZnSO_4{\cdot}7H_2O$	9·00	9·00	9·00
$FeSO_4{\cdot}7H_2O$	6·00	6·00	6·00
H_3BO_3	3·00	3·00	3·00
$MnCl_2{\cdot}4H_2O$	1·50	1·50	1·50
$Na_2MoO_4{\cdot}2H_2O$	0·77	0·77	0·77
$CoCl_2{\cdot}6H_2O$	0·85	0·85	0·85
$CuSO_4{\cdot}5H_2O$	0·59	0·59	0·59
KI	0·20	0·20	0·20
Vitamins			
d-Biotin	0·05	0·05	0·05
p-Aminobenzoic acid	0·20	0·20	0·20
Nicotinic acid	1·00	1·00	1·00
Calcium pantothenate	1·00	1·00	1·00
Pyridoxine HCl	1·00	1·00	1·00
Thiamine HCl	1·00	1·00	1·00
myo-Inositol	25·00	25·00	25·00

NI, not included.

Growth test on agar plates

Sterile, 90-mm-diameter Petri dishes were used for growth test on solid medium. Prepared yeast inocula were resuspended in sterile NaCl solution (8·5 g l^{-1}) to an initial OD_{600} = 0·1 and serial diluted to obtain 10^3- and 10^4-fold dilutions. From each dilution, 2 μl was inoculated on Delft + P, Delft − P and Delft + Phy agar plates, cultivated at 25°C for 72 h and, thereafter, photographed.

Growth test in microtitre plates

Sterile flat-bottom, 96-well tissue culture plates (92096, TPP) were used for growth test in liquid medium. Microtitre plate wells were filled in triplicate with 200 μl of corresponding medium (Delft + P, Delft − P or

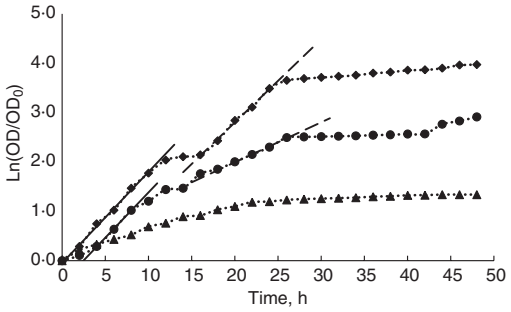

Figure 1 Growth curve of *Saccharomyces cerevisiae* KVL013 in Delft + P (phosphate) medium, ◆; Delft + Phy (phytic acid dipotassium salt) medium, ●; and Delft – P (phosphate free) medium, ▲. Cell concentration is measured every 2 h by optical density (OD) at 600 nm. Cell growth is represented by $Ln(OD/OD_0)$, where OD_0 is the initial OD. The maximum slope (solid line, in respiro-fermentative phase; dashed line, in respiratory phase) represents the maximum specific growth rate (μ). The interaction of the maximal slope with the *x*-axis represents the lag time (λ).

Delft + Phy), inoculated with 2 μl of prepared yeast inocula and cultivated at 25°C for 48 h. Yeast growth was monitored as optical density at 600 nm (OD_{600}) using a Microplate reader (M965 AcuuReader; Metertech; Food Diagnostics, Grenaa, Denmark). Before OD measurement, the plate was agitated for 3 s. Measurements were taken every 2 h. For experiments performed in the Delft + P and Delft + Phy media, the duration of the lag phase (λ), as well as the maximum specific growth rate (μ) and the maximum specific growth rate ratio (μ_{phy}/μ_P) in the respiro-fermentative and respiratory growth phases, was calculated (see Fig. 1).

Phytase extraction

Each yeast culture was prepared in a 500-ml Erlenmeyer flask, containing 100 ml of Delft + Phy medium. Medium was inoculated with prepared yeast inocula and cultivated in a shaking water bath (170 rev min^{-1}) at 30°C for 48 h. Inoculation level was set to $OD_{600} = 0{\cdot}1$. Each yeast strain was cultured in triplicate.

The number of culturable cells after cultivation was determined in triplicate, by counting colony-forming units (CFU) on YPG agar plates after 48-h incubation at 25°C.

Extracellular enzyme extract was prepared as follows: After cultivation, cells were harvested by centrifugation (5000 ***g*** for 10 min, 4°C), supernatants were collected, filtered (0·2-μm Minisart filters) and kept on ice (max 1 h) until activity measurements were performed.

Intracellular (cytoplasmic and/or cell-wall bound) enzyme extract was prepared as follows: The pellets were washed three times with 20-ml sterile ultrapure water and resuspended in 5-ml ice-cold buffer (0·2 mol l^{-1} sodium acetate/HCl, pH 5·5). One gram of glass beads (425–600 μm; Sigma) was added and vortexed for 1 min, then held on ice for 1 min, a total of five times. Homogenate was centrifuged at 5000 ***g*** for 20 min, 4°C, filtered (0·2-μm Minisart filters) and kept on ice (max 20 min) until activity measurements were performed.

Determination of phytase activity

Phytase activity was assayed combining the methods of Quan *et al.* (2002) and Olstorpe *et al.* (2009): 0·8 ml of phytic acid dipotassium solution (3 mmol l^{-1} phytic acid dipotassium (P5681; Sigma-Aldrich, Broendby, Denmark) in 0·2 mol l^{-1} sodium acetate/HCl buffer, pH 5·5) was preincubated at 30°C for 5 min, and 0·2 ml of enzyme extract was added, mixed and incubated at 30°C. Samples were taken at different intervals (0, 15, 30 and 45 min) during assaying, and the reaction was stopped immediately by adding 1 ml of 10% trichloroacetic acid (TCA). Enzyme blank was prepared from sodium acetate buffer mixed with enzyme extract and TCA.

Measurements of the liberated inorganic phosphate from the phytic acid dipotassium salt was modified according to Heinonen and Lahti (1981). To the assay tubes, containing 0·4 ml of sample, 3·2 ml of freshly prepared acid molybdate reagent (1 volume of 10 mmol l^{-1} ammonium molybdate, 1 volume of 2·5 mol l^{-1} sulfuric acid and 2 volume of acetone) was added. Absorbance of the yellow colour was measured at 355 nm in a spectrophotometer (Agilent 8453; Agilent Technologies, Horsholm, Denmark), using sodium acetate buffer with TCA as a blank.

Phosphate standard curve was prepared with KH_2PO_4 (Sigma-Aldrich, P5655), dissolved in 0·2 mol l^{-1} sodium acetate/HCl buffer (pH 5·5) and measured under the same conditions as the enzyme sample. The sensitivity of the phytase assay was estimated to be from 0 to 2 μmol ml^{-1} of inorganic phosphate.

One unit of phytase activity was defined as the amount of phytase that liberates 1 μmol ml^{-1} inorganic phosphate per minute from a 3 mmol l^{-1} K-phytate solution at pH 5·5 and a temperature of 30°C. This temperature and pH value were considered optimal for bread dough leavening (Haros *et al.* 2001).

Two phytase activities were calculated: volumetric and specific. Volumetric activities of extra- (E) and intracellular (I) phytase were expressed as unit per ml of enzyme extract (U ml^{-1}). Extracellular phytase activities were measured in culture supernatants, and intracellular (cytoplasmic and/or cell-wall bound) phytase activities were measured in crushed pellet supernatants. The phytase activity was measured at several time points (0, 15, 30

and 45 min) and calculated from the steepest part of the activity curve.

$$\mathrm{U\,ml^{-1}} = \frac{\Delta c_{\mathrm{PO_4}}}{\Delta t} \cdot \frac{V_t}{V} \quad (1)$$

where V_t is the total sample volume (2 ml); V, volume of extra- or intracellular enzyme extract (0·2 ml); $\Delta c_{\mathrm{PO_4}}$, measured changed phosphate concentration during time (μmol ml^{-1}); Δt, reaction time (min).

Specific activity of extracellular phytase (E) was expressed as unit per 10^{10} CFU [U (10^{10} CFU)$^{-1}$].

$$\mathrm{U\,(10^{10}CFU)^{-1}} = \frac{\frac{\Delta c_{\mathrm{PO_4}}}{\Delta t} \cdot \frac{V_t}{V}}{c_{\mathrm{CFU}}} \cdot 10^{10} \quad (2)$$

where c_{CFU} is the concentration of CFU (n ml^{-1});

Specific activity of intracellular phytase (I) was expressed as unit per milligram of total protein (U mg^{-1} total protein).

$$\mathrm{U\,mg^{-1}\,total\,protein} = \frac{\left(\frac{\Delta c_{\mathrm{PO_4}}}{\Delta t} \cdot \frac{V_t}{V}\right)}{c_{\mathrm{mg\ total\ protein}}} \quad (3)$$

where $c_{\mathrm{mg\ protein}}$ is the total protein concentration (mg ml^{-1}).

Protein determination

Protein concentrations in intracellular enzyme extracts were determined by measuring the absorbance of intracellular enzyme extract at 600 nm using the Bio-Rad Colorimetric Protein assay, kit II (Bio-Rad Laboratories Inc., Sundbybergm, Sweden). Bovine serum albumin (BSA) was used as protein concentration standard.

Results

Yeast growth on solid Delft + Phy medium

To check the ability of yeasts to grow on phytic acid as the only phosphorus source in a solid medium, yeast strains were cultivated on minimal Delft medium plates, supplemented with phytic acid dipotassium salt (Delft + Phy). As controls, yeast strains were cultivated on phosphate-containing (Delft + P, positive control) and phosphate-free (Delft − P, negative control) minimal Delft medium plates.

All 39 tested yeast strains grew on Delft + Phy as well as on Delft + P plates after 72-h incubation at 25°C (data not shown). For most of the tested strains, no major differences were noted in colony sizes between the two different media (data not shown). However, smaller colonies were observed on Delft + Phy plates in comparison with colonies on Delft + P plates for *S. cerevisiae* strains P3, P4 (Fig. 2), P5, P6 and *C. krusei* strain K3 (data not shown). Differences in colony sizes and growth intensities were observed between species. With the exception of *C. krusei* K3, intensive growth and big colonies on Delft + Phy and Delft + P were species-specific traits for *C. krusei*. As an example, *C. krusei* K190 colony growth is shown in Fig. 2. Noticeably, slower growth and smaller colonies were observed for *S. pastorianus* and *S. cerevisiae* strains (Fig. 2). With the exception of *A. adeninivorans*, very slight growth of yeast colonies was observed on Delft − P plates (Fig. 2).

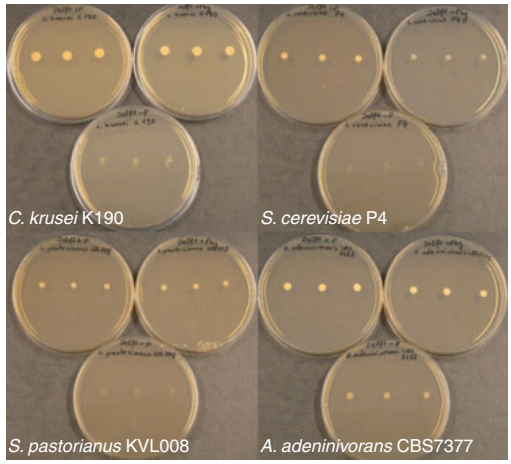

Figure 2 Growth of *Candida krusei* K190, *Saccharomyces cerevisiae* P4, *Saccharomyces pastorianus* KVL008 and *Arxula adeninivorans* CBS 7377 on Delft + P (phosphate) medium (top left); Delft + Phy (phytic acid dipotassium salt) medium (top right); and Delft − P (phosphate free) medium (bottom) plates after 72-h incubation.

Yeast growth in liquid Delft + Phy medium

Growth in liquid medium was quantified by measuring OD_{600} of the culture in microtitre plates over 48 h. The capabilities of the species to grow in medium, where the only source of phosphorus was phytate, were determined by comparing the growth parameters of the yeasts (i.e. final OD_{600}, λ and $\mu_{\mathrm{Phy}}/\mu_{\mathrm{P}}$) in Delft + Phy with those of positive (Delft + P) and negative (Delft − P) controls (Olstorpe *et al.* 2009).

All strains were able to grow in liquid Delft + Phy to varying extents (Fig. 3). Of the tested yeast strains, 37% grew very well in Delft + Phy and reached 93–98% of the

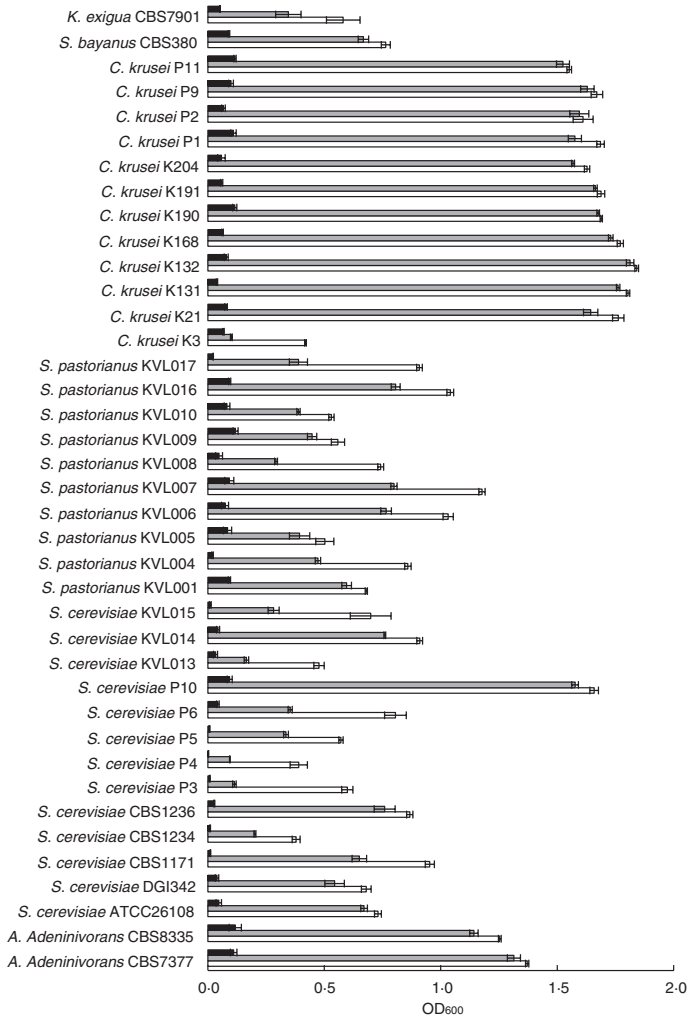

Figure 3 Optical density values (OD_{600}, horizontal axis) of yeast cell growth at 25°C in Delft − P (black bars), Delft + Phy (grey bars) and Delft + P (white bars) medium after 48-h cultivation. Error bars represent standard error from three separate growth analyses.

final optical density in Delft + P. This intensive growth in Delft + Phy was a species-specific trait for *C. krusei* and *A. adeninivorans* (Fig. 3). For *S. cerevisiae*, the final optical density ranged from 19% (strain P3) to 95% (strain P10), for *S. pastorianus* from 40% (strain KVL008) to 80% (strain KVL009), for *S. bayanus* it was about 86% and for *K. exigua* it was 60% (Fig. 3). Minimal growth occurred for all strains in Delft − P with OD_{600} ranging from 0·01 to 0·1 (Fig. 3).

The data in Table 3 show that no lag phase was observed for 20 strains of 39 grown in Delft + P medium, while in Delft + Phy medium, only eight strains did not show a lag phase. The lag phase for all strains was longer in Delft + Phy medium except for three strains of *S. cerevisiae*, four strains of *S. pastorianus* and one strain of *C. krusei*, which did not show a lag phase when either grown in Delft + Phy or in Delft + P media (Table 3). Of all tested strains, *S. pastorianus* KVL008 had the longest lag phases in both media. The μ_{Phy}/μ_P was larger than one for *c.* 33 and 50% of the strains in the respiro-fermentative phase and in the respiratory phase, respectively (Table 3). These findings indicate that the growth of these yeast strains is more rapid in the Delft + Phy medium than in the Delft + P medium, especially in the respiratory phase.

Table 3 Growth parameters for the yeast strains grown in modified defined minimal medium Delft + P (phosphate) and in Delft + Phy (phytic acid dipotassium salt)

			Respiro-fermentative phase			Respiratory phase		
Strains	λ_P*, h	λ_{Phy}†, h	μ_P‡, h^{-1}	μ_{Phy}§, h^{-1}	μ_{Phy}/μ_P¶	μ_P, h^{-1}	μ_{Phy}, h^{-1}	μ_{Phy}/μ_P
CBS7377	1·0	3·5	0·30 ± 0·03	0·24 ± 0·08	0·80	0·15 ± 0·03	0·16 ± 0·01	1·07
CBS8335	2·0	3·0	0·29 ± 0·01	0·25 ± 0·02	0·86	0·06 ± 0·01	0·08 ± 0·02	1·33
K3	–**	2·0	0·20 ± 0·01	0·18 ± 0·02	0·90	0·22 ± 0·01	0·02 ± 0·00	0·09
K21	0·5	3·0	0·23 ± 0·05	0·21 ± 0·03	0·91	0·09 ± 0·00	0·12 ± 0·01	1·33
K131	1·0	3·0	0·49 ± 0·02	0·46 ± 0·01	0·94	0·16 ± 0·02	0·20 ± 0·03	1·25
K132	1·0	2·0	0·30 ± 0·02	0·32 ± 0·05	1·07	0·09 ± 0·03	0·11 ± 0·02	1·22
K168	1·0	1·5	0·26 ± 0·02	0·26 ± 0·01	1·00	0·18 ± 0·01	0·21 ± 0·02	1·17
K190	–	–	0·27 ± 0·02	0·29 ± 0·01	1·07	0·11 ± 0·01	0·14 ± 0·01	1·27
K191	1·0	2·0	0·29 ± 0·04	0·20 ± 0·03	0·69	0·11 ± 0·01	0·17 ± 0·01	1·55
K204	1·0	3·5	0·30 ± 0·02	0·29 ± 0·01	0·97	0·12 ± 0·01	0·09 ± 0·00	0·75
P1	2·0	2·5	0·33 ± 0·02	0·31 ± 0·02	0·94	0·10 ± 0·01	0·10 ± 0·01	1·00
P2	0·5	3·5	0·29 ± 0·03	0·34 ± 0·05	1·17	0·09 ± 0·00	0·07 ± 0·00	0·78
P9	1·0	3·5	0·25 ± 0·01	0·33 ± 0·00	1·32	0·11 ± 0·01	0·13 ± 0·01	1·18
P11	–	3·0	0·26 ± 0·02	0·36 ± 0·02	1·39	0·11 ± 0·01	0·13 ± 0·02	1·18
CBS7901	–	10·0	0·20 ± 0·03	0·25 ± 0·01	1·25	0·22 ± 0·01	0·06 ± 0·01	0·27
CBS380	3·0	4·5	0·35 ± 0·04	0·30 ± 0·03	0·86	0·13 ± 0·03	0·11 ± 0·03	0·85
ATCC26108	1·0	3·0	0·46 ± 0·12	0·37 ± 0·14	0·80	0·12 ± 0·01	0·18 ± 0·03	1·50
DGI342	1·0	2·5	0·53 ± 0·06	0·38 ± 0·10	0·72	0·12 ± 0·01	0·14 ± 0·04	1·17
CBS1171	–	–	0·15 ± 0·02	0·10 ± 0·01	0·67	0·22 ± 0·09	0·32 ± 0·03	1·45
CBS1234	–	2·0	0·18 ± 0·01	0·16 ± 0·02	0·89	0·18 ± 0·01	0·18 ± 0·01	1·00
CBS1236	0·5	1·5	0·38 ± 0·14	0·22 ± 0·00	0·58	0·19 ± 0·04	0·19 ± 0·02	1·00
P3	–	2·5	0·27 ± 0·01	0·17 ± 0·01	0·63	0·17 ± 0·01	0·06 ± 0·01	0·35
P4	3·0	7·0	0·08 ± 0·01	0·08 ± 0·02	1·00	0·19 ± 0·01	0·18 ± 0·01	0·95
P5	–	3·0	0·20 ± 0·00	0·21 ± 0·02	1·05	0·15 ± 0·02	0·02 ± 0·00	0·13
P6	1·0	1·5	0·35 ± 0·02	0·26 ± 0·01	0·74	0·14 ± 0·01	0·05 ± 0·00	0·36
P10	–	1·0	0·34 ± 0·03	0·33 ± 0·13	0·97	0·13 ± 0·01	0·12 ± 0·02	0·92
KVL013	–	2·0	0·19 ± 0·04	0·18 ± 0·07	0·95	0·17 ± 0·01	0·08 ± 0·01	0·47
KVL014	–	–	0·24 ± 0·02	0·24 ± 0·01	1·00	0·14 ± 0·03	0·15 ± 0·01	1·07
KVL015	–	–	0·15 ± 0·01	0·11 ± 0·01	0·73	0·21 ± 0·05	0·20 ± 0·01	0·95
KVL001	–	3·0	0·23 ± 0·04	0·22 ± 0·08	0·96	0·12 ± 0·02	0·02 ± 0·01	0·17
KVL004	–	1·0	0·33 ± 0·02	0·12 ± 0·02	0·36	0·11 ± 0·01	0·18 ± 0·02	1·50
KVL005	–	–	0·15 ± 0·01	0·16 ± 0·01	1·07	0·18 ± 0·04	0·14 ± 0·02	0·78
KVL006	–	–	0·20 ± 0·01	0·14 ± 0·02	0·70	0·26 ± 0·00	0·18 ± 0·03	0·69
KVL007	–	3·0	0·18 ± 0·02	0·25 ± 0·02	1·34	0·16 ± 0·01	0·17 ± 0·02	1·06
KVL008	5·0	14·0	0·23 ± 0·04	0·27 ± 0·01	1·18	0·09 ± 0·02	0·09 ± 0·01	1·00
KVL009	–	–	0·23 ± 0·05	0·19 ± 0·04	0·82	0·16 ± 0·01	0·14 ± 0·04	0·86
KVL010	–	2·0	0·23 ± 0·03	0·09 ± 0·01	0·39	0·12 ± 0·02	0·20 ± 0·02	1·67
KVL016	–	1·0	0·25 ± 0·01	0·29 ± 0·02	1·16	0·12 ± 0·01	0·12 ± 0·01	1·00
KVL017	–	–	0·16 ± 0·01	0·14 ± 0·01	0·86	0·30 ± 0·03	0·13 ± 0·02	0·43

Results are expressed as the mean of three replicated measurements and standard deviation (±SD).
*Duration of the lag phase for the yeast strains grown in Delft + P.
†Duration of the lag phase for the yeast strains grown in Delft + Phy.
‡The maximum specific growth rate for the yeast strains grown in Delft + P.
§The maximum specific growth rate for the yeast strains grown in Delft + Phy.
¶The maximum specific growth rate ratio calculated from the mean values.
**No lag phase was observed.

Extra- and intracellular phytase activities

On the basis of the results mentioned previously, 14 yeast strains were selected for further determination of extracellular and intracellular phytase activity. Four *S. cerevisiae* strains, i.e. ATCC26108, DGI342, CBS1236 and KVL015, and one *C. krusei* strain K132, were selected as representative strains within species with higher μ_{phy}/μ_P values in the respiratory phase than in the respiro-fermentative phase, and with values close to one (KVL015) or higher.

The strains *S. cerevisiae* P10 and *S. pastorianus* KVL016 were selected as those with the highest final optical density within these two species. Four *C. krusei* strains, i.e. P1, P2, P11 and K204, were selected as representative strains within a species for their rapid growth in liquid Delft + Phy medium. *Saccharomyces cerevisiae* KVL013 and *S. pastorianus* KVL008 were chosen as negative controls for their very low final optical density in liquid Delft + Phy when compared with Delft + P. Moreover, KVL008 had the longest lag phase in both media in comparison with all other strains. *Arxula adeninivorans* CBS7377 was chosen as the positive control for general comparison, because this species is well known to exhibit high extra- and intracellular phytase activities (Sano *et al.* 1999; Olstorpe *et al.* 2009).

The 14 selected strains were pregrown in Delft + Phy, containing 3 mmol l^{-1} phytic acid dipotassium salt, to activate phytase production (Andlid *et al.* 2004; Veide and Andlid 2006; In *et al.* 2008). There is no internationally recognized unit defining phytase activity, which depends on assay conditions, *e.g.* substrate source and concentration, assay temperature and pH, reaction time (Selle and Ravindran 2007). In this study, one phytase activity unit is defined as the amount of enzyme that liberates 1 μmol ml^{-1} inorganic orthophosphate per minute from 3 mmol l^{-1} K-phytate at pH 5·5 and a temperature of 30°C. Two activities were calculated – volumetric and specific.

The extracellular specific and volumetric phytase activities differed dramatically between the two *S. pastorianus* strains (Table 4). Both activities were larger in *S. pastorianus* KVL008 than in *S. pastorianus* KVL016. Strain KVL016 had 1% of specific activity and 4% of volumetric activity of activities for strain KVL008. The extracellular phytase activities also differed among *S. cerevisiae* strains. The lowest specific activity was observed in strain CBS1236, which had 4% activity of the strain with the highest activity (KVL015). The lowest volumetric activity was observed in two *S. cerevisiae* strains: CBS1236 and KVL013. They had 22% activity of strain KVL015. Five strains of *C. krusei* could be grouped according to their extracellular specific activities into two groups: first group with activity of *c.* 110 mU $(10^{10}$ CFU$)^{-1}$ (strains K204 and P1) and second group with activity of *c.* 521 mU $(10^{10}$ CFU$)^{-1}$ (strains P2, K132 and P11). There were no differences in specific activities between the two *C. krusei* strains in the first group. Specific activities among *C. krusei* strains in the second group were almost equal and similar to those observed for *A. adeninivorans* (Table 4). Surprisingly, our results show that the strain with the highest extracellular specific and volumetric phytase activity was the negative control *S. pastorianus* KVL008 (a lager beer strain), followed by *S. cerevisiae* KVL015 (an ale beer strain) and *C. krusei* P2 (isolated from sorghum beer).

The intracellular specific and volumetric phytase activities within two species, *S. pastorianus* and *C. krusei*, were almost equal, except *C. krusei* strain P1, which had threefold higher specific and volumetric activities than other strains. The intracellular specific and volumetric activities, however, differed among *S. cerevisiae* strains (Table 4).

Table 4 Volumetric (mU ml^{-1}) and specific extracellular [mU $(10^{10}$ CFU$)^{-1}$] and intracellular (mU mg^{-1} total protein) phytase activities from yeast strains of *Saccharomyces cerevisiae*, *Saccharomyces pastorianus*, *Candida krusei* and *Arxula adeninivorans*

		Phytase activities*			
		Extracellular		Intracellular	
Species	Strains	mU ml^{-1}	mU $(10^{10}$ CFU$)^{-1}$	mU ml^{-1}	mU mg^{-1} total protein
S. cerevisiae	ATCC26108	9 ± 1	281 ± 22	6 ± 1	4 ± 1
	DGI342	10 ± 1	62 ± 7	6 ± 1	3 ± 1
	CBS1236	3 ± 1	28 ± 2	20 ± 3	12 ± 1
	P10	28 ± 1	457 ± 52	14 ± 2	8 ± 1
	KVL013	3 ± 1	83 ± 28	30 ± 5	17 ± 3
	KVL015	67 ± 1	732 ± 8	36 ± 1	22 ± 3
S. pastorianus	KVL008	76 ± 6	1981 ± 20	14 ± 2	8 ± 1
	KVL016	3 ± 1	26 ± 3	19 ± 3	11 ± 0
C. krusei	K132	30 ± 5	509 ± 62	18 ± 1	11 ± 2
	K204	20 ± 3	111 ± 18	17 ± 2	10 ± 2
	P1	14 ± 2	110 ± 5	46 ± 2	27 ± 3
	P2	50 ± 14	595 ± 58	19 ± 5	11 ± 2
	P11	35 ± 3	460 ± 53	15 ± 1	9 ± 2
A. adeninivorans	CBS7377	61 ± 5	519 ± 38	6 ± 1	3 ± 1

*Results are expressed as the mean of three replicated measurements and standard error of the mean (±SEM).

For all of the tested yeast strains, the extracellular specific phytase activities were significantly higher than the intracellular phytase-specific activities (Table 4). The extracellular volumetric activities, however, were higher than the intracellular volumetric activities for ten strains of 14 tested strains. The intracellular volumetric phytase activities were higher than the measured extracellular volumetric phytase activities for the following strains: *S. cerevisiae* KVL013 (10-fold), *S. cerevisiae* CBS1236 and *S. pastorianus* KVL016 (sevenfold) and *C. krusei* P1 (threefold).

Discussion

Yeast strains with high extracellular phytase activity might be used directly in bread making for the production of bread with high content of bioavailable minerals because of the activity of phytase during dough fermentation. Although phytase activity of baker's yeast has been widely documented, both under bread dough leavening conditions (Harland and Harland 1980; Harland and Frolich 1989; Turk *et al.* 1996, 2000; Andlid *et al.* 2004) and other conditions (Nakamura *et al.* 2000; Haraldsson *et al.* 2005; Veide and Andlid 2006; In *et al.* 2008, 2009), there seems to be no high phytase-active yeasts available for bread industry today. Our objective therefore was to study the extra- and intracellular phytase activities of yeast strains, isolated from various grain-based food and beers, under conditions optimal for bread dough leavening in order to identify strains that could be used in baking industry to increase the bioavailability of minerals in bread.

Strains belonging to *S. cerevisiae*, *K. exigua* and *C. krusei* have often been isolated from bread leavens (Hansen 2006). In this study, one *K. exigua* strain CBS7901 isolated from sourdough, eight *C. krusei* strains isolated from kenkey and three baker's yeast strains (*S. cerevisiae*) were tested. Furthermore, 24 yeast strains isolated from beer and belonging to *S. cerevisiae*, *S. bayanus*, *S. pastorianus* and *C. krusei* were also tested.

Inorganic phosphate is an important nutrient required in millimolar concentrations in yeast for the synthesis of phospholipids, nucleic acids and cellular metabolites (Wykoff and O'Shea 2001). As phosphate is often present in only low amounts in the environment, some yeast cells may produce phytase. Phytase is an inducible enzyme in most micro-organisms and its expression is subjected to a complex regulation (Greiner 2005).

To investigate whether the yeast species and strains in our study produce phytase, the simple and rapid, growth-based test on solid Delft + Phy medium was used (Howson and Davis 1983; Lambrechts *et al.* 1992; Bae *et al.* 1999). However, because of the fact that some micro-organisms may grow on solid phytate medium but not in liquid medium and *vice versa* (Tseng *et al.* 2000), growth test in liquid Delft + Phy medium in parallel with the test on solid medium was performed. Intensive growth of *A. adeninivorans* on Delft + Phy plates after 3 days of cultivation is in agreement with previous results describing this yeast species as phytase positive (Sano *et al.* 1999). Interestingly, growth of *S. cerevisiae* P10 (isolated from pito) was conspicuously intensive in comparison with other *S. cerevisiae* strains, and the growth curve was similar to that of *C. krusei* (data not shown), which has been described as the most prominent species for phytase production (Quan *et al.* 2001; Hellstrom *et al.* 2010). Expectedly, very slight growth of yeasts colonies was observed on Delft − P plates, except for *A. adeninivorans*, indicating that this yeast species may possess an unusual large storage pool of phosphate in the form of e.g. polyphosphate in the vacuoles (Shirahama *et al.* 1996).

Yeast phytases have been found to be mostly extracellular (Nayini and Markakis 1984; Sano *et al.* 1999; Nakamura *et al.* 2000), with the exception of *C. krusei* (Quan *et al.* 2001), *Pichia anomala* (Vohra and Satyanarayana 2001), *Schwanniomyces castellii* (Lambrechts *et al.* 1992), which are cell-wall bound. Our results show that, for all the tested strains, the extracellular phytase activity is higher than the intracellular phytase activity. In fact, the intracellular phytase activity of almost all the strains is very low. From an industrial point of view, the extracellular phytase activity would be more important for bread making than the intracellular phytase activity, because the yeast cells should be intact in the dough in order to ensure a good fermentation. In this case, the intracellular phytase will not have access to phytate in the dough.

Our results demonstrate that the phytase activity of *S. cerevisiae* and *C. krusei* is strain dependent. These data agree with those of Turk *et al.* (2000) and Hellstrom *et al.* (2010), respectively, and they stress the fact that yeast should be considered at a strain level, and not only at a species level, when selecting high phytase-active yeasts for bread making. Interestingly, among the six *S. cerevisiae* strains and five *C. krusei* strains tested, the highest extra- and intracellular phytase activities are found for KVL015 (an ale beer strain) and P2 (isolated from sorghum beer), respectively.

To the best of our knowledge, lager beer yeast (*S. pastorianus*) has never been described as phytase positive. In this study, we have screened ten strains of *S. pastorianus* for phytase production. After the preliminary screening, we chose two of them for further investigations. *Saccharomyces pastorianus* KVL016 shows very low extracellular specific phytase activity. Unexpectedly,

S. pastorianus KVL008, which was chosen as a negative control, shows the highest extracellular specific and volumetric phytase activities among all the strains screened in this study. In fact, the specific extracellular phytase activity of *S. pastorianus* KVL008 is *c.* 3-times higher than the activity of the other screened strains. At present, we do not have a clear explanation for this phenomenon. However, this finding shows that slow yeast growth in liquid medium, supplemented with phytate, does not necessarily indicate low phytase activity.

In conclusion, the three yeast strains, i.e. KVL008, KVL015 and P2, with the highest extracellular specific and volumetric phytase activities are beer strains. *Saccharomyces pastorianus* KVL008 is a lager beer strain, *S. cerevisiae* KVL015 is an ale beer strain and *C. krusei* P2 is isolated from sorghum beer. In our days, brewer's yeast was not used for bread making. However, before the commercial production of baker's yeast began, ale-barm or brewer's yeast was used for this purpose (Davidson 1999). The high phytase activities of the yeast strains mentioned earlier, observed under conditions optimal for bread dough leavening (i.e. pH 5·5 and 30°C), suggest that these yeast strains may be a particularly interesting source of phytase for the production of wholegrain bread with high content of bioavailable minerals.

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Paper 2

Isolation and identification of phytase-active yeasts from sourdoughs

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Isolation and identification of phytase-active yeasts from sourdoughs

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ABSTRACT

Identification of high phytase-active yeasts is necessary in order to find prominent candidates for the production of wholemeal bread with high content of bioavailable minerals. Tested yeasts were isolated from Danish and Lithuanian sourdoughs, since high phytase-active yeasts adapted to grow in sourdough matrix would be a good choice for bread industry. Isolated species were; *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Pichia occidentalis*, *Candida humilis* and *Kazachstania exigua*. Studies of phytase-positive isolates were carried out at conditions optimal for leavening of bread dough (pH 5.5 and 30 °C). All the tested yeasts isolated from sourdoughs exhibited phytase activities. The most prominent isolates for extracellular phytase production were found to be *S. cerevisiae* L1.12 with a specific extracellular activity of 10.6 U/10^{10} CFU, followed by *S. cerevisiae* L6.06 with a specific extracellular activity of 8.2 U/10^{10} CFU. Some other isolates of *S. cerevisiae*, one of *C. humilis*, and one of *P. kudriavzevii* also had high specific extracellular activities of *c.* 4–7 U/10^{10} CFU. These isolates may be potential candidates for improving mineral bioavailability in whole grain bread.

1. Introduction

Nowadays, whole grain bread is gaining popularity across the world due to increasing awareness of its nutritional benefits. Several epidemiological studies found that intake of whole grain food protects against certain type of cancers, cardiovascular disease, type 2 diabetes and obesity (Slavin, 2003), due to a wide range of nutrients and biologically active constituents, such as dietary fibre, vitamins and minerals (Spiegel et al., 2009). Whole grain food contains, however, considerable amounts of phytic acid, which is the most recognized and documented antinutritional factor that chelates divalent minerals such as iron and zinc (Brune, Rossanderhulten, Hallberg, Gleerup, & Sandberg, 1992; Sandberg & Svanberg, 1991). Phytic acid forms insoluble complexes with these minerals into phytate and thus reduces their bioavailability (Brune et al., 1992; Lopez, Leenhardt, Coudray, & Remesy, 2002). Efficient reduction of phytate can be achieved by enzymatic degradation during food processing, either by increasing the activity of endogenous phytase, or by addition of enzyme preparations (Sandberg et al., 1999). Several studies have shown that a reduction of the phytate content, based on enzymatic hydrolysis, causes an increased absorption of minerals, such as zinc, iron, calcium and magnesium (Brune et al., 1992; Reinhold, Faradji, Abadi, & Ismailbeigi, 1976).

Phytate degrading enzymes – phytases - are enzymes naturally found in cereals (Eeckhout & DePaepe, 1994) and microorganisms (Lambrechts, Boze, Moulin, & Galzy, 1992; Olstorpe, Schnurer, & Passoth, 2009; Ullah & Gibson, 1987). Wheat and rye exhibit high phytase activities with values ranging from 900 to 2886 U/kg dry matter in wheat and from 4100 to 6100 U/kg dry matter in rye (Eeckhout & DePaepe, 1994; Greiner & Egli, 2003). However, the activities in wheat were considered insufficient to notably improve the mineral bioavailability in whole grain wheat bread (Harland & Harland, 1980; Turk & Sandberg, 1992).

The amount of hydrolysed phytate in different bread types varied between 13 and 100% (Lopez et al., 2001) and depends on various factor, including phytase activity, temperature, pH, and fermentation time (Turk & Sandberg, 1992) A few studies have shown that the content of phytate decreasesby 25–60% of the initial level during wheat bread making from high extraction flour (Bartnik & Florysiak, 1988; Daniels & Fisher, 1981). On the other hand, phytate is fully degraded during commercial rye bread production due to the higher phytase activity in rye and long processing time as compared to wheat bread making (Nielsen, Damstrup, Dal Thomsen, Rasmussen, & Hansen, 2007).

Many studies have shown that the addition of sourdough during the bread making can reduce the phytate content in bread (Lopez et al., 2001; Turk, Carlsson, & Sandberg, 1996). Moreover, Mckenzieparnell and Davies (1986) reported that the highest levels of phytate remained in unleavened bread. Most wheat bread even whole wheat bread is produced with short fermentation times and

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the bread is leavened by use of baker's yeast instead of sourdough fermentation. The reason for this might be the time-consuming sourdough preparation.

Phytase activity in baker's yeast has been studied previously under bread dough leavening conditions (Harland & Harland, 1980; Turk et al., 1996; Turk, Sandberg, Carlsson, & Andlid, 2000). Harland and Harland (1980) showed that phytate hydrolysis may be increased up to 25% by adding the double amount of yeast. In contrast, very low phytase activity from baker's yeast was observed during wheat bread making in experiments of Turk et al. (1996).

To our knowledge, there are no high phytase-active yeasts available for bread industry today. Our objective therefore was to isolate and identify yeasts from different sourdoughs and to study their phytase activity under conditions optimal for leavening of wheat bread dough (30 °C and pH 5.5) in order to identify species and/or strains with high activities that might be used in the baking industry.

2. Materials and methods

2.1. Sourdough sampling

13 sourdoughs samples were collected from three Danish (A, B and C) and three Lithuanian (D, E and F) bakeries. Ten out of the 13 sourdoughs were made from rye flour, two from wheat flour, and one from spelt flour (Table 1). The two Danish rye sourdoughs (DS-1, DS-2) and the Lithuanian sourdough LS-2 were made from high extraction rye flour. Moreover, amylolytic enzymes were added to the sourdough LS-2.

The sourdough samples from the Danish bakeries were collected and delivered to our laboratory within 1 h, where they were stored at 4 °C until they were characterized (within 8 h).

The sourdough samples from the Lithuanian bakeries were collected and promptly frozen, and stored at −20 °C until shipped to our laboratory. Afterwards, these sourdoughs were refreshed according to the bakery recipes and characterized.

2.2. pH and total titratable acidity (TTA)

Ten grams of each sourdough samples were suspended in 90 ml sterile ultrapure water and homogenized in a stomacher (STOMACHER 400, VWR Bie & Berntsen, Herlev, Denmark) at normal speed for 1 min. The pH value was recorded with a Knick Portamess pH meter (Elscolab, Heiloo, Nederland). Subsequently, the suspension was titrated with 0.1 mol/L NaOH to a final pH of 8.5 ± 0.1. The TTA was expressed as the amount (mL) of 0.1 mol/L NaOH required to neutralize 10 g of sample. Two independent measurements were performed for each sample.

2.3. Isolation of yeasts and enumeration

Ten grams of each sourdough samples were suspended in sterile 90 mL saline peptone solution (NaCl, 8.5 g/L; Na_2HPO_4, 0.3 g/L; peptone, 1.0 g/L; pH 5.5) and homogenized in a stomacher at normal speed for 1 min. Further, decimal dilutions were prepared and 0.1 mL of each were spread onto YPG agar (D-(+)-glucose, 10 g/L; yeast extract, 3 g/L; peptone 5 g/L; pH 5.5) supplemented with 50 mg/L chlortetracycline and 100 mg/L chloramphenicol to inhibit bacterial growth. Incubation was carried out at 30 °C for 72 h. Two independent counts of colony forming units (CFU) were performed for each sample. Further, 20 colonies were randomly selected from plates with 50–200 colonies, re-cultivated in 10 mL YPG broth at 30 °C for 24 h, and purified by streaking onto YPG agar. Purified yeast isolates for long-term storage were stored at −80 °C in YPG broth containing 2.75 mol/L glycerol.

2.4. DNA extraction, molecular typing and identification

A total of 221 isolates were initially grouped according to their rep-PCR (repetitive DNA sequence-based polymerase chain reaction) fingerprinting patterns as described below. Initially, DNA was extracted using InstaGene DNA extraction kit (Bio-Rad Laboratories, Sundbyberg, Sweden) following the instructions of the manufacturer. The rep-PCR reaction was carried out in a 25 μL volume containing 2.5 μL 10 × PCR reaction buffer (Fermentas, Vilnius, Lithuania), 0.2 μL *Taq* polymerase (5 U/μL, Fermentas), 1.5 mmol/L $MgCl_2$ (Fermentas), 200 μmol/L of dNTP-mix (Fermentas), 0.8 μmol/L of primer GTG_5 (DNA Technologies, Aarhus, Denmark), 1.5 μL of DNA template, and sterile MilliQ water to adjust to 25 μL (Nielsen, Teniola, et al., 2007). The reaction was performed in an automatic thermal cycler (GeneAmp PCR System 9700, Perkin-Elmer) and was basically carried out as described by Nielsen, Teniola, et al. (2007) with slight changes: 5 min of initial denaturation at 94 °C, 30 cycles of 94 °C for 30 s, 45 °C for 60 s, 65 °C for 8 min followed by a final elongation step of 65 °C for otherwise 16 min. The PCR products were separated by 1.5% agarose gel

Table 1
Main characteristics of sourdoughs.

Bakeries	Sourdough sample	Flour type	Fermentation temperature, °C	pH	TTA, mL[a]	Dry matter, %	log CFU/g sample
Danish bakeries							
A	DS-1	Rye	32	3.75 ± 0.01	13.3 ± 0.1	36.1 ± 0.1	7.3 ± 0
B	DS-2	Rye	21	3.80 ± 0.00	20.1 ± 1.3	47.4 ± 0.1	6.9 ± 0.1
C	DS-3	Wheat	22	3.80 ± 0.01	7.7 ± 0.4	34.8 ± 0.5	7.8 ± 0
	DS-4	Spelt	22	3.65 ± 0.00	13.9 ± 1.3	35.9 ± 1.2	6.9 ± 0.1
Lithuanian bakeries							
D	LS-1	Rye	28–32	4.07 ± 0.01	14.9 ± 0.1	55.1 ± 0	7.4 ± 0
	LS-2	Rye	43–45	3.37 ± 0.01	28.1 ± 0.3	33.3 ± 1.4	Nd[b]
E	LS-3	Rye	24–26	4.20 ± 0.01	10.0 ± 0.3	49.1 ± 0	7.9 ± 0
	LS-4	Rye	24–26	4.17 ± 0.01	11.3 ± 0.1	38.1 ± 0	7.4 ± 0
F	LS-5	Wheat	34–35	3.60 ± 0.01	7.5 ± 0.2	18.7 ± 0	Nd
	LS-6	Rye	34–35	3.47 ± 0.01	18.3 ± 0.3	26.0 ± 0	7.7 ± 0
	LS-7	Rye	34–35	3.46 ± 0.01	11.9 ± 0.1	30.0 ± 0	7.5 ± 0
	LS-8	Rye	34–35	3.43 ± 0.00	11.0 ± 0.1	27.8 ± 0.2	7.5 ± 0
	LS-9	Rye	34–35	3.91 ± 0.01	10.9 ± 0.1	25.5 ± 0.1	6.7 ± 0.1

Results are expressed as the mean of two replicated measurements and standard deviation (±SD).
[a] the TTA was expressed as the amount (mL) of 0.1 mol/L NaOH required to neutralize 10 g of sample.
[b] not detected.

electrophoresis (5 h, 120 V) in 1 × TBE buffer using a Generuler 1 kb DNA ladder (Fermentas) as reference. Subsequently, gels were stained with ethidium bromide, destained in sterile MilliQ and documented using an AlphaImager HP (Alpha Innotech, Kem-En-Tec A/S, Taastrup, Denmark). Electrophoretic patterns were compared using the pattern analysis software package BioNumerics version 4.5 (Applied Maths NV, Sint-Martens-Latem, Belgium). For cluster analysis, the similarity among digitized profiles was calculated on the basis of the Dice's coefficient. The dendrogram was constructed according to the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA).

Based on the cluster analysis results, a representative number of isolates within each of the different clusters were identified to species level by sequencing of the D1/D2 region of the 26S rRNA gene. For amplification of the D1/D2 domain, the NL-1(5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL-4 (5′-GGT CCG TGT TTC AAG ACG G-3′) primers (DNA Technologies, Aarhus, Denmark) were used (Jespersen, Nielsen, Honholt, & Jakobsen, 2005; Kurtzman & Robnett, 1998). The reaction was performed in an automatic thermal cycler (GeneAmp PCR System 9700) and was basically carried out as described by Jespersen et al. (2005) with some changes: 5 min of initial denaturation at 94 °C, 30 cycles of 94 °C for 90 s, 53 °C for 30 s, 72 °C for 90 s followed by a final extension at 72 °C for 7 min. The amplified products were purified and sequenced by Macrogen Inc., South Korea. The sequences were assembled by use of CLC Main Workbench 5.7.1 (CLC bio A/S, Aarhus) and compared to the sequences reported in GenBank using the BLAST algorithm. Furthermore, the same isolates were identified on the basis of carbohydrate assimilation profiles using the API ID 32 C kit (BioMerieux, Marcy-L'Etolie, France).

2.5. Nucleotide accession numbers

The nucleotide sequences determined in this study have been assigned GenBank Accession Nos. JQ585726–JQ585744.

2.6. Growth test for phytase-active yeasts

Three different defined minimal media were prepared according to Nuobariene, Hansen, Jespersen, and Arneborg (2011) and used to investigate the ability of the yeast strains to grow on media with phytate as the only phosphor source: Delft + P (positive control; phosphate containing medium), Delft-P (negative control; phosphate free medium) and Delft + Phy (phytate medium; K-phytate (phytic acid dipotassium salt; P5681, Sigma–Aldrich, Broendby, Denmark) as phosphate source). Due to the fact that some microorganisms may grow on solid phytate medium but not in liquid medium and *vice versa* (Tseng, Fang, & Tseng, 2000), growth tests were performed both on solid and in liquid medium.

2.7. Extracellular and intracellular phytase activities

Extracellular and intracellular (cytoplasmic and/or cell-wall bound) phytase extracts were prepared according to Nuobariene et al. (2011) as well as measurements of the phytase activity with some modifications. Briefly, 40 μL of substrate solution (3 mmol/L K-phytate in 0.2 mol/L sodium acetate/HCl buffer, pH 5.5) was preincubated in 1.5-ml eppendorf tube (VWR International ApS, Herlev, Denmark) at 30 °C for 3 min, and 10 μL of enzyme extract was added, mixed and incubated at 30 °C. Samples were taken at different time intervals (0, 15, 30 and 45 min) during assaying, and the reaction was stopped immediately by adding 50 μL TCA (trichloroacetic acid solution; 10 g of TCA dissolved in sterile ultrapure water to a final volume of 100 mL). Enzyme blank was prepared from sodium acetate buffer mixed with enzyme extract and TCA. Substrate blank was prepared from substrate solution mixed with TCA. Afterwards, the preassay mixture was mixed with 800 μL of freshly prepared acid molybdate reagent (1 volume of 10 mmol/L ammonium molybdate, 1 volume of 2.5 mol/L sulphuric acid and 2 volume of acetone) and incubated at room temperature for 15 min. Liberated inorganic phosphate from K-phytate was measured using a 96-well quartz microtiter plate (VWR Bie & Berntsen, Herlev, Denmark). A total volume of 225 μL test solution was transferred into each well. The plate was placed in the Microplate reader chamber (M965 AcuuReader; Metertech; Food Diagnostic, Grenaa, Denmark), shaken for 10 s, and the absorbance was read at 355 nm.

One unit of phytase activity was defined as the amount of phytase that liberates 1 μmol/L inorganic phosphate per minute from a 3 mmol/L K-phytate solution at pH 5.5 and a temperature of 30 °C. This temperature and pH value were considered as optimal for bread dough leavening (Haros, Rosell, & Benedito, 2001).

Volumetric and specific activities were calculated as described previously (Nuobariene et al., 2011). Volumetric activities were expressed as unit per ml of enzyme extract (U/mL). Extracellular specific activities were measured in culture supernatants and expressed as unit per 10^{10} CFU (U/10^{10} CFU). Intracellular specific activities were measured in crushed pellet supernatants and expressed as unit per milligram of total protein (U/mg total protein). The total protein content of intracellular enzyme extracts were determined as described in Nuobariene et al. (2011).

3. Results

3.1. Sourdough characteristics

Results from the characterization of the 13 sourdoughs collected from bakeries in Denmark and Lithuania are shown in Table 1. The pH values were very similar in all samples collected from Denmark ranging from 3.65 to 3.80, while the pH values in sourdoughs from Lithuania varied between 3.37 and 4.20. The lowest TTA values of 7.7 mL and 7.5 mL were observed in wheat sourdoughs DS-3 (Denmark) and LS-5 (Lithuania), respectively. The highest TTA value of 28.1 mL was observed in rye sourdough LS-2 made from high extraction flour. The very high TTA may be due to a higher content of fermentable carbohydrates as a result of the added amylolytic enzymes.

3.2. Enumeration and yeast identification

The numbers of yeast were about the same in the Danish (6.9–7.8 log CFU/g sample) and the Lithuanian (6.9–7.9 log CFU/g sample) sourdoughs (Table 1). No yeast was isolated from rye sourdough LS-2 and it could be explained by fact that sourdough yeasts may not tolerate high temperatures (43–45 °C). No yeast was either isolated from the liquid wheat sourdough LS-5; i.e. the sourdough with the lowest dry matter content (Table 1), so the yeasts might not have survived the freeze storage during transportation.

In total, 221 yeast colonies were isolated and purified from 11 out of 13 sourdough samples. All the isolates were subjected to the rep-PCR analysis. Based on results from cluster analysis, isolates were grouped according to their fingerprint pattern. For a representative number of isolates within each group; i.e. 19 isolates in total, the identifications to species level were further done by sequencing of the D1/D2-region of the 26S rRNA gene (Fig. 1). Based on results from sequencing, five species were identified; i.e. *Candida humilis*, *Pichia occidentalis* (formerly named as *Issatchenkia occidentalis*), *Pichia kudriavzevii* (formerly named as *Issatchenkia orientalis*, anamorph *Candida krusei*), *Saccharomyces cerevisiae* and *Kazachstania exigua* (formerly named as *Saccharomyces*

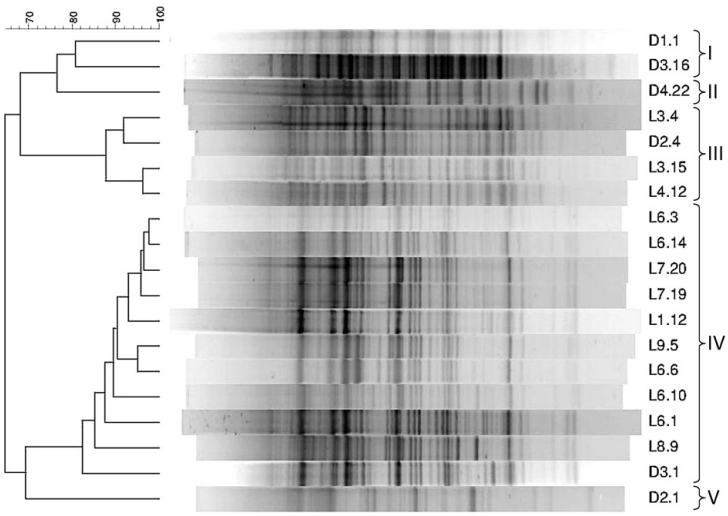

Fig. 1. rep-PCR-based dendrogram and gel image representing the fingerprint patterns of isolates, belonging to the following species; I – *Candida humilis*, II – *Pichia occidentalis*, III – *Pichia kudriavzevii*, IV – *Saccharomyces cerevisiae*, V – *Kazachstania exigua*. For cluster analysis the similarity among digitized profiles was calculated on the basis of the Dice's coefficient and the tree was generated using UPGMA (unweighted pair group with arithmetic averages).

exiguus (Kurtzman, 2003)). All sequenced isolates showed high similarities (100%) to sequences in the GenBank (Table 2). The identification results obtained by API 32C ID system was in agreement with those obtained by molecular identification for all isolates, except one; i.e. *P. occidentalis* which was attributed to *Candida sake* (data not shown).

Table 2
Identification of yeast species by sequencing of the D1/D2 region of the 26S rRNA gene.

Groups[f]	Isolate	Sequencing of the D1/D2 region			
		Length D1/D2	Identities	Similarity to GenBank sequence, %	Closest related yeast species
I	D3.16	582	570/570	100.0	*C. humilis*[a]
	D1.1	576	571/571	100.0	*C. humilis*
II	D4.22	573	573/573	100.0	*P. occidentalis*[b]
III	L3.4	579	579/579	100.0	*P. kudriavzevii*[c]
	D2.4	590	585/585	100.0	*P. kudriavzevii*
	L3.15	578	578/578	100.0	*P. kudriavzevii*
	L4.12	580	580/580	100.0	*P. kudriavzevii*
IV	L6.3	584	584/584	100.0	*S. cerevisiae*[d]
	L6.14	582	582/582	100.0	*S. cerevisiae*
	L7.20	599	596/596	100.0	*S. cerevisiae*
	L7.19	581	581/581	100.0	*S. cerevisiae*
	L1.12	584	584/584	100.0	*S. cerevisiae*
	L9.5	590	590/590	100.0	*S. cerevisiae*
	L6.6	582	582/582	100.0	*S. cerevisiae*
	L6.10	581	581/581	100.0	*S. cerevisiae*
	L6.1	567	567/567	100.0	*S. cerevisiae*
	L8.9	590	590/590	100.0	*S. cerevisiae*
	D3.1	589	589/589	100.0	*S. cerevisiae*
V	D2.1	586	573/573	100.0	*K. exigua*[e]

[a] *Candida humilis.*
[b] *Pichia occidentalis* (formerly named as *Issatchenkia occidentalis*).
[c] *Pichia kudriavzevii* (formerly named as *Issatchenkia orientalis*, anamorph *Candida krusei*).
[d] *Saccharomyces cerevisiae.*
[e] *Kazachstania exigua* (formerly named as *Saccharomyces exiguus*, anamorph *Candida holmii*).
[f] Based on results from sequencing of the D1/D2 region of the 26S rRNA gene, isolates were divided into five groups.

Table 3 gives an overview of yeast species found in the individual Danish and Lithuanian sourdoughs. *C. humilis* was the predominant species (54% of isolates) in Danish sourdoughs and was isolated from three out of four sourdoughs, followed by *K. exigua* (22% of isolates) and *S. cerevisiae* (19% of isolates) which were isolated from one and two sourdoughs, respectively. Other isolated species; i.e. *P. kudriavzevii* and *P. Occidentalis*, accounted for 7% of the isolates.

The dominant yeast species isolated from Lithuanian sourdoughs were *S. cerevisiae* (72% of isolates) which was the dominant species in five out of seven sourdoughs followed by *P. kudriavzevii* (29% of isolates). The Lithuanian sourdoughs were dominated by one yeast species where a combination of two yeast species was found in three out of four Danish sourdoughs.

3.3. Phytase-active yeasts growth test

All 19 sequenced isolates grew very well on/in Delft + Phy as well as on/in Delft + P with the exception of *C. humilis* D1.01. Smaller colonies for this strain were observed on Delft + Phy plates in comparison with colonies on Delft + P plates (data not shown). In liquid Delft + Phy, *C. humilis* D1.01 reached 56% of the final optical density in Delft + P. For the rest of the tested strains, growth in liquid Delft + Phy was as intensive as in Delft + P (Fig. 2). Minimal growth in liquid Delft-P occurred for all strains with final OD ranging from 0.02 to 0.07 (Fig. 2).

3.4. Extra- and intracellular phytase activities

In this study two activities; i.e. volumetric and specific, were calculated for extra- and intracellular phytases, and the results are listed in Table 4. The highest extracellular activities of all isolates were observed in *S. cerevisiae* L1.12 isolated from Lithuanian rye sourdough with a volumetric activity of 55 mU/mL and a specific

Table 3
Yeasts isolated from Danish and Lithuanian sourdoughs.

Sample	Number of isolates	% of yeast population				
		Candida humilis	*Kazachstania exigua*	*Pichia kudriavzevii*	*Pichia occidentalis*	*Saccharomyces cerevisiae*
DS-1	20	100				
DS-2	20		80	20		
DS-3	20	85				15
DS-4	23	35			9	56
LS-1	20					100
LS-2	Nd[a]					
LS-3	20			100		
LS-4	18			100		
LS-5	Nd					
LS-6	20					100
LS-7	20					100
LS-8	20					100
LS-9	20					100

Results are expressed as the mean of two independent counts of colony forming units (CFU) and standard deviation (±SD).
[a] not detected.

activity of 10.6 U/10^{10} CFU. Extracellular specific phytase activities among other *S. cerevisiae* isolates differed very much and varied between 0.9 and 8.2 U/10^{10} CFU. Also, the extracellular volumetric and specific activities were larger for the *C. humilis* isolate D3.16 than for D1.01. The specific extracellular phytase activity values within the four *P. kudriavzevii* isolates ranged from 1.1 to 4.6 U/10^{10} CFU, and the highest activity was observed in isolate L3.04, isolated form Lithuanian rye sourdough, being in the same level as observed for the *C. humilis* isolate D3.16. *K. exigua* and *P. occidentalis* had similar specific extracellular activities with values of *c.* 2 U/10^{10} CFU.

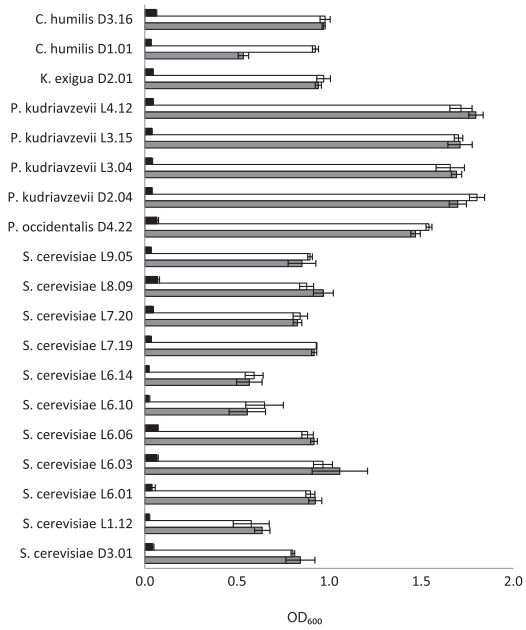

Fig. 2. Optical density values (OD_{600}, horizontal axis) of yeast cell growth at 30 °C in Delft-P (black bars), Delft + Phy (grey bars) and Delft + P (white bars) medium after 48 h cultivation. Error bars represent standard deviation from three separate growth analyses.

4. Discussion

The objectives of this work were to (i) isolate and identify yeasts from sourdough, and (ii) to test them for phytase activity under conditions optimal for bread dough leavening (pH 5.5, and 30 °C). The idea is that high phytase-active yeast adapted to grow in a sourdough matrix might be directly used in baking industry to increase the bioavailability of minerals in bread.

In the present study, four out of the five identified yeast species; i.e. *S. cerevisiae*, *C. humilis*, *K. exigua*, *P. kudriavzevii*, have been described in sourdough previously (Gullo, Romano, Pulvirenti, & Giudici, 2003; Succi et al., 2003; Zhang et al., 2011). *P. occidentalis* has been related to pulp of tropical fruits (Trindade, Resende, Silva, & Rosa, 2002), vineyard and winery (Sabate, Cano, Esteve-Zarzoso, & Guillamon, 2002) as well as to table olives (Arroyo-Lopez, Duran-Quintana, Ruiz-Barba, Querol, & Garrido-Fernandez, 2006).

Table 4
Volumetric (U/ml) and specific extracellular (U/10^{10} CFU) and intracellular (U/mg protein) phytase activities from yeast strains of *Candida humilis*, *Kazachstania exigua*, *Pichia kudriavzevii*, *Pichia occidentalis* and *Saccharomyces cerevisiae*.

Species	Strains	Phytase activities[a]			
		Extracellular		Intracellular	
		mU/mL	U/10^{10} CFU	mU/mL	mU/mg total protein
C. humilis	D3.16	37 ± 9	4.7 ± 0	12 ± 1	11 ± 1
	D1.01	1 ± 0	2.3 ± 0.2	12 ± 2	15 ± 3
K. exigua	D2.01	34 ± 2	2.0 ± 0.1	22 ± 0	19 ± 0
P. kudriavzevii	L4.12	36 ± 10	1.1 ± 0	24 ± 6	63 ± 1
	L3.15	46 ± 14	1.4 ± 0.1	13 ± 2	32 ± 3
	L3.04	92 ± 6	4.6 ± 0.1	52 ± 7	107 ± 5
	D2.04	51 ± 18	2.8 ± 0	42 ± 11	90 ± 17
P. occidentalis	D4.22	30 ± 3	2.1 ± 0.1	32 ± 1	32 ± 1
S. cerevisiae	L9.05	47 ± 7	3.2 ± 0.2	32 ± 1	63 ± 1
	L8.09	5 ± 2	0.9 ± 0.2	36 ± 2	47 ± 8
	L7.20	23 ± 4	2.7 ± 0	31 ± 3	45 ± 2
	L7.19	30 ± 1	4.1 ± 0.4	74 ± 5	114 ± 2
	L6.14	19 ± 1	3.5 ± 0.2	51 ± 19	50 ± 1
	L6.10	39 ± 4	6.4 ± 0.1	38 ± 2	47 ± 2
	L6.06	56 ± 5	8.2 ± 0.2	29 ± 1	41 ± 11
	L6.03	36 ± 7	7.3 ± 0	44 ± 2	49 ± 4
	L6.01	12 ± 3	1.7 ± 0.2	18 ± 1	31 ± 16
	L1.12	55 ± 2	10.6 ± 0.5	31 ± 6	42 ± 8
	D3.01	14 ± 3	2.3 ± 0.1	48 ± 0	36 ± 0

[a] Results are expressed as the mean of three replicated measurements and standard error of the mean (±SEM).

According to our study, the species composition differs between Lithuanian and Danish sourdoughs as well as within bakeries. A pure culture of *S. cerevisiae* is found in 71% of Lithuanian sourdoughs with fermentation temperature of 30–35 °C. These data agree with the observation made by Meroth, Hammes, and Hertel (2003), showing that *S. cerevisiae* is the dominating yeast species in sourdough with fermentation temperature of 30 °C. Other authors have also reported the dominance of *S. cerevisiae* in Italian (Succi et al., 2003), Turkish (Gul, Ozcelik, Sagdic, & Certel, 2005) and Chinese (Zhang et al., 2011) sourdoughs. *Saccharomyces cerevisiae* is found in association with *C. humilis* in two out of four Danish sourdoughs in this investigation. *C. humilis* has previously been reported to be a dominant yeast species in sourdoughs for the production of bread made from durum wheat bran (Gullo et al., 2003). Several other studies have also confirmed the presence of *C. humilis* in sourdoughs (Barnett, Payne, & Yarrow, 2000; Gullo et al., 2003). *P. kudriavzevii* is found as a non-dominant yeast species in one out of four Danish sourdoughs in association with *K. exigua*, while *P. kudriavzevii* is found as a pure yeast culture in two Lithuanian sourdoughs. Whereas, the occurrence of *K. exigua* in sourdough is well-documented (Pulvirenti, Solieri, Gullo, De Vero, & Giudici, 2004; Valmorri, Tofalo, Settanni, Corsetti, & Suzzi, 2010), only a few studies have, as yet, reported on the isolation of *P. kudriavzevii* from artisan Belgian (Vrancken et al., 2010) and Chinese (Zhang et al., 2011) sourdoughs.

Using the simple and rapid growth tests on solid and in liquid media, containing K-phytate as the main phosphorus source, we have seen that all tested isolates are able to grow very well; except for the *C. humilis* isolate D1.01. This is in agreement with our previous results describing some yeast strains as phytase positive microorganisms (Nuobariene et al., 2011). However, from growth tests, based on monitoring of cells growth in phytate rich environment, it is impossible to predict which yeast isolates will have higher or lower phytase activities (Nuobariene et al., 2011). Therefore, it is essential also to perform phytase activity assays on each isolate.

Phytase activity is detected in the extracellular as well as in the intracellular (crushed cells suspension) medium of all tested isolates. These results are in agreement with previous studies, indicating that yeast phytases are extracellular (Nakamura, Fukuhara, & Sano, 2000) and intracellular (cell-wall bound) (Lambrechts et al., 1992) enzymes. However, our results show that the specific extracellular phytase activity of all the tested yeast is at least 20-fold higher than the specific intracellular activity, and they are in agreement with our previous findings (Nuobariene et al., 2011).

All the tested isolates exhibit different phytase activities. Taken together with the fact that the yeasts are isolated from different sourdoughs from different countries and bakeries, it may be postulated that the different isolates represent different strains. Phytase activity was also shown to be strain dependent in the studies of Turk et al. (2000) and Nuobariene et al. (2011).

In this study we used a microtiter plate method to assess phytase activity instead of the single cuvette method described in Nuobariene et al. (2011). We find that the microtiter plate method is more rapid, and that the results are comparable with those of the single cuvette method (data not shown). In our previous work (Nuobariene et al., 2011) we have shown that the specific extracellular phytase activity in yeasts isolated from grain-based food and beer range from 0.03 to 2.0 $U/10^{10}$ CFU. In the current study, we show that the phytase activities of yeasts isolated from sourdoughs are at least five times higher. The reason for this difference may be that the yeasts isolated from sourdoughs are adapted to grow in phytate rich medium and thus produce more phytase. This, however, remains to be investigated.

The most prominent isolates for extracellular phytase production are *S. cerevisiae* L1.12 with a specific extracellular activity of 10.6 $U/10^{10}$ CFU, followed by *S. cerevisiae* L6.06 with a specific extracellular activity of 8.2 $U/10^{10}$ CFU. Three other isolates of *S. cerevisiae*, one of *C. humilis*, and one of *P. kudriavzevii* also have high specific extracellular activities of *c.* 4– $U/10^{10}$ CFU. Whereas *S. cerevisiae* and *P. kudriavzevii* are well known to be good phytase producers (Nuobariene et al., 2011), *C. humilis* has, to the best of our knowledge, never been described as phytase positive. All these isolates may be potential high phytase-active yeast starters for the use in the baking industry. Taking into consideration, however, the fact that phytate in a whole grain dough matrix may be found in aggregation with different compounds, such as protein, starch and dietary fibre, further studies are necessary to establish the significance of high phytase-active yeast to degrade phytate during bread making.

In conclusion, the two *S. cerevisiae* isolates with the highest extracellular specific and volumetric activities; i.e. L1.12 and L6.06, may be ideal candidates for improving mineral bioavailability in whole grain bread, as *S. cerevisiae* are generally recognized as safe (GRAS) and as baker's yeasts are strains of *S. cerevisiae*.

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Paper 3

Isolation and identification of phytase-active lactic acid bacteria from sourdoughs

Isolation and identification of phytase-active lactic acid bacteria from sourdoughs

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Running title: **Phytase-active LAB from sourdoughs**

Abstract

Identification of high phytase-active lactic acid bacteria is necessary in order to find prominent candidates for the production of wholemeal bread with high content of bioavailable minerals. Tested lactic acid bacteria were isolated from Lithuanian sourdough, since high phytase-active yeasts adapted to grow in sourdough matrix would be a good choice for bread industry. Isolated species were; *Lactobacillus frumenti*, *Lactobacillus reuteri*, *Lactobacillus fermentum* and *Pediococcus pentosaceus*. Studies of phytase positive-isolates were carried out at conditions optimal for leavening of dough bread (pH 5.5 and 30°C). Phytase activities of tested lactic acid bacteria have been found to be extra- and intra-cellular. The most prominent isolates for extracellular phytase production were found to be two *L. frumenti* isolates 2.5 and 2.1 with a volumetric phytase activity of 56-60 mU/mL. Herein, for the first time, *L. frumenti* isolates isolated from sourdough are reported as phytase-positive strains.

Keywords: phytate; phytase activity; *Lactobacillus frumenti*; *Lactobacillus fermentum*; sourdough

1. Introduction

The consumption of whole grain products is recommended to be increased, because several epidemiological studies show protective effects of whole grain products against several food related diseases (Slavin, 2004). Grain products, such as a whole-grain bread has been recommended because of their high content of dietary fiber, B-vitamins, vitamin E and several minerals of which P, Mg, Fe, Cu, Ca and Zn are the most important. Each component may partly account for its beneficial effects on human health (De Munter et al., 2007; Slavin, 2003). However, the main part of minerals in cereals are complexly bound to phytic acid (myo-inositol 1,2,3,4,5,6-hexaphosphate) as phytate, a phosphor-rich component used by all cereal grains as mineral store (Febles et al., 2002; Kumar et al., 2010). In wheat the content of phytic acid is 0.4-1.4%, while the content is 0.5-1.5% in rye (Reddy, 2002).

Phytic acid is the most recognized and documented antinutritional factor due to formation of the insoluble complexes with minerals and thus reduced bioavailability (Cheryan, 1980; Erdman, 1981). Several studies have shown that a reduction of phytate content in grain-based food causes an increased absorption of minerals, such as calcium (Lonnerdal et al., 1989; Reinhold et al., 1974), iron (Brune et al., 1992; Hallberg, 1987), zinc (Erdman, 1981) and magnesium (Pallauf et al., 1998). Reduction of phytate can be achieved by enzymatic degradation during bread making, either by increasing the activity of endogenous cereal phytase, or by addition of phytase-active lactic acid bacteria (LAB), yeasts, or other microorganisms (Nayini and Markakis, 1983;Türk and Sandberg, 1992). However, there seems to be no high phytase-active yeasts or LAB available for bread industry today.

Phytase [*myo*-inositol hexakis (dihydrogenphosphate) phosphohydrolase] is a common term used to describe enzymes that hydrolyses phosphomonoester bonds from phytate into *myo*-inositol and phosphoric acid via penta- to mono-phosphate, thus decreasing or eliminating its antinutritional effect (Konietzny and Greiner, 2002). The activity of phytases increase available phosphate, minerals and a non-chelator compound *myo*-inositol. Phytases are widely distributed among cereals and microorganisms. In wheat, the phytase values ranging from approx. 900 to 2900 U/kg dry matter, while the phytase activity in rye varies from 4100 to 6100 U/kg dry matter (Eeckhout and DePaepe, 1994; Greiner and Egli, 2003; Steiner et al., 2007).

Generally, the activity of cereal phytase is considered to be insufficient to significantly decrease the amount of phytate in whole-wheat flour during wheat bread making (Türk and Sandberg, 1992). On the other hand in commercial rye bread making with addition of sourdough, the phytate is fully degraded (Nielsen et al., 2007). This may be due to the higher phytase activity in rye and long processing time compared to wheat bread making even the rye dough pH is lower than the pH-optimum (pH 6) for rye phytase (Greiner and Egli, 2003; Nielsen et al., 2008). Some of the LAB or yeast from the sourdough may also contribute with phytase activity during sourdough fermentation. Reale et al (2004) showed that the phytate content in whole-wheat dough decreased about 80-90% after 12 h fermentation using LAB as starter culture, whereas only approx. 50% of phytate was hydrolysed when the dough was fermented with yeast under the same conditions.

The phytase activities have been found in microorganisms, such as yeast and LAB, used in food fermentation. A few studies have shown yeast phytase to be mostly extracellular and their activities ranging from 14 to 566 mU/mL (Lambrechts et al., 1992; Nuobariene et al., 2011, 2012; Olstorpe et al., 2009). However, only few strains

of LAB, i.e. *Lactobacillus plantarum, L.brevis, L. curvatus*, *L. sanfranciscensis, L. fermentum, L. plantarum* were shown to express intracellular (cytoplasmic and/or cell-wall bound) phytase activity in phytate rich medium (De Angelis et al., 2003; Lopez et al., 2000; Reale et al., 2004).

The use of selected LAB with high phytase activity could find use as starters to increase the mineral bioavailability in whole-meal bread fermented in short time in combination with yeast as alternative to yeast fermented bread.

So far, only few strains of LAB, i.e. *Lactobacillus amylovorus* and *L. plantarum* present in a wide variety of microbial systems of plant origin, showed consistent extracellular phytase activity (Sreeramulu et al., 1996). However, Reale et al. (2007) were unable to confirm these findings using the same *L. amylovorus* strain under identical growth conditions.

No LAB with high phytase activity is available for the bread industry today to increase the mineral availability in production of whole meal bread. Our objective was therefore to isolate LAB from different sourdoughs and to screen them for phytase activity under conditions optimal for bread dough leavening (pH 5.5, and 30°C).

2. Material and methods

2.1 Sourdough sampling

Seven sourdoughs samples were collected from two Lithuanian bakeries (A and B). Six out of the seven sourdoughs were made from rye flour, while one was made from wheat flour (Table 1). The sourdough LS-2 was made from high extraction rye flour,

while sourdoughs S5 and S6 were made from low extraction flour. Moreover, amylolytic enzymes were added to this sourdough. All five sourdoughs from bakery B were made with addition of scalded flour.

The sourdough samples from the bakeries were collected and promptly frozen, and stored at -20°C until shipped to laboratory at University of Copenhagen. Afterwards, the sourdoughs were refreshed according to the bakery recipes and characterized by pH, total titratable acids (TTA), dry matter content and total number of LAB (log CFU). For this purpose commercial whole-meal and wheat flours retailed on the local market (Copenhagen, Denmark) were used. During refreshment the sourdoughs were fermented for 24 h at optimal sourdough temperatures as determined from the bakeries (Table 1).

2.2 pH and total titratable acidity (TTA)

Ten grams of each sourdough sample were suspended in 10 ml 96% ethanol and 80 mL sterile ultrapure water and homogenized in a stomacher (STOMACHER 400, VWR Bie & Berntsen, Herlev, Denmark) at normal speed for 1 min. The pH value was recorded using a Knick Portamess pH meter (Elscolab, Heiloo, Nederland). Subsequently, the suspension was titrated with 0.1 M NaOH to a final pH of 8.5±0.1. The TTA was expressed as the amount (mL) of 0.1 NaOH required to neutralize 10 g of sample. Two independent measurements were performed for each sample.

2.3 Isolation and enumeration of lactic acid bacteria

Ten grams of each sourdough sample were suspended in sterile 90 mL physiological saline (0.9% NaCl) and homogenized in a stomacher at normal speed for 1 min. Further, decimal dilutions were prepared and 0.1 mL of each were spread onto

modified MRS (mMRS) agar, pH 5.5 (Table 2). Incubation was carried out at 30°C for 72 h under anaerobic conditions in the anaerobic jar (Sigma-Aldrich, 28029) with anaerobic atmosphere generation bags (Sigma-Aldrich, 68061) placed in. Two independent colony forming units (CFU) counts were performed for each sample. Furthermore, 24 colonies were randomly selected from plates with 50-200 colonies, re-cultivated in 10 mL mMRS broth at 30°C for 48 h, and purified by streaking onto mMRS agar. Purified LAB isolates for long-term storage were stored at -80°C in mMRS broth containing 50% (v/v) glycerol.

2.4 Growth media and growth test for phytase-active lactic acid bacteria

A total of 168 isolates and five LAB strains, belonging to *Lactobacillus sakei* (KTU05-6), *Pediococcus acidilactici* (KTU05-7) and *P. pentosaceus* (KTU05-10, KTU05-8, KTU05-9) previously isolated from Lithuanian sourdoughs (Digaitiene et al., 2005) were screened using the method described by Nuobariene et al. (2011). Three different Chemically Defined Media (CDM), modified from Hébert et al. (2004) were used to investigate the ability of the strains to grow on media supplemented with phytate as the only phosphorus source: CDM+P (positive control; phosphate containing medium), CDM-P (negative control; phosphate free medium), and CDM+Phy (phytate medium; phytic acid dipotassium salt as phosphate source) (Table 2).

Amino acids, vitamins and ferrous sulphate were prepared separately as 50-fold concentrated stock solution, filter sterilized (0.2 μm Minisart filters), and stored at 4°C. Potassium, magnesium, and manganese salts were prepared separately as 50-fold concentrated stock solution, autoclaved at 121°C for 15 min, and stored at 4°C. Heat-sensitive phytic acid dipotassium salt was prepared according to Nuobariene et

al. (2011). To stabilize the pH during LAB cultivation, 50 mmol/L succinic acid/NaOH buffer, pH 5.5, was used.

Solid medium and LAB inocula for growth test in/on CDM as well as intra- and extracellular phytase extraction were prepared as described by Nuobariene et al. (2011) with some modifications. Briefly, for growth test of isolated LAB on agar plates and growth test in microtiter plates, CDM media were used (Table 2). LAB inocula were prepared from cells grown overnight in mMRS at 30°C under anaerobic conditions.

2.5 DNA extraction, molecular typing and identification

Based on preliminary screening of the isolated LAB for phytase activity 21 out of 168 isolates were chosen and initially grouped according to their repetitive DNA sequence-based polymerase chain reaction (rep-PCR) fingerprint patterns as described below. Initially, DNA was extracted using GenElute^{TM}Bacterial Genomic kit (Sigma-Aldrich, Broendby, Denmark) following the instructions of the manufacturer. The rep-PCR reactions were carried out with repetitive primer pair Rep-1R-Dt (5'-III NCG NCG NCA TCN GGC-3')/Rep2-Dt (5'-NCG NCT TAT CNG GCC TAC-3'), where N is any nucleotide and I is inosine (Versalovic et al., 1994) purchased from Eurofins MWG Operon (Ebersberg, Germany) in a thermal cycler (GeneAmp PCR System 9700, Perkin-Elmer) as described previously (Christiansen et al., 2006). Electrophoretic patterns were compared using the pattern analysis software package BioNumerics version 4.5 (Applied Maths NV, Sint-Martens-Latem, Belgium). For cluster analysis, the similarity among digitized profiles was calculated on the basis of the Dice's coefficient. The dendrogram was constructed according to the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA).

Based on the cluster analysis results, a representative number of isolates within each of the different clusters were identified to species level. Total extracted DNA as mentioned above was used for amplification of the 16S rRNA gene using the primers 16S-27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16S-1492R (5'-GGT TAC CTT GTT ACG ACT T-3') as previously described (Tuma et al., 2007).

The amplified products were purified and cycle sequenced in both directions by Macrogen Inc., South Korea. The primers 16S-27F, 16S-1492R, 16S-518F (5'-CCG GCA GCC GCG GTA ATA CG-3'), and 16S-800R (5'- TAC CAG GGT ATC TAA TCC-3') were used to sequence almost complete 16S rRNA. The sequences were assembled by use of CLC Main Workbench 5.7.1 (CLC bio A/S, Aarhus) and compared to the sequences reported in GenBank using the BLAST algorithm.

2.6 Nucleotide accession numbers

The nucleotide sequences determined in this study have been assigned GenBank Accession Nos. JX272054-JX272063

2.7 Determination of phytase activity

Volumetric and specific activities of intra- and extracellular phytase were determined as described previously (Nuobariene et al., 2011). Briefly, 0.8 mL of substrate solution (3 mmol/L K-phytate (Sigma-Aldrich, P5681) in 0.2 mol/L sodium acetate/HCl buffer, pH 5.5), was preincubated at 30°C for 5 min, 0.2 mL of enzyme extract was added, mixed, and incubated at 30°C. Samples were taken at different intervals (0, 15, 30, and 45 min) during assaying, and the reaction was stopped immediately by adding 1 mL 10% trichloroacetic acid (TCA). Enzyme blank was prepared from sodium acetate buffer mixed with enzyme extract and TCA. Substrate

blank was prepared from substrate solution mixed with TCA. Afterwards, the 0.4 mL of preassay mixture was mixed with 3.2 mL of freshly prepared acid molybdate reagent (1 volume of 10 mmol/L ammonium molybdate, 1 volume of 2.5 mol/L sulphuric acid and 2 volume of acetone) and incubated at room temperature for 15 min. Liberated inorganic phosphate from phytic acid dipotassium salt was measured using a 96-well quartz microtiter plate (VWR Bie & Berntsen, Herlev, Denmark). A total volume of 200 µL test solution was transferred into each well. The plate was placed in the Microplate reader chamber (M965 AcuuReader; Metertech; Food Diagnostic, Grenaa, Denmark), shaken for 10 s, and the absorbance was read at 355 nm.

One unit of phytase activity was defined as the amount of phytase that liberates 1 µmol/L inorganic phosphate per minute from a 3 mmol/L K-phytate solution at pH 5.5 and at 30°C. Volumetric activities were expressed as unit per ml of enzyme extract (U/mL). Extracellular specific activities were measured in culture supernatants and expressed as unit per 10^{10} CFU (U/10^{10} CFU). Intracellular specific activities were measured in crushed pellet supernatants and expressed as unit per milligram of total protein (U/mg total protein). The total protein content of intracellular enzyme extracts were determined by measuring the absorbance of intracellular enzyme extract at 600 nm using the Bio-Rad Colorimetric Protein assay, Kit II (Bio-Rad Laboratories Inc). BSA (bovine serum albumin) was used as protein concentration standard.

3. Results

3.1 Sourdough characteristics

Results from the characterization of the seven sourdoughs collected from bakeries in Lithuania are shown in Table 3. The pH values in the five samples collected from bakery B varied only from pH 3.43 to 3.91, while the pH values in the two sourdoughs from bakery A varied more from pH 3.37 to 4.07. The lowest TTA value of 7.5 mL was observed in wheat sourdough S3, and the highest TTA value of 28.1 mL was observed in rye sourdough S2 made from high extraction flour. The very high TTA of S2 may be due to higher content of fermentable carbohydrates as amylolytic enzymes were added into this sourdough.

3.2 Enumeration and growth test for phytase-active LAB

The numbers of LAB were about the same in all sourdoughs (8.9 – 9.5 log CFU/g sample) except in rye sourdough S1 which had a lower viable number of 7.3 log CFU/g sample. This sourdough had also the highest pH indicating that the fermentation was not finished (Table 3).

All 168 tested isolates grew well on CDM+P plates, whereas only 21 isolates grew on CDM+Phy plates after four days incubation at 30°C. For nine out of 21 isolates, no major differences were noted in colony sizes between the two different media (data are not shown). However, smaller colonies were observed on CDM+Phy plates in comparison with colonies in CDM+P for 12 out of 21 isolates. Very slight growth or no growth was observed on CDM–P plates.

Similar results were found when the 21 selected LAB isolates were growing in liquid media (Fig 1). 20% of tested LAB isolates grew very well in CDM+Phy and reached 83-96% of the final optical density in CDM+P. This intensive growth in CDM+Phy was a species-specific trait for *L. frumenti* (Fig 1). For *L. fermentum* the final optical density in CDM+Phy ranged from 28% (isolate 5.2) to 77% (isolate 7.2)

of the final optical density in CDM+P. Growth of *L. reuteri* in CDM+Phy was very weak and reached only 7-28% of the final optical density in CDM+P. Minimal growth in liquid CDM-P occurred for isolates with final OD ranging from 0.01 to 0.10, except for *L. frumenti* isolates (0.061 – 0.074).

3.3 Identification of LAB

In total, 168 LAB colonies were isolated, purified and tested for phytase activity. Based on results from growth test, 21 isolate which show growth in/on liquid/solid CDM+Phy media were subjected to the rep-PCR analysis. Based on results from cluster analysis, isolates were grouped according to their fingerprint pattern into four groups (Table 4). For a representative number of isolates within each group, i.e. 9 isolates in total, the identification to species level were further done by sequencing the 16S rRNA gene. Based on results from sequencing, four species were identified; i.e. *Lactobacillus fermentum*, *L. reuteri*, *L. frumenti* and *P. pentosaceus*. All sequenced isolates showed high similarities (99.9-100%) to sequences in the GenBank (Table 4).

3.4 Extracellular ad intracellular phytase activities

Two activities for extra- and intracellular phytases were calculated – volumetric and specific (Table 5).

The extracellular volumetric and specific phytase activities differed both among species and between strains within the same species. The highest extracellular volumetric activities of all isolates were observed in two *L. frumenti* isolates (56-60 mU/mL) whereas the highest extracellular specific activities were observed in *P. pentosaceus* isolates (23-30 U/10^{10}CFU). The extracellular volumetric and specific activities differed between the four *L. fermentum* isolates from 3 to 46 mU/mL and

from 1 to 10 U/10^{10} CFU, respectively (Table 5). Both activities were highest in *L. fermentum* 5.1 whereas the activities for other two *L. fermentum* isolates 6.1 and 7.2 were lower but equal. The extracellular phytase activities also differed among *L. reuteri* and *P. pentosaceus* isolates.

The intracellular volumetric and specific activities differed among the species (Table 5). The highest intracellular activities of all isolates were observed in *L. fermentum* 7.2 isolated from rye sourdough with a volumetric activity of 61 mU/mL and a specific activity of 362 mU/10^{10} CFU followed by two other isolates of *L. fermentum* (6.1 and 5.2), two *L. frumenti* isolates (2.1 and 2.5) and a *P. pentosaceus* isolate (KTU05-9).

4. Discussion

In order to produce bread with high content of bioavailable minerals, the phytate should be hydrolysed during dough fermentation. According to Lopez et al. (2000) and Reale et al. (2004) the phytate content in wheat bread dough was reduced 60% and 80-90% after 5 h and 12 h of fermentation, respectively. However, long dough fermentation time resulted in more sour tasting bread.

LAB strains with high extracellular phytase activity might be used to produce bread with high content of bioavailable minerals at shorter dough fermentation time which will result in less acid and therefore more acceptable bread taste. Although cytoplasmic and cell-bound phytase activity of LAB isolated from sourdough has been documented (De Angelis et al., 2003; Lopez et al., 2000), there seems to be no high extracellular phytase active LAB available for bread industry today. Moreover, to the best of our knowledge, no phytase active LAB has been tested under bread

dough leavening conditions jet. Therefore, the objectives of this study were to (i) isolate and identify phytase active LAB from sourdoughs, and (ii) to test them for phytase activity under conditions optimal for bread dough leavening (pH 5.5, and 30°C).

In the present study, the four identified LAB species have also been isolated from sourdoughs previously; i.e. *P. pentosaceus* (Faid et al., 1994), *L. frumenti* (Muller et al., 2000), *L. reuteri* (Hamad et al., 1992) and *L. fermentum* (Spicher and Schröder, 1978). Despite these LAB strains are frequently found in sourdough systems, the investigation of phytase active LAB isolated from sourdough under conditions optimal for bread dough leavening (pH 5.5, 30°C) is limited.

In this study, we have investigated the ability of 168 LAB isolates to grow on/in solid/liquid CDM+Phy media, where phytate was the only source of phosphorus and 13% of isolates produced phytase under both solid and liquid conditions. This is consistent with a resent investigation of 150 LAB strains, where less than 25% tested strains were able to hydrolyze phytate on/in solid/liquid media (Anastasio et al., 2010).

After the initial LAB growth test on/in media supplemented with phytate, isolates were tested for extra- and intracellular phytase activity. LAB phytases have been reported to produce significant intracellular or cell bound phytase activities rather than extracellular activities (Palaciosa et al., 2008; De Angelis et al., 2003). However, our results demonstrate that the phytase activities of tested LAB strains are extra- and intracellular. These data agree with those of Sreeramulu et al. (1996), and he reported that nine out of 19 tested LAB of the genera *Lactobacillus* and *Streptococcus* exhibit the extracellular phytase activity. From an industrial point of view, the extracellular phytase activity would be more important for bread making

than the intracellular phytase activity, since the LAB cells should be intact in the dough in order to ensure a good fermentation. The intracellular phytase will not have access to degrade the phytate in the dough.

In this study, the highest volumetric extracellular activities (56-60 mU/mL) were found among *L. frumenti* isolates. Moreover, to the best of our knowledge, *L. frumenti* has never been described as a phytase positive species. Observed extracellular phytase activities in *L. fermentum* isolate 5.1 and *P. pentosaceus* isolate 1.2 are consistent with Songre-Ouattara et al. (2008) report showing that *L. fermentum* isolated from fermented pearl-millet slurries produce extracellular phytase in MRS broth. Additionally, we found that *L. fermentum* also produces intracellular phytase. In contrast, Zamudio et al. (2001) found, that *L. fermentum* and *P. pentosaceus* at pH 4.5 and 37°C exhibit very low extracellular phytase activity (less than 6.3 mU/mL) and do not produce intracellular phytase activity. Moreover, Coda et al. (2011) reported the phytase activity value of 2.5 U (nmol/mL per minute) in sourdough when *P. pentosaceus* and *Lactobacillus plantarum* were used as starter culture. Another study showed the hydrolysis of phytate in wheat flour medium (pH 4.3, 30°C) by using whole cells of *Lactobacillus acidophilus*, *L. plantarum* and *Leuconostoc mesenteroides* isolated from sourdough (Lopez et al., 2000).

The most prominent isolates for extracellular phytase production would be two *L frumenti* isolates (i.e., 2.5 and 2.1) with a volumetric phytase activity of 56-60 mU/mL, followed by *P. pentosaceus* KTU05-9 with a volumetric phytase activity of 54 mU/mL and *L. fermentum* 5.1 with a volumetric phytase activity of 51 mUmL.

To determine the real benefits of fermenting whole-grain-wheat dough with selected LAB, it would be necessary to investigate the hydrolytic break down of phytate during dough fermentation and to check the concentration of free

microelements, such as Fe^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, Ca^{2+}, in dough matrix. This study provides a preliminary testing of LAB isolates to identify the best strains for further studies.

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Table 1 Sourdough type and fermentation conditions

Bakeries	Sourdough sample	Rye flour type	Wheat flour type	Fermentation temperature, °C	Dough yield	Firmness	Additions
A	S1	1370[a]	NI[e]	28-32	167	Firm	NI
	S2	1740[b]	NI	43-45	276	Fluid	Amylolytic enzymes
B	S3	NI	550 C[d]	34-35	250	Fluid	Scalded flour
	S4	1370	NI	34-35	258	Fluid	Scalded flour with malt
	S5	700[c]	NI	34-35	250	Fluid	Scalded flour with malt
	S6	700	NI	34-35	250	Fluid	Scalded flour
	S7	1370	NI	34-35	250	Fluid	Scalded flour

[a]Ash content of 1.31 to 1.60%
[b]Ash content of 1.61 to 1.80%
[c]Ash content of 0.61 to 0.75%
[d]Ash content of 0.51 to 1.63%
[e]NI: not included

Table 2 Medium used in the experiment: modified MRS (mMRS), Chemically Defined Medium (CDM): CDM+P (positive control; phosphate containing medium), CDM-P (negative control; phosphate free medium), and CDM+Phy (phytate medium; phytic acid dipotassium salt as phosphate source)

Component	Medium composition, g/L			
	mMRS	CDM+P	CDM-P	CDM+Phy
D-glucose	7.00	7.00	7.00	7.00
Fructose	7.00	7.00	7.00	7.00
Maltose	7.00	7.00	7.00	7.00
Tryptone	10.00	NI	NI	NI
Meat extract	5.00	NI	NI	NI
Yeast extract	3.00	NI	NI	NI
Sodium acetate	5.00	5.00	5.00	5.00
Sodium gluconate	2.00	2.00	2.00	2.00
Ammonium citrate	NI	1.00	1.00	1.00
Phytic acid dipotassium salt	NI	NI	NI	0.74
$K_2HPO_4 \cdot 3\ H_20$	2.6	6	NI	NI
KCl	NI	NI	3.28	3.21
$FeSO_4 \cdot 4H_20$	NI	0.02	0.02	0.02
$MgSO_4 \cdot 7H_20$	0.10	0.10	0.10	0.10
$MnSO_4 \cdot 4H_20$	0.05	0.05	0.05	0.05
Cysteine-HCl·H_20	0.50	0.50	0.50	0.50
Tween 80	0.001	0.001	0.001	0.001
Bases				
Adenine	NI	0.01	0.01	0.01
Xanthine	NI	0.01	0.01	0.01
Thymine	NI	0.01	0.01	0.01
Uracil	NI	0.01	0.01	0.01
Guanine	NI	0.01	0.01	0.01
Vitamins				
D-Biotin	NI	0.01	0.01	0.01
Vitamin B_{12}	NI	0.001	0.001	0.001
p-Aminobenzoic acid	NI	0.01	0.01	0.01
Nicotinic acid	NI	0.001	0.001	0.001
Calcium pantothenate	NI	0.001	0.001	0.001
Pyridoxine HCl	NI	0.002	0.002	0.002
Riboflavin	NI	0.001	0.001	0.001
Thiamine HCl	NI	0.001	0.001	0.001
Folic acid	NI	0.001	0.001	0.001
Inosine	NI	0.005	0.005	0.005
Amino acids				
L-Alanine	NI	0.10	0.10	0.10
L-Arginine	NI	0.10	0.10	0.10
L-Asparagine	NI	0.20	0.20	0.20
L-Aspartic acid	NI	0.20	0.20	0.20
L-Cysteine	NI	0.20	0.20	0.20
L-Glutamine	NI	0.20	0.20	0.20
L-Glutamic acid	NI	0.20	0.20	0.20
Glycine	NI	0.10	0.10	0.10
L-Histidine	NI	0.10	0.10	0.10
L-Isoleucine	NI	0.10	0.10	0.10

L-Leucine	NI	0.10	0.10	0.10
L-Lysine	NI	0.10	0.10	0.10
L-Methionine	NI	0.10	0.10	0.10
L-Phenylalanine	NI	0.10	0.10	0.10
L-Proline	NI	0.10	0.10	0.10
L-Serine	NI	0.10	0.10	0.10
L-Threonine	NI	0.10	0.10	0.10
L-Tryptophan	NI	0.10	0.10	0.10
L-Tyrosine	NI	0.10	0.10	0.10
L-Valine	NI	0.10	0.10	0.10

*NI: not included

Table 3 Main characteristics of sourdoughs

Bakeries	Sourdough sample	pH	TTA	Dry matter, %	LAB log CFU/ g sample
A	S1	4.07±0.01	14.9±0.1	55.1±0	7.3
	S2	3.37±0.01	28.1±0.3	33.3±1.4	9.2
B	S3	3.60±0.01	7.5±0.2	18.7±0	9.2
	S4	3.47±0.01	18.3±0.3	26.0±0	9.5
	S5	3.46±0.01	11.9±0.1	30.0±0	9.1
	S6	3.43±0.00	11.0±0.1	27.8±0.2	9.1
	S7	3.91±0.01	10.9±0.1	25.5±0.1	8.9

Results are expressed as the mean of two replicated measurements and standard deviation (±SD)

Table 4 Identification of lactic acid bacteria species by sequencing of the 16S rRNA gene

Groups[e]	Isolate	Sequencing of the 16S rRNA gene			
		Length	Identities	Similarity to GenBank sequence, %	Closest related yeast species
I	1.2	1501	1491/1493	99.9	[a]*P. pentosaceus*
II	2.1	1370	1369/1370	99.9	[b]*L. frumenti*
	2.5	1370	1369/1370	99.9	*L. frumenti*
III	2.3	1371	1369/1371	99.9	[c]*L. reuteri*
	8.1	1512	1512/1512	99.9	*L. reuteri*
IV	5.1	1444	1444/1444	100.0	[d]*L. fermentum*
	5.2	1518	1513/1515	99.9	*L. fermentum*
	6.1	1485	1484/1485	99.9	*L. fermentum*
	7.2	1508	1507/1509	99.9	*L. fermentum*

[a] *Pediococcus pentosaceus*

[b] *Lactobacillus frumenti*

[c] *Lactobacillus reuteri*

[d] *Lactobacillus fermentum*

[e] Based on results from sequencing of the 16S rRNA gene, isolates were divided into four groups

Table 5 Volumetric (mU/mL), specific extracellular (U/10^{10} CFU) and specific intracellular (mU/mg total protein) phytase activities of *Lactobacillus sakei*, *Pediococcus acidilactici*, *P. pentosaceus*, *L. frumenti*, *L. reuteri* and *L. fermentum*

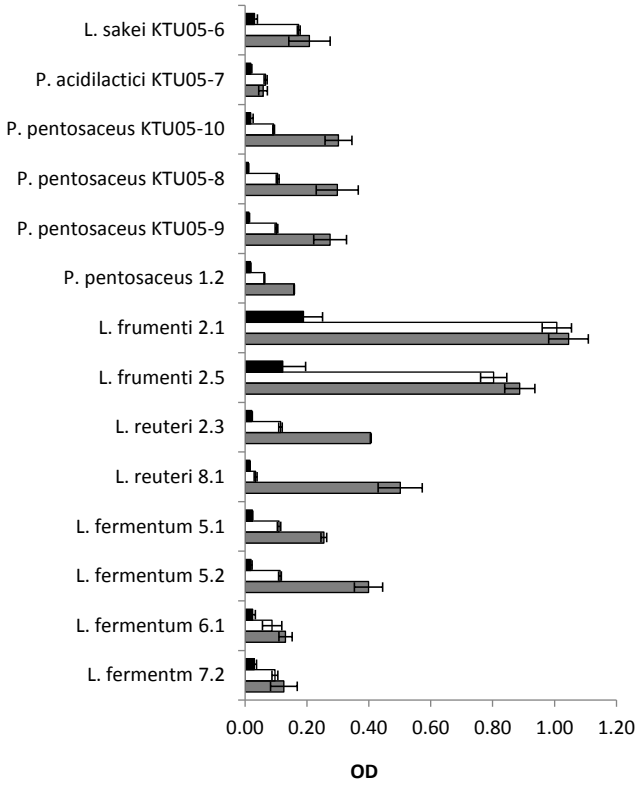
Species	Strains	Phytase activities[a]			
		Extracellular		Intracellular	
		mU/mL	U/10^{10} CFU	mU/mL	mU/mg total protein
L. sakei	KTU05-6	25±0	24±3	14±1	67±6
P. acidilactici	KTU05-7	11±1	13±3	17±0	87±2
P. pentosaceus	KTU05-10	21±4	23±4	12±2	60±1
	KTU05-8	32±1	30±3	8±3	42±8
	KTU05-9	54±6	25±4	28±3	124±15
	1.2	43±2	28±3	7±3	35±1
L. frumenti	2.1	56±8	13±2	13±2	138±35
	2.5	60±3	16±1	16±1	126±19
L. reuteri	2.3	6±1	12±4	13±2	77±14
	8.1	19±2	7±1	61±4	279±35
L. fermentum	5.1	46±7	10±3	10±3	62±18
	5.2	3±0	1±0	35±10	168±40
	6.1	11±3	3±1	52±6	240±24
	7.2	16±9	6±3	61±9	362±50

[a] Results are expressed as the mean of three replicated measurements and standard error of the mean (±SEM)

Phytase activity (U), is the amount of enzyme required to liberate 1 μmol of phosphate per min under the assay conditions.

Figure legends

Figure 1 Optical density values (OD_{600}, horizontal axis) of lactic acid bacteria cell growth at 30°C in Chemically Defined Medium (CDM): CDM-P (black bars; phosphate free medium), CDM+Phy (white bars; phytic acid dipotassium salt containing medium) and CDM+P (grey bars; phosphate containing medium) medium after 48 h cultivation. Error bars represent standard deviation from three separate growth analyses.


L. sakei KTU05-6
P. acidilactici KTU05-7
P. pentosaceus KTU05-10
P. pentosaceus KTU05-8
P. pentosaceus KTU05-9
P. pentosaceus 1.2
L. frumenti 2.1
L. frumenti 2.5
L. reuteri 2.3
L. reuteri 8.1
L. fermentum 5.1
L. fermentum 5.2
L. fermentum 6.1
L. fermentm 7.2
0.00
0.20
0.40
0.60
0.80
1.00
1.20
OD

Paper 4

Purification and characterization of phytase produced by yeast Saccharomyces cerevisiae isolated from rye sourdough

Purification and characterization of phytase produced by yeast *Saccharomyces cerevisiae* isolated from sourdough

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Running title: **Characterization of *Saccharomyces cerevisiae* phytase**

Abstract

Purification and characterization of high-active extracellular phytase produced by generally recognized as safe (GRAS) yeast species *S. cerevisiae* is necessary in order to find prominent candidate for the production of wholemeal bread with high content of bioavailable minerals. Phytase was purified to homogeneity using ultrafiltration and affinity chromatography (Con-A) techniques. The purified enzyme was most active at pH 4.0 and 35°C. The study revealed that *S. cerevisiae* phytase is quite stable at pH range from 3.5 to 5.5 and temperature range from 25°C to 40°C. The Michaelis-Menten constant K_m and the maximum reaction velocity V_{max} of the purified phytase on potassium phytate calculated from the Lineweaver-Burk plot were 0.421 mM and 527mmol/min, respectively.

1. Introduction

Nowadays, whole-grain bread is gaining popularity across the world due to increasing awareness of its nutritional benefits. Several epidemiological studies found that intake of whole-grain foods protect against certain type of cancers, cardiovascular disease, type 2 diabetes and obesity (Slavin, 2003). Grain products, such as a whole-grain bread has been recommended because of their high content of dietary fiber, B-vitamins, vitamin E and several minerals of which P, Mg, fe, Cu, Ca and Zn are the most important. Moreover, each component may partly account for its beneficial effects on human health (De Munter et al., 2007; Slavin, 2003). It was shown that 100 g of whole grains contain 20-70% of the dietary requirements of the essential minerals (Cordain, 1999). Despite that whole grains are relatively rich in minerals, their absorption in human gut are very low (Cheryan, 1980) due to antinutritional phytic acid salts (phytate), and this may lead to mineral deficiencies. In wheat the content of phytic acid have been reported to be 0.4-1.4%, while the content on rye is 0.5-1.5% (Reddy, 2002).

Phytic acid, also known as *myo*-inositol 1,2,3,4,5,6-hexakis phosphate, consist of a *myo*-inositol ring with six phosphate groups esterified to each carbon atom. Due to its chemical structure, phytic acid is a very stable, high negatively charged molecule over a wide pH range. Thus, phytic acid acts as a strong chelator of cations and binds minerals, such as iron, zinc, calcium, magnesium (Raboy, 2003). Formed phytate complexes are insoluble at physiological pH, and, therefore, minerals and phosphate are unavailable for absorption in the human intestine (Lopez et al., 2002; Brune et al., 1992). To increase the bioavailability of minerals, enzymatic degradation of phytate and its dephosphorylated isomer is needed (Sandberg et al., 1999).

Phytase [*myo*-inositol hexakis (dihydrogenphosphate) phosphohydrolase] is a common term used to describe enzymes that hydrolyse phosphomonoester bonds from phytate into *myo*-inositol and phosphoric acid via penta- to mono-phosphate, thus decreasing or eliminating its antinutritional effect (Konietzny and Greiner, 2002). The activity of phytases increase available phosphate, minerals and a non-chelator compound *myo*-inositol.

Reduction of antinutritional phytate during bread making can be achieved by enzymatic degradation in two ways. First, the endogenous phytase activity which present in whole-grain flour can be increased during bread processing. In wheat, the phytase values ranging from approx. 900 to 2900 U/kg dry matter, while the phytase activity in rye varies from 4100 to 6100 U/kg dry matter (Steiner et al., 2007; greiner and Egli, 2003; Eeckhout and Depaepe, 1994). Generally, the activity of cereal phytase is considered to be insufficient to significantly decrease the amount of phytate in whole-wheat flour during wheat bread making (Türk and Sandberg, 1992). On the other hand in commercial rye bread making with addition of sourdough, the phytate is fully degraded (Nielsen et al., 2007). This may be due to the higher phytase activity in rye and long processing time compared to wheat bread making even the rye dough pH is lower than pH-optimum for rye phytase (Nielsen et al., 2008; Greiner and Egli, 2003). Second, phytase are naturally found in microorganisms, such as yeast and lactic acid bacteria (Nuobariene et al., 2012; Hellstrom et al., 2010; Olstorpe et al., 2009; Ullah and Gibson, 1987). Hence, high-phytase active yeasts as well as lactic acid bacteria could be added as starter culture to improve mineral bioavailability in whole-grain bread fermented in short time as alternative to sourdough bread and/or yeast fermented bread. A few studies have shown yeast phytase to be mostly extracellular and their activities ranging from 14 to 566 mU/ml (Nuobariene et al.,

2012; Nuobariene et al., 2011; Olstorpe et al., 2009; Lambrechts et al, 1992). Studies on phytase activity from lactic acid bacteria identified few strains of *Lactobacillus* spp. to express intracellular activities only (Reale et al., 2004; De Angelis et al., 2003; Lopez et al., 2000).

Few reports have been published on the enzymatic properties of yeast phytases. Most of the extracellular yeast phytases have been found to be optimally active in the pH range between 3.6 and 4.6, and in the temperature range between 35 to 75°C (In et al., 2009¸Quan et al., 2002; Vohra and Satyanarayana, 2001; Segueilha et al., 1992). A phytase with a pH and temperature profile that is ideally suited for maximal activity in whole-grain wheat dough is desirable for bread making with increased amount of bioavailable minerals. There is therefore an ongoing interest in isolating and characterizing new and safe microbial strains that produce efficient phytases.

In this study extracellular phytase produced by *Saccharomyces cerevisiae* L1.12 strain, previously isolated from rye sourdough (Nuobariene et al., 2012), has been purified and characterized.

2. Materials and methods

2.1 Microorganism

Saccharomyces cerevisiae L1.12 has previously been isolated from rye sourdough and identified as a phytase-positive yeast strain (Nuobariene et al., 2012). The yeast strain was propagated onto YPG (D-(+)-glucose, 10 g/l; Bacto yeast extract, 3 g/l; Bacto peptone, 5 g/l; pH 5.5) agar plates at 30°C for 48 h. Further, for the short term storage

(max four weeks) culture was maintained at 4ºC. For the long term storage, the culture was stored at -80ºC in YPG medium containing 20% glycerol.

2.2 Phytase production

One loop of two-day-old *S. cerevisiae* L1.12 cells was picked from the plate and transferred into the 10 ml sterile YPG medium. Afterwards, inoculated cell culture tubes were incubated in a shaking water bath (170 rev/min) at 30°C overnight. Yeast cells from overnight culture were harvested by centrifugation (Sorvall RT6000D; Bush & Holm, Herlev, Denmark) at 5000 *g* for 10 min, 4°C, washed three times with 20 ml sterile ultrapure water and diluted to an initial OD_{600}=1.

For phytase production an aliquot of prepared yeast suspension was transferred into 500 ml Erlenmeyer flask, containing 150 ml Delft+Phy medium (defined minimal medium, containing phytic acid dipotassium salt as phosphate source) and grown in a shaking water bath (170 rpm) at 30°C for 72 h (Nuobariene et al., 2011). Inoculation level for the main culture growth was set to OD_{600} = 0.1.

2.3 Phytase purification

The extracellular phytase produced by *S. cerevisiae* L1.12 was purified using ultrafiltration and affinity chromatography. All purification steps were performed at 4ºC.

After cultivation, the yeast cells from the fermented broth were removed by centrifugation (5000 *g* for 10 min, 4°C); culture supernatant with extracellular protein fractions was collected and filtered (0.2 μm Minisart filters; Bie & Berntsen, Herlev, Denmark). 100 ml of prepared extracellular protein extract, as described above, was

desalted and concentrated to 40 ml using Centriprep 3K centrifugal filter device with Ultracel YM membrane (the nominal molecular weight limit 3.000).

The presence of glycan on the yeast phytase protein (Segueilha et al., 1992) facilitated further purification of the desalted extract. Concanavalin A (Cona A) sepharose, a lectin-conjugate resin that binds high glucose and mannose groups, was used for affinity chromatography. The sample was loaded at flow rate of 0.1 ml/min onto Con A sepharose column (20 mm diameter × 30 mm) equilibrated with buffer A (0.02 M Tris-HCl and 0.5 M NaCl, pH 7.2) and, afterword, washed extensively (3 column volumes) to remove unbound proteins. Bound glycoproteins were competitively eluted with 0.25 M methyl-α-D-mannopyranoside solubilized in buffer A. Aliquots of eluent (1 ml) were applied to the column at interval of 30 min. Collected fractions (1 ml each) which show phytase activities were combined as a single fraction and assays for phytase activity.

2.4 Extracellular phytase standard assay

The extracellular phytase activity was assayed according to Nuobariene et al. (2012). Briefly, 40 µl of substrate solution (3 mmol/l phytic acid dipotassium salt (K-phytate) in 0.2 mol/l sodium acetate/HCl buffer, pH 5.5) was preincubated in 1.5-ml eppendorf tube (VWR International ApS, Herlev, Denmark) at 30°C for 3 min, afterwards, 10 µl of enzyme extract was added, gently mixed by inverting tube and incubated at 30°C for 15 min. The reaction was stopped by adding 50 µl TCA (trichloroacetic acid solution; 10 g of TCA dissolved in sterile ultrapure water to a final volume of 100 ml). A separate enzyme blank was prepared from sodium acetate buffer mixed with enzyme extract and TCA. Substrate blank was prepared from substrate solution mixed with TCA.

100 µl of preassay mixture was mixed with 800 µl of freshly prepared acid molybdate reagent (1 volume of 10 mmol/l ammonium molybdate, 1 volume of 2.5 mol/l sulphuric acid and 2 volume of acetone) and incubated at room temperature for 15 min. Liberated inorganic phosphate from K-phytate was measured using a 96-well quartz microtiter plate (VWR Bie & Berntsen, Herlev, Denmark). A total volume of 225 µl test solution was transferred into each well. The plate was placed in the Microplate reader chamber (M965 AcuuReader; Metertech; Food Diagnostic, Grenaa, Denmark), shaken for 10 s, and the absorbance was read at 355 nm.

Phosphate standard curve was prepared with KH_2PO_4 (Sigma-Aldrich, P5655), dissolved in 0.2 mol/l sodium acetate/HCl buffer (pH 5.5), and measured under the same conditions as the enzyme sample.

One unit of phytase activity was defined as the amount of phytase that liberates 1µmol/ml inorganic phosphate per minute from a 3 mmol/l K-phytate solution at pH 5.5 and a temperature of 30°C. This temperature and pH value was considered optimal for bread dough leavening (Haros et al., 2001b) .

2.5 Protein quantification

Protein concentration was determined by measuring the absorbance of enzyme extract at 600 nm using the Bio-Rad Colorimetric Protein assay, Kit II (Bio-Rad Laboratories Inc). BSA (bovine serum albumin) was used as protein concentration standard.

2.6 Estimation of the isoelectric point

The p*I* of purified phytase was estimated with an isoelectric focusing system on a 5% polyacrylamide (PAA) gel containing 6.25% pharmalyte (pH 3-10). Proteins with a p*I*

ranging from 4.45 to 9.6 (Bio-Rad Laboratories) were used as markers. The gel was staining with 0.1% Coomassie Blue G-250 in aqueous solution of 25% methanol and 5% acetic acid.

2.7 Effects of pH and temperature on phytase activity and stability

The effect of pH on the phytase activity was determined by incubating the enzyme in K-phytate solution at various pH (pH 3.0 to 7.0) using the standard assay conditions. The buffers used for reaction were 0.2 M sodium acetate-HCl (pH 3.0-6.0) or 0.2M Tris-HCl (pH 6.0-7.0). The maximum activity was taken as 100% and percentage relative activity plotted against different pH values.

The effect of pH on phytase stability was studied by incubating the purified enzyme at 30°C for 15 min at various pH values ranging from 3.0 to 7.0 followed by measuring the residual activity under the standard assay conditions.

The phytase assays for determination of temperature optimum were performed as earlier outlined, except for the variation of the incubation temperature. For this purpose, activity of the phytase was assayed over a temperature range of 25°C to 60°C at 5°C intervals. The maximum activity was taken as 100% and percentage relative activity were plotted against different temperatures.

Thermostability of the phytase was tested by incubating enzyme extract without substrate addition at a temperature range of 25ºC to 65ºC (with 10ºC intervals) for 2 h and sampled every 15 min. Residual activities where than measured as indicated above, at a fixed temperature of 30°C and pH 5.5.

2.8 Determination of kinetic parameters

The kinetic parameters for the hydrolysis of K-phytate by purified phytase from *S. cerevisiae* L1.12 were determined at optimal conditions for phytase activity (pH 4.0 and 35°C). The substrate concentrations were varied from 0.5 to 4.0 mM to generate saturation curve to determinate the kinetic enzyme parameters (K_m, Michaelis-Menten constant, and V_{max}, the velocity of the enzyme reaction at maximum concentration of substrate). The enzymatic reactions were initiated by mixing 40 µl purified enzyme with 160 µl different concentrations of substrate (K-phytate, pH 4.0). Reactions were timed once enzyme was added to the preincubated substrate solution followed by incubation at 35°C. Aliquot (10 µl) was taken from reaction mixtures every 45 seconds for 15 min and added to a prepared stop solution (10 µl 10% TCA) in 96-well micro plate. Afterwards, in to the preassay mixture 160 µl of freshly prepared acid molybdate reagent (1 volume of 10 mmol/l ammonium molybdate, 1 volume of 2.5 mol/l sulphuric acid and 2 volume of acetone) was added and incubated at room temperature for 15 min. The liberated inorganic phosphate was detected by measuring the absorbance at 355 nm and quantitated by comparing the absorbance to a phosphate standard curve. The calculated concentrations of liberated phosphate were plotted against the reaction time. The slope of the resulting reaction progress curve yielded the initial velocity (V_0) of the enzyme reaction. Afterwards, double reciprocal transformation was performed to make a Lineweaver-Burk plot ($1/V_0$ vs. 1/[S]) and to calculate K_m and V_{max}.

3. Results and discussions

3.1 Cultivation of the *S. cerevisiae* isolate L1.12 for phytase production

The activity of phytase in response to phytic acid dipotassium salt and the growth of *S. cerevisiae* isolate L1.12 during 72 h of cultivation are shown in Figure 1. The yeast cells reached their stationary growth phase after 42 h of cultivation. Increase in phytase activity during the exponential growth phase was very slow. However, rapid increase in phytase activity was observed in the late exponential growth phase (36-42 h of cultivation) and was continuously increasing during early stationary growth phase. It can be seen from the Figure 1 that the maximum phytase activity (0.062 ± 0.005 U/mL) was obtained when yeast cells reach the stationary phase and remained almost constant for the next 12 h of cultivation. Afterword, slight decrease in phytase activity was observed in late stationary growth phases. This finding is in agreement with Quan et al. (2001) findings which showed that phytase production by *Candida krusei* occurred in the late stage of exponential growth phase and that phytase activity increased gradually with increasing incubation time. Moreover, in bacteria such as *Lactobacillus sanfranciscensis* (De Angelis et al., 2003), *Bacillus* spp. (Choi et al., 2001), phytase production have also been reported to be growth associated, and that phytase activity increases markedly at stationary growth phase.

Figure 1:

3.2 Phytase purification

Up to now, there have been only few reports on the yeast extracellular phytases purification and enzymatic properties. During the last decade, extracellular phytases from yeast, including the wild strain of *Schwanniomyces castellii* (Segueilha et al.,

1992), marine yeast *Kodamaea ohmeri* (Li et al., 2008), and *S. cerevisiae*, isolated from the mash of traditional Korean traditional wine (In et al., 2009) have been purified to homogeneity. However, phytases produced by yeast species, mentioned above mostly are attributed as potential for commercial applications in the feed industry. Therefore, purification and characterization of *S. cerevisiae* isolate isolated from sourdough which may be an ideal candidate for improving mineral bioavailability in whole grain bread (Nuobariene et al., 2012) is of importance.

The extracellular phytase from *S. cerevisiae* isolate L1.12 was purified using ultrafiltration and affinity chromatography. The results of purification of phytase are summarized in Table 1. The first purification step, ultrafiltration, leads to an increase in purity of only 1.11-fold, but recover nearly all the protein in the original extract, given that the yield is 99.7%. This finding is in agreement with In et al. (2009) findings which showed that after ultrafiltration the purity of extracellular enzyme extract increase in 1.68-fold with a yield of 99.9%. However, ultrafiltration of intracellular enzyme extract resulted in 10.3-fold purification with a yield of 57.6% (Quan et al., 2002).

A Con-A affinity chromatography step in the phytase purification procedure took advantage of the presence of high glucose and mannose side chains. This resulted in separation of phytase from major non-glycosylated proteins. The phytase was purified 8.09-fold, with a yield of 3.66% to a maximum specific activity of 42.85 U/mg. To the best of our knowledge, Con-A affinity chromatography has never been used for yeast phytase purification. However, Guimarães et al. (2004) using ion-exchange chromatography followed by Con-A affinity chromatography purified phytase from *Aspergillus caespitosus* resulting in 12.7-fold purification with 12.3% yield.

Table 1:

3.3 Estimation of the isoelectric point

The p*I* value determined by isoelectric focusing analysis was 6.5 (Figure 2). This was higher than the previously reported values (p*I* 4.5-5.5) for yeast phytases. Moreover, Guo et at. (2008) reported p*I* values for purified phytase from *Pichia pastoris* to be between 3.4 and 4.2, while p*I* for phytase from *C. krusei* was found to be 5.5. Generally, the phytase from various fungal and bacterial strains display acidic isoelectric point, below 6.0, while the phytases from some fungi, including *Aspergillum fumigatus* and *Escherichia coli* have p*I* about 8.6 and 6.0-7.4, respectively (Golovan et al., 2000; Pasamontes et al., 1997).

Figure 2:

3.4 Effect of pH and temperature on phytase activity

Figure 3 demonstrates the effect of pH variation on phytase activity produced by *S. cerevisiae* isolate L1.12 at 30°C (relative activities). The pH optimum at 4.0 for *S. cerevisiae* phytase is in agreement with Vohra and Satyanarayana (2001). The optimum pH for extracellular phytase produced by baker's yeast (*S. cerevisiae*) and *S. cerevisiae* strain isolated from the mash of traditional Korean yakju was found to be 3.5 (In et al., 2009; Türk et al., 1996), whereas Nayini and Markakis (1984) reported the optimum pH 4.6 for intracellular phytase produced by baker's yeast. Moreover, In

et al. (2009) reported that yeast phytase had sharp decline in activity as the pH value moved towards the neutral range more than 0.5 pH-units from optimum. In contrast, we observed less than 15% decrease in relative phytase activity when the pH value was moved towards the neutral or acidic range more than 0.5 pH-units from optimum. This can be explained by the fact that the pH of rye dough is about 4.3-4.6, except for the sourdough where pH is around 3.7 (Nielsen et al., 2007) and that phytase purified from rye sourdough yeast is well adapted for current conditions. However, in the whole wheat sourdough where pH varies between 5.28 and 5.52 (Haros et al., 2001a), the activity of yeast phytase seems to be only about 50-70% of optimum activity.

The effect of pH on the enzyme stability indicates that the *S. cerevisiae* extracellular phytase is active in the pH range 3.5-5.5, with more than 80% of the initial activity remaining (Figure 3). However, sharp decline in activity was observed when the enzyme was incubated at pH 6.0 as well as in more neutral pH.

Figure 3:

Figure 4 shows the effect of temperature variation on yeast phytase activity at pH 5.5 (relative activities). Maximum extracellular phytase activity from *S. cerevisiae* was exhibited at 35°C with 98% and 78% of residual activity at temperature of 30°C and 40°C, respectively. Further, the residual activity at 55°C was found to be only 7%. Comparison of the optimal temperature of phytases suggested that the extracellular phytase from *S. cerevisiae* isolate L1.12 had slightly lower optimal temperature than those of *S. cerevisiae* isolated from the mash of traditional Korean yakju and *C. krusei* isolated from soil (40°C) (In et al., 2009; Quan et al., 2002).

Figure 4:

During an incubation period of 2 h, no loss of activity was observed at temperature below 40 °C, while about 8% of the activity was maintained after 15 min incubation at 55°C. No phytase activity was detected when enzyme was incubated at 65°C (Figure 4). Although phytases from *Schwanniomyces castellii* and *Pichia anomala* have been reported to be thermostable at temperature above 70°C (Vohra and Satyanarayana, 2001; Segueilha et al., 1992), our findings are in agreement with thous of Quan et al. (2002). They observed that *C. krusei* phytase is easily inactivated at temperature above 50°C. Moreover, our tested phytase activity at 45°C displays an increasing trend in activity (Table 2) during two hours incubation. Even though the phytase of isolate L1.12 has a low activity at this temperature, seems that it is very stable at 45°C.

Table 2

3.5 Determination of kinetic parameters

The Michaelis-Menten constant K_m and the maximum reaction velocity V_{max} of the purified phytase on potassium phytate calculated from the Lineweaver-Burk plot were 0.421 mM and 527mmol/min, respectively (Figure 5). Calculated K_m value was significantly lower than reported for purified phytase of *S. cerevisiae* isolated from the mash of traditional Korean yakju (0.66 mM). In contrast, the K_m values of the

other yeast phytases that have been studied were considerably lower and varies from 0.038 mM for purified phytase of *Schwanniomyces castellii* () (Segueilha et al., 1992) to 0.03 mM for purified phytase of *Candida krusei* (Quan et al., 2002). Sano et al. (1999) found the K_m value for partly purified phytase of *Arxula adeninivorans* to be 0.230.

Figure 5

3. Conclusions

Extracellular phytase produced by yeast *S. cerevisiae*, isolated from rye sourdough was purified and characterized. Our results suggest that studied yeast isolate may be ideal candidate for improving mineral bioavailability in whole grain bread, as *S. cerevisiae* are generally recognized as safe (GRAS) and as baker's yeasts are strains of *S. cerevisiae*.

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Table 1 Purification of the extracellular phytase from *S. cerevisiae* isolate L1.12

Step	Total activity, U	Total protein, mg	Specific activity, U/mg	Purification (fold)	Yield, %
Culture supernatant	24.60	4.62	5.32	1	100
Ultrafiltration	24.53	4.12	5.96	1.12	99.7
Con-A Sepharose	0.90	0.021	42.85	8.05	3.7

Table 2 Temperature stability (% residual activity compared to the activity at standard assay conditions was taken as 100%) of *Saccharomyces cerevisiae* isolate L1.12 phytase

Temp., °C	Time in minutes							
	15	30	45	60	75	90	105	120
25	96	100	115	136	133	129	115	98
30	100	98	96	124	131	112	101	105
35	99	97	100	98	115	100	99	103
40	82	87	96	112	110	98	103	99
45	34	31	37	61	66	57	58	58
55	10	9	9	8	12	7	7	6
65	ND*	ND	ND	ND	ND	ND	ND	ND

*ND; not detected

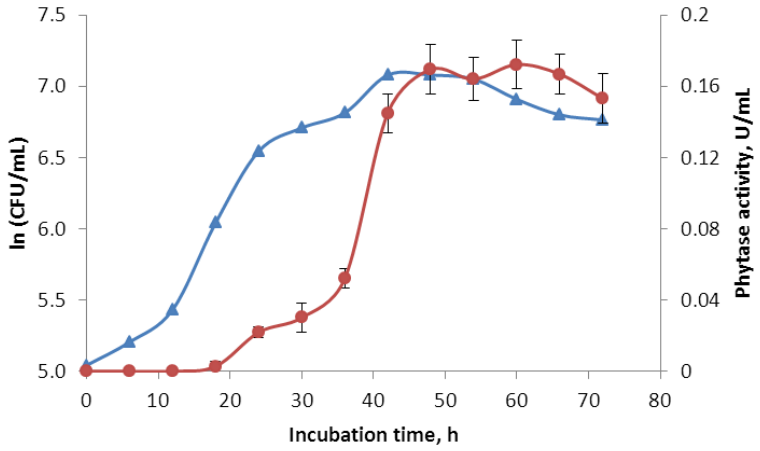

Figure 1 Phytase activity and yeast growth at different time points; (circles) phytase activity, U/mL; (triangles) yeast growth, ln(CFU/mL).

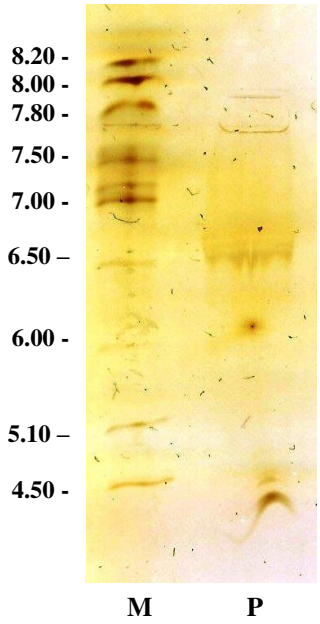

Figure 2 Isoelectric focusing of *Saccharomyces cerevisiae* phytase. Lane P, Coomassie Blue stained isoelectric focusing gel of the purified phytase; lane M, isoelectric focusing marker

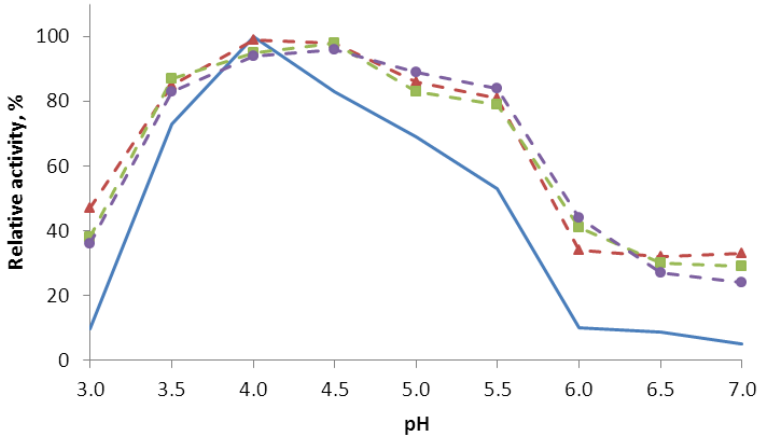

Figure 3 Effect of pH on the activity (solid line) and stability (dashed line) of extracellular phytase from *Saccharomyces cerevisiae* isolate L1.12. The maximum activity was taken as 100% and percentage relative activity plotted against different pH values. To determinate pH stability, purified enzyme was incubated in each buffer at 30°C for 1h (triangles), 2h (squares) and 4h (circles). The residual activity was measured under the standard assay conditions.

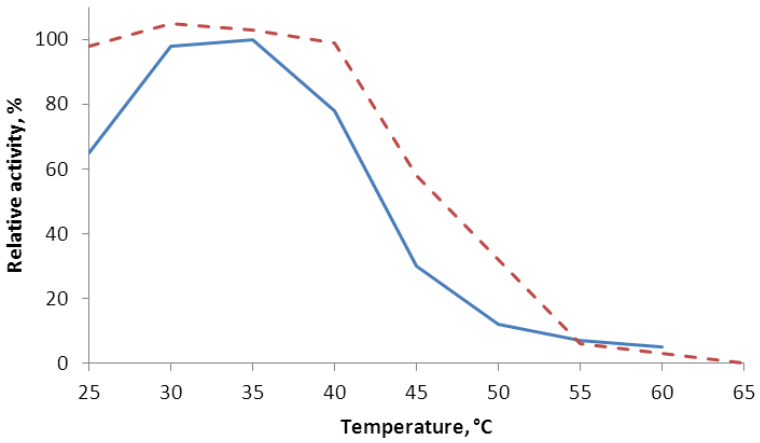

Figure 4 Effect of temperature on the activity (solid line) and stability (dashed line; after enzyme incubation for 2 h) of extracellular phytase from *Saccharomyces cerevisiae* isolate L1.12. The maximum activity was taken as 100% and percentage relative activity plotted against different pH values.

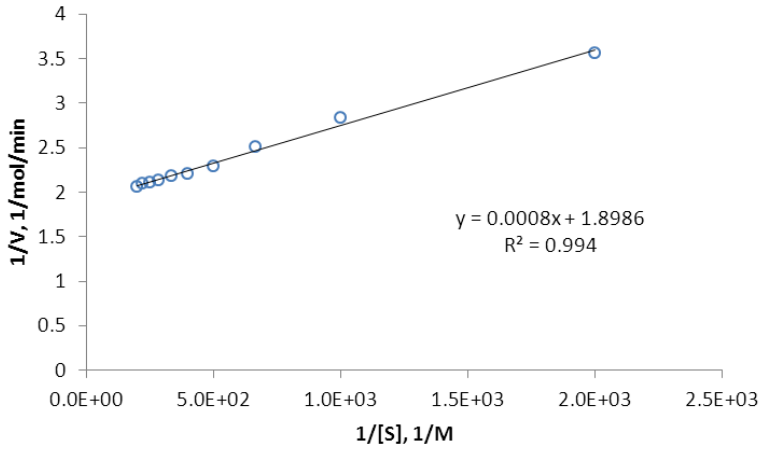

Figure 5 The double-reciprocal plot for the hydrolysis of potassium phytate by the extracellular phytase from the *S. cerevisiae* isolates L1.12. Reaction conditions; pH 4.0 and 35°C.

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LINA NUOBARIENE

Phytases in Yeast and Lactic Acid Bacteria Isolated from Grain-based Food

Nowadays, the consumption of whole-grain breads is gaining popularity across the world due to increasing awareness of its nutritional benefits. Whole grains contain wide range of nutrients, such as dietary fibre, vitamins and minerals. Despite that whole grains contain 20-70% of the daily requirements of the minerals, their absorption in human gut are very low due to antinutritional phytic acid salt (phytate), and this may lead to mineral deficiencies. To increase the bioavailability of minerals, enzymatic degradation of phytate and its dephosphorylated isomers is needed.

The aims of the thesis were therefore: (i) to isolate and identify phytase active yeasts and lactic acid bacteria strains to be used in bread making to increase the content of bioavailable minerals in bread; (ii) to purify and characterize the phytase isolated from the most prominent yeast strain for phytase production.

The highest specific extracellular phytase activities were observed in *Saccharomyces cerevisiae* L1.12 (10.6 U/10^{10} CFU), *S. cerevisiae* L6.06 (8.2 U/10^{10} CFU), *Lactobacillus frumenti* 2.1 (13 U/10^{10} CFU), and *L. frumenti* 2.5 (16 U/10^{10} CFU). Furthermore, *Saccharomyces pastorianus, Candida humilis,* and *L. frumenti* have for the first time been recognized as phytase positive strains.

Phytase purification resulted in 8.1-fold purification with a yield of 3.6% to a maximum specific activity of 42.9% U/mg. The optimum pH and temperature were found to be 4.0 and 35°C, respectively.

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