THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

# On yeasts from traditional fermented foods

## Characterization, phytate degradation, strain improvement and applications

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ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

Food and Nutrition Science
Department of Biology and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2016

Microbial Ecology
Department of Agricultural Sciences
BOLOGNA UNIVERSITY
Bologna, Italy 2016

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ISBN: 978-91-7597-473-6


Doktorsavhandlingar vid Chalmers tekniska högskola
Löpnummer: 4154
ISSN: 0346-718X

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Cover: The study of yeasts in fermented milk and flour based food items (by Linnea Qvirist).

Printed by Chalmers Reproservice
Gothenburg, Sweden 2016

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# ABSTRACT

Plant materials naturally contain minerals of iron, zinc and calcium [1]. However, plants also contain a compound called phytic acid, which can chelate the minerals and form insoluble complexes. Minerals from plant foods are unavailable for intestinal uptake when they are bound in phytate complexes [2-4]. Cereal-based diets low in meat, can thereby lead to micronutrient deficiencies of e.g. iron and zinc.

Yeasts were isolated from food fermentations, and studied with the aim to find starter strains able to degrade phytate in cereal-based matrices. The overall phytate degrading ability of different strains was studied, followed by analysis of release of the phytase (phytate degrading) enzyme to the surrounding media by the two strains *Pichia kudriavzevii* TY13wt and TY1322, and of factors affecting the phytase release. Yeasts isolated from the traditionally fermented goat milk from the Yaghnob valley, Tajikistan, were genetically identified by ITS1-4 sequencing and restriction fragment length polymorphism. Phenotypic characterization was done by studying e.g. growth at various pH and temperatures, on different carbon sources, in presence of ox bile, ethanol or lactic acid, under osmotic and oxidative stress. Selected strains were used as starters for fermentation of some plant-based substrates.

A prominent phytate degradation was observed from the strain *P. kudriavzevii* TY13wt, previously isolated from Tanzanian Togwa. Through mutagenesis by ultraviolet light exposure of strain TY13wt, mutant strain TY1322 was acquired, having improved phytate degrading ability. Strain TY1322 showed about eight times higher phytate degradation compared to wild type strain TY13wt under certain conditions. The phytase synthesis from strain TY1322 was not repressed by high phosphate levels (26mM), as opposed to the wild type strain. Both TY13wt and TY1322 were able to release phytase to the surrounding media, which was induced by yeast extract medium. Strains TY13wt and TY1322 could grow at pH 2, at 46°C, in presence of 2% ox bile or 6% ethanol, and under osmotic stress. The phytase produced showed two pH optima (3.5 and 5.5) and one temperature optimum (55°C). Yeast isolation from fermented goat milk of the Yaghnob valley resulted in 52 isolates belonging to *Kluyveromyces marxianus* (29)*, Pichia fermentans* (12), *Saccharomyces cerevisiae* (10) and *Kazachstania unispora* (1). Characterization of the strains revealed i) two genetically different groups among the *K. marxianus* strains, ii) Yaghnob *S. cerevisiae* strains forming a separate cluster in a phylogenetic tree constructed of 350 previous *S. cerevisiae* isolates of various origins, iii) phenotypically intriguing traits of several strains, e.g. *S. cerevisiae* strains able to grow at elevated temperatures, 37°C (all ten strains), 40°C (9), 42°C (2), and 46°C (1). The phenotypic intra-species variations found among some of the Yaghnob strains may potentially indicate isolation of some new species. Finally, application of selected yeasts and lactic acid bacteria for soy milk and soy flour fermentations showed strong phytate degradation in fermentations containing strain *P. kudriavzevii* TY1322, and the phytate degrading effect of TY1322 was improved in co-cultures with Yaghnob strains *K. marxianus* AL2 or BL8.

Traditional food fermentations constitute a valuable source of microbial strains. This work demonstrates the usefulness of phytase-active strains for phytate degradation in plant-based substrates, and new strain isolation revealed several strains able to grow under stress conditions that may occur during fermentation, or inside the gastrointestinal tract. This work can contribute to future strain selection for food and feed processes.

**Keywords:** *Pichia kudriavzevii*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, Food Fermentation, Phytase, Phytate, Phenotypic Characterization, Goat Milk, Yaghnob Valley

# LIST OF PUBLICATONS

The following publications constitute the basis for this thesis.

**I.** Hellström, A., **Qvirist, L.**., Svanberg U., Veide Vilg J., Andlid T. *Secretion of non-cell-bound phytase by the yeast Pichia kudriavzevii TY13.* Journal of Applied Microbiology, 2015. **118**(5): p. 1126-36. Doi: 10.1111/jam.12767

**II.** **Qvirist L.**, Carlsson N-G., Andlid T. *Assessing phytase activity – methods, definitions and pitfalls*. Journal of Biological Methods, 2015. **2**(1). Doi: 1 0.14440/jbm.2015.58

**III.** **Qvirist L.**, Vorontsov E., Veide Vilg J., Andlid T. *Strain improvement of Pichia kudriavzevii TY13 for raised phytase production and reduced phosphate repression*. Microbial Biotechnology, 2016. Online. doi:10.1111/1751-7915.12427

**IV.** **Qvirist L.**, De Filippo C., Strati F., Stefanini I., Sordo M., Andlid T., Felis G., Mattarelli P., Cavalieri D. *Isolation, identification and characterization of yeasts from fermented goat milk of the Yaghnob Valley in Tajikistan*. Frontiers in Microbiology, 2016. **7**(1690). Doi: 10.3389/fmicb.2016.01690

**V.** **Qvirist L.**, Patrignani F., Modesto M., Andlid T., Lanciotti R., Mattarelli P. *Yeasts and LAB from fermented Yaghnob goat milk and Tanzanian Togwa; Applicability as fermentation starters for soy milk, soy, buckwheat and rye/wheat flour for decreased phytate content.* **MANUSCRIPT**

# CONTRIBUTION REPORT

The author of this thesis contributed to the following Papers accordingly:

**Paper I:** Linnea Qvirist (**LQ**) was shared first author and equal contributor to this Paper, together with A. Hellström. LQ was responsible for planning, performing and analyzing approximately half of the experiments, and was responsible for writing the manuscript.

**Paper II:** LQ planned and performed the experiments, analyzed the data, and wrote the manuscript.

**Paper III:** LQ planned and performed the experiments and analyzed the data, except for the proteomics analysis, and was responsible for writing the manuscript.

**Paper IV:** LQ planned and performed the experiments and analyzed the data, except for the microsatellite analysis, and was responsible for writing the manuscript.

**Paper V:** LQ planned and performed the experiments and analyzed the data, and was responsible for writing the manuscript.

# ABBREVIATIONS

| | |
|---|---|
| WHO | World Health Organization |
| LAB | Lactic Acid Bacteria |
| UHF | Unknown Health Factor |
| FDA | Food and Drug Administration |
| $InsP_6$ | Phytic acid (phytate when in its salt form) |
| InsP's | Inositol Phosphates |
| Pi | Inorganic Phosphate |
| HPLC | High Performance Liquid Chromatography |
| YNB | Yeast Nitrogen Base |
| YPD | Yeast extract, Peptone, Dextrose |
| CSM | Complete Supplement Mixture |
| ATP | Adenosine Tri Phosphate |
| ADP | Adenosine Di Phosphate |
| AMP | Adenosine Mono Phosphate |
| ITP | Inosine Tri Phosphate |
| F6P | Fructose 6-phosphate |
| GMO | Genetically Modified Organism |
| EMS | Ethyl Methane Sulfonate |
| UV | Ultra Violet |
| G, C, A, T | Nucleotides Guanine, Cytosine, Adenine, Thymine |
| BCIP | 5-Bromo-4-Chloro-3-Inodyl Phosphate |
| PCR | Polymerase Chain Reaction |
| DNA | Deoxyribonucleic Acid |
| ITS | Internal Transcribed Spacer |
| BLAST | Basic Local Alignment Search Tool |
| NCBI | National Centre for Biotechnology Information |
| RFLP | Restriction Fragment Length Polymorphism |
| SSR | Simple Sequence Repeats |

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# 1. INTRODUCTION

With the continuously increasing human population on Earth, comes an increased need to provide safe and nutritious foods for everyone. Already today, a large part of Earth's human population live under starvation and/or conditions of malnutrition [5, 6]. Mineral deficiencies may result from a low food intake or from eating a one-sided diet that does not provide all essential nutrients. In many parts of the world, plant and cereal-based diets are common. Plant-based foods naturally contain several minerals of e.g. iron, zinc, calcium and magnesium [1, 7, 8], but due to the presence of various antinutrients, these minerals may be unavailable for intestinal uptake once consumed. One well-studied antinutrient is phytic acid, which acts by chelating metal ions, forming insoluble complexes and aggregates that monogastric animals, including humans, are unable to degrade in the gastrointestinal tract. This makes the naturally present minerals in plant and cereal-based foods unavailable for intestinal absorption.

For many people in developing countries, nutritional deficiencies can become life threatening due to the negative effects on the immune defense, and something as simple as a diarrheal condition can become lethal. The most common mineral deficiencies are of iron and zinc, but deficiencies of magnesium and calcium are also rather prevalent in some parts of the world [9]. Those deficiencies can cause a range of different health impairments; lowered immune defense, increased risk of infections, delayed physical and mental development, halted growth in children, increased risk of heart failures, depression, loss of appetite and low fertility [6]. In the Western world, the most common mineral deficiency is iron deficiency [5], especially among people with vegan or vegetarian diets and in menstruating individuals. Iron deficiency in the Western world is rarely lethal but can cause severe health problems because of the increased risk of infections and increased stress on the heart; in the worst case, this can lead to heart failure [10].

One way to battle mineral deficiencies is by fortification and supplementation. However, mineral supplementation has been associated with some potential risks. Supplementation may increase the risk of metal overdosing, which can cause harmful effects in the body [11]. Overdosing of iron, for example, may increase the risk of intestinal cancers [12]. Further, fortification and supplementation requires a well-functioning economy and infrastructure, and is for that reason not applicable in all parts of the world. Alternative solutions are hence needed.

One alternative to fortification and supplementation is to increase the availability of the minerals that are already present in plant-based foods, which can be accomplished by microbiological degradation of the antinutrient phytate through fermentation. Food fermentations has been used for a very long time, and there is evidence of wine fermentations dating back to about 3000 years BC [13]. Traditional fermented foods are for example kefir and yoghurts (from dairy substrates), sourdoughs and other plant/cereal-based fermentations, and alcoholic drinks such as wine and beer. Fermentation is applied to improve the properties of the raw material, for example to achieve improved organoleptic and structural properties, and to prolong shelf life. During fermentation, the metabolism and actions of the microorganisms that are present can contribute to the nutritional properties of the food. The composition of species in traditional fermentations is often unknown. Different species and strains may have different metabolic patterns, i.e. they are able to produce or degrade different compounds. Some examples are the production of some vitamins, degradation of antinutrients, production of compounds preventing the growth of spoilage organisms etc. Furthermore, the relationship between

the human gut microbiota and our health has recently achieved increased attention, and consumption of “good” microbes has become more and more popular.

In many traditional food fermentations, the microbiota in the fermentation is maintained over generations by back-slopping, i.e. using a previous batch sample as inoculum for a new batch. Long before the discovery of microorganisms and the knowledge about their presence and role in fermentations, positive effects from fermenting the food was observed and encouraged people to continue with the process. Today, the research community possesses many tools and has obtained a great deal of knowledge about food fermentation processes. This can now enable us to identify many microorganisms from fermentations, and to investigate their effects on the fermented food and/or beneficial effect of the fermented food for the people who consume it.

Traditional food fermentations have largely disappeared in the Western world, perhaps as a result of our lifestyles where we consume larger amounts of industrially produced foods and fast foods, and less homemade and traditional foods. By exploring the microbial composition of traditional food fermentations, we can identify interesting strains and enable re-introduction of those strains into our food fermentation processes, and perhaps regain some of our lost food tradition.

The aim of this thesis is to study various yeasts, originating from one cereal-based and one dairy based traditional food fermentation, and to characterize the strain’s i) ability to degrade phytate, ii) growth under various conditions, and iii) fermentation capacity in model foods together with selected lactic acid bacteria, with the goal to identify promising microorganisms for future industrial and household-scale food fermentations for a decreased level of phytic acid.

# 2. OBJECTIVES

The overall aim of this thesis was to study yeasts from traditional food fermentations, in order to identify potential starter culture(s) with the capacity to degrade phytate in plant-based substrates. The yeast strains were characterized by their ability to degrade the antinutrient phytate, and their growth under various conditions (different carbon sources, pH, temperatures, presence of ox bile, ethanol or lactic acid, and under oxidative and osmotic stress). Finally, fermentations with selected strains in a few plant-based substrates (soy milk and different flours) were investigated.

The specific objectives were:

- To investigate the ability of yeasts to degrade phytate (Papers I, III and IV)
- To study yeasts' ability to release non-cell-bound phytase enzyme to the surrounding medium and mechanisms affecting this (Papers I and III)
- To develop/improve the phytate degrading yeast strain *Pichia kudriavzevii* TY13wt by:
    - Increasing the overall phytate degrading ability (Paper III)
    - Improving the resistance to phosphate repression (Paper III)
- To evaluate quantification of phytase activity versus phosphatase activity (Paper II)
- To identify and characterize yeasts from a traditional food fermentation:
    - To study the phenotypic variations between strains (Paper IV)
    - To find strains potentially useful for future food fermentations (Paper V)
- To make fermentations with selected yeasts and bacteria:
    - In soy milk to evaluate strain survival, phytate degradation and volatile profiles (Paper V)
    - In bread dough to evaluate strains' leavening ability, their effect on the bread product appearance, and the ability to degrade phytate during leavening (Paper V)
    - In fermentations of soy bean flour or buckwheat flour, and in a mix of wheat and rye flour, to study the phytate degradation (Paper V)

# 3. BACKGROUND

Many different yeast species, and strains, have been studied in various ways to find a set of strains for use in application tests. To minimize the risk of working with potentially harmful microorganisms, only microorganisms isolated from traditionally fermented foods (sections 3.3.2-3.3.4) that have been frequently consumed over the years were used. Microorganisms present in a food item that has been consumed over a long period of time may be considered safe (section 3.2.2). Further, microorganisms isolated from traditional food fermentations have potentially developed the ability to efficiently degrade and ferment that specific food raw material (section 3.3.1), hence isolation from relevant matrices can be beneficial. Genetic identification (section 3.2.3) of all strains used in this work was done to prevent using pathogenic microorganisms. All strains were assessed for various phenotypic characteristics in order to gain an estimation or idea about the strains' potential ability to ferment various raw materials. The microbial degradation of phytate (section 3.5) from plant materials, and the release of phytases (phytic acid degrading enzymes, section 3.6) were especially studied. Yeast viability was tested in conditions mimicking those that might occur in the gastrointestinal tract, e.g. various pH, elevated temperatures and presence of bile.

## 3.1 Nutritional deficiencies

Nutritional deficiencies are widespread in most parts of the world, mainly as an effect of poor nutritional intake or poor gastrointestinal uptake. The World Health Organization (WHO) points at iron deficiency as the most prevailing mineral deficiency, affecting people all over the world irrespective of socio-economic class [5]. The human population is expected to continue to increase over the coming years, and one of the major challenges in this aspect will be to provide safe and nutritional foods for everyone, which is already a tremendous challenge. Some of the most common mineral deficiencies in the world are of iron and zinc, especially for humans eating one-sided cereal-based diets low in red meat. However, both those minerals are found, at various levels, in plant-based materials. The problem is the coincident presence of antinutrients, which inhibit the metal ion absorption from plant materials. Microbial degradation of the metal absorption inhibitor phytic acid, through fermentation of plant-based substrates, may be one way to improve the mineral uptake from plat based diets.

### 3.1.1 Iron deficiency

Iron deficiency is the only significant nutritional deficiency in the industrialized world [5, 6]. About 50% of pregnant women and 40% of preschool children in developing countries are estimated to be anemic, in total corresponding to about 30% of the global world population [5, 6]. Iron deficiency can be caused by malnutrition, but it can also be an effect of malabsorption, especially in combination with infectious diseases such as tuberculosis, HIV or malaria. The effects of iron deficiency vary from exhaustion and reduced cognitive ability, to more severe health conditions, sometimes leading to premature death. Less noticeable cases of iron deficiency can cause poor performance in school or at work as a result of exhaustion, apathy and tiredness [10], which generally do not prompt people to seek medical attention. In men, a relation between poor iron status and an increased rate of suicide attempt has also been seen, which was not the case for the females in that study [14]. In developing countries, different approaches are being taken to improve the iron status in populations. The approaches include i) increasing the iron intake, ii) increasing the intestinal uptake, and iii) controlling the infectious diseases known to be related to iron deficiency anemia [5, 6].

Vegetarian and vegan diets are gaining interest in the industrialized world for health, ethical and environmental reasons. Reducing meat intake and increasing the intake of legumes and whole grains may result in a reduced intake and absorption of both iron and zinc [15], although reducing meat and

increasing the vegetable intake in the diet is in line with the general nutritional recommendations in many industrialized countries. It is not always the case that meat-free diets contain overall less iron, but, due to the presence of antinutrients such as phytic acid and polyphenols in legumes and whole grains, the gastrointestinal uptake of the minerals that are present in plant-based food is often reduced [16, 17]. Especially in children and pre-menopausal women, the nutritional recommendations for iron intake from a vegetarian diet may be difficult to achieve; even when the recommended intake is reached, the uptake may be inadequate [15]. Meat contains up to 40% of its iron as the easily absorbed form *heme* iron, while vegetarian iron sources contain most of their iron as the less easily absorbed *non-heme* iron. The absorption of heme iron is about 15-40% while the corresponding percentage for non-heme iron is about 1-15%, greatly depending on the iron status of the individual as well as on the composition of the meals [18-22]. In an iron deficient person, non-heme iron absorption is upregulated to a higher extent as compared to the upregulation of heme iron uptake, i.e. resulting in a similar or higher uptake rate of non-heme iron compared to heme iron, depending on the meal [19].

A review by Hunt [15] gives some important inhibitors of on non-heme iron, including phytic acid (found in whole grains, legumes, nuts, lentils), tannins and chlorogenic acids (found in tea, red wine, coffee and some varieties of cereals, vegetable, spices), calcium, soy protein and eggs. Polyphenols are also known to inhibit non-heme iron uptake [23]. The uptake enhancers of non-heme iron, as described in the review, are mainly ascorbic acid and the so called meat factor (found in meat, poultry and fish) [15].

### 3.1.2 Mineral fortification

Mineral and vitamin fortification of cereal-based products such as flours and breakfast cereals, are done in several countries to reduce nutritional deficiencies. For economic and infrastructural reasons, this is not always applicable. Although fortification can reduce the prevalence of nutritional deficiencies, several studies have also pointed to the risks of overusing the supplementation of minerals, and have indicated that overdose of some metals can lead to an induced risk of some cancers and of other health issues [11, 12]. Sweden, for example, has decided not to apply any general fortification program, in contrast to e.g. the USA, although a number of fortified products can also be found on the Swedish market. A possible alternative to fortification is to improve the availability of nutrients – especially minerals – that are already present in the food raw-material. For cereal-based foods, this can be achieved through microbial fermentation, as fermentation of cereal-based foods has been shown to result in degradation of the metal absorption inhibitor phytate [24-32] and thereby to improve the mineral bioavailability of e.g. zinc [4] and iron [2, 33, 34]. Phytate degradation by microorganisms in general, and by yeasts in particular, has shown promising phytate degradation results in previous studies using various media and matrices [24, 25, 30, 35-38].

## 3.2 Yeasts

Yeasts are unicellular eukaryotic microorganisms, belonging to the fungus group. Yeasts have been isolated from varying habitats such as plants [39], soil [40], water [41], glaciers [42], geothermal regions [43] and intestinal tracts of animals[44, 45] and insects [46]. It is estimated that about 1% of all yeast species has been identified today, corresponding to about 1500 species [47, 48]. Yeast cells are typically in the µm size range, although, under certain circumstances, some yeasts are able to form multicellular-like forms, so called pseudohyphae. Pseudohyphae are chains of connected single cells that are a result of incomplete budding, making the total yeast size many-fold longer. Some fungi are able to grow both as yeasts and as filamentous fungi; the latter stage is characterized by the formation of so called true hyphae, having a cytoplasmic connection between the cells [49]. Mold growth is a

different growth form, and these fungi are referred to as dimorphic (two forms). Yeasts are named according to genus - species - strain, for example for *Saccharomyces cerevisiae* CBS7764, where the genus is *Saccharomyces*, the species is *Saccharomyces cerevisiae* and the strain name is CBS7764.

### 3.2.1 Yeast diversity

Yeasts are today well known for their use in bread, beer and wine production, and evidence has been found from about 3000 years BC of yeast fermented wine [13]. The most studied yeast species is *Saccharomyces cerevisiae*, which is also known as baker's yeast. However, many different yeast species are used today, both in research and in industrial applications, for production of fuels, chemicals, enzymes, vitamins and much more. Different species and strains can possess different phenotypic and morphological characteristics, conferring potential application in different fields [50-52]. Some examples of various phenotypic characteristics are the ability to grow at various temperatures and at various pH, the production of certain desired compounds (e.g. carbon dioxide to raise a bread or ethanol for wine production), and their ability to degrade undesired compounds such as antinutrients.

The number of unexplored yeasts is estimated to be huge [53], and isolation from unexplored habitats may thus result in the identification of species and strains with interesting novel biotechnological potentials. Different phenotypic traits are of interest depending on the intended use of a newly isolated yeast strain. For example, yeasts intended for use in human consumption need a more thorough safety assessment than yeasts used in bioethanol production. There are also variations in the safety assessments needed for yeasts consumed in a viable state (e.g. in homemade yoghurts) compared to yeasts that are consumed in a not-viable state (e.g. in a baked bread). Various legislations and guidelines exist for various industries, which must be consulted depending on the intended end-use.

### 3.2.2 GRAS yeast and QPS qualification

Generally Recognized As Safe (GRAS) is a term founded by the U.S Food and Drug Administration (FDA, www.fda.gov, retrieved in 2016) for additives and compounds intended for use in food. The meaning of the term includes:

*"Any substance that is intentionally added to food is a food additive, that is subject to premarket review and approval by FDA, unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, or unless the use of the substance is otherwise excluded from the definition of a food additive."*

And:

*"The use of a food substance may be GRAS either through scientific procedures or, for a substance used in food before 1958, through experience based on common use in food."*

In 2002, the International Dairy Federation (IDF) and European Food and Feed Cultures Association (EFFCA) summarized a list of microorganisms traditionally used in food and feed production, mainly based on species already used in various starter cultures. More recently, in 2011, the list was updated with additional species and with the latest taxonomy, summarized in "Inventory of Microbial Food Cultures" presented by Bourdichon and coworkers [54]. The list includes, among others, all the yeast species included in the work reported in this thesis: *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Kazachstania unispora*, *Pichia fermentans* and *Pichia kudriavzevii*.

The European Food Safety Authority (EFSA) has assessed several biological agents and their safety for use as food or feed additives. Their assessment aims at providing biological agents with or without a Qualified Presumption of Safety (QPS) status. In the EFSA list from 2013 (appendix B Table 1) both *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* are listed as having QPS status [55].

### 3.2.3 Microorganism identification

In microbiological research, knowing the identity of the microorganism under investigation will allow the researcher to obtain information from previous studies of the same microorganism. Some different approaches available for yeast identification. Before the invention of genetically based methods and the polymerase chain reaction (PCR) technique, it was common to use phenotypic characterization for identification. The drawbacks of using solely phenotypic characteristics for identification is that it is time consuming, requires a great deal of material and, most of all, that the results are not specific enough to distinguish between closely related (or phenotypically similar, but not related) isolates [56]. In recent years, the use of genetic analyses for identification has become the more commonly used method.

Ribosomes are molecular machineries involved in the protein synthesis of all cells. They are made up of proteins and so called ribosomal RNA (rRNA), the latter being encoded by so called rDNA found in special regions of the genome. One of those regions, the ribosomal Internal Transcribed Spacer (ITS) region, is commonly used for yeast identification [57]. After amplifying a highly variable sequence in this region, the so called ITS1-4 region [58], using PCR and designated primers, the nucleotide sequence can be determined by sequencing. By comparing the sequence with sequences present in online data bases, such as the Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI) [59], similarity and coverage values can be obtained in order to identify the studied isolates on a species level. Therefore, taxonomic approach using both phenotypic and genotypic characterization is essential for distinguishing strain variations within species. Without investigating the phenotypic traits of isolates, it is not possible to assess the strains' different performance under various conditions.

## 3.3 Traditional food fermentations

Fermentation of foods has been done for centuries, worldwide, as a way to prepare and/or preserve foods. Without knowing the actual mechanism of the fermentation process, people probably realized that the fermentation processes improved the taste and texture, and prolonged the shelf-life of the foods, and therefore continued the practice of fermentation. Today the mechanisms behind those effects are to a large extent known, for example the microbial production of gas, which improves the texture and volume of breads, or the production of alcohol, acids and bacteriocins, which reduces the risk of unwanted growth of spoilage or pathogenic microorganisms, thereby improving the storage durability [60]. Microbes can also contribute positively by producing various nutrients and flavoring components [60].

Looking at various news and social media in the Western world (newspapers such as The Huffington Post and The Telegraph, TV programs such as BBC News, numerous blogs and magazines), instructions and guidelines for how to prepare in-house made food fermentations such as beer, yoghurt, sourdough breads, pickled vegetables etc. have seemed to increase over the last years. This indicates an increased interest in re-introducing food fermentations, and the accompanying beneficial effects of fermentations, into our daily lives.

### 3.3.1 Microorganisms in traditional food fermentation processes

Spontaneous food fermentations have been done for a very long time [13] and are in many areas still used as the main method for food preparation. In spontaneous fermentations, there is no external control of physical conditions, such as pH or temperature, and the inoculum is of an unknown composition. An inoculation method called back-slopping is often used in this type of fermentation. Back-slopping is based on using an aliquot from a previous batch of fermented food to initiate the fermentation of a new batch [61]. By this technique, an unknown amount and composition of microorganisms are continuously transferred from one batch to the next. The microorganism present in this type of repeated fermentation has been allowed to adapt well to the substrate of the fermentation over several generations, allowing a natural selection of microorganisms well adapted to the specific food matrix.

Various yeast species are often isolated from natural food fermentations, and some examples of previously reported species are; *Candida lusitania*, *Cryptococcus laurentii*, *Saccharomyces cerevisiae* and *Candida kefyr* from traditionally fermented Saudi Sameel milk [62]; *Issatchenkia orientalis, Kazachstania unisporus, Rhodotorula mucilaginosa, Candida pararugosa, Torulaspora delbrueckii, Geotrichum sp., Kazachstania unisporus, Geotrichum fragrans, Debaryomyces hansenii, Yarrowia lipolytica, Trichosporon gracile* and *Pichia membranifaciens* from fermented milks of the Tibetan plateau in China [63]; *Saccharomyces cerevisiae*, *Candida humilis*, *Saccharomyces servazzii*, *Saccharomyces bayanus*, *Kazachstania sp*., and *Torulaspora delbrueckii* from liquid sourdoughs [64]; and *Saccharomyces cerevisiae*, *Trichosporum mucoides*, *Candida famata*, and *Candida albicans* from traditionally fermented milk of Kenya [65]. This thesis work explored yeasts isolated from traditional spontaneous fermentations of a cereal-based food called Togwa, from Tanzania, and one dairy based food from the Yaghnob Valley in Tajikistan.

### 3.3.2 Tanzanian Togwa

Togwa is a cereal-based, porridge-like food produced in Tanzania, where it is used as a weaning food for younger children an at prolonged fermentation stages, as a refreshment drink for adults [66]. The preparation is usually made with either sorghum or maize. The method of inoculation is back-slopping, as described previously, by using a small aliquot of a previous Togwa batch as inoculum for the new batch. It may be hypothesized that implementing this technique in Togwa fermentations has allowed a natural selection of microbes with a high ability to degrade the plant-based raw materials used in Togwa, including the metal absorption inhibitor phytate. The fermentation also naturally includes several lactic acid bacteria and yeasts [35, 67, 68], and the product is consumed while still containing the viable microbes.

### 3.3.3 Yaghnobi-fermented goat milk

In Tajikistan, there is a valley called the Yaghnob valley, where an ethnical minority human population lives. Due to the mountainous areas surrounding this valley, the influence from the “modern world” has been limited, and the Yaghnob population has maintained an ancient language and a unique lifestyle and culture [69]. The diet of this population is very low in fruits and vegetables, and the traditionally homemade fermented goat milk constitutes one of its staple foods. This type of traditional food fermentation is done by a kind of back-slopping. The fermented milk contains both lactic acid bacteria and yeasts and is consumed containing viable microorganisms. The microorganisms in fermented milks, prepared in geographically isolated areas such as the Yaghnob valley, will potentially also represent the microbiota of that geographical area (see section 3.3.5). The yeast community of the Yaghnob-fermented milk has not been studied previously and, due to the geographically isolated

nature of this area, the yeast community in this fermented food is of great interest from a food fermentation point of view and from a microbiological evolutionary and ecological point of view.

In 2012, a group of scientists visited the Yaghnob valley to document the language and culture of the population living there as part of the Italian missions to the Yaghnob valley (for a recent review on the two missions, the reader is referred to a Paper by Adam Benkato in 2015 [69]). Although the mission did not include microbiological work, the visitors could bring back samples of the fermented goat milk to Italy, from which yeast and bacteria were isolated.

### 3.3.4 Sourdough

The positive properties of sourdough bread compared to conventional bread are reported as e.g. improved taste (slightly more acidic than regular bread), texture (improved tenancy and toughness) and improved nutritional bioavailability and gastrointestinal mineral uptake [70]. It is thus not surprising that household sourdough preparation has recently also attained increased interest in Sweden. Both yeast and lactic acid bacteria (LAB) are present during sourdough fermentation and contribute to the organoleptic and nutritional properties of the product, e.g. through phytate degradation [24, 29, 71-74]. Sourdough preparation is done by using a mother-dough, often originating from a liquid mixture of flour and water, where the endogenous LAB and yeasts from the flour become active and are allowed to enumerate. The mother-dough is maintained over time by regular addition of water and flour. When a new sourdough is prepared, an aliquot of the mother-dough is used as inoculum to the new dough, transferring an unknown composition and amount of microorganisms for the fermentation. In some cases the whole motherdough is used as inoculum, and a small aliquot of the new sourdough is then taken out after mixing (before the addition of spices or similar) and maintained as a new mother-dough for future sourdough preparations [75]. The microorganisms of the mother-dough are allowed to naturally evolve and adapt to the substrate, and a natural selection of microorganisms takes place. At the end of the sourdough preparation, the bread is baked and the microbes that have been present are no longer viable.

### 3.3.5 Flux of microorganisms in nature

There is a continuous flux of microbes in natural environments, between nature, humans and animals etc. One example of how such microbe movement is enabled is given in a study by Stefanini and coworkers [46], showing how the yeast *Saccharomyces cerevisiae* is spread by wasps. Human activities have also been shown to have an impact on the evolution of the yeast community, and a study of the evolution of *Saccharomyces cerevisiae* gives the presence of humans as one of the enablers behind the cross-breeding and development of mosaic strains from different ancestors in the *S. cerevisiae* species [76].

It is not uncommon that the same species of yeast is isolated both from fermented foods and from human fecal samples. The question is whether i) the yeast is found in the feces as a result of consumption of fermented foods that have enumerated this specific species in the intestinal microbiota or ii) the species is found in the food because of poor hygienic procedures that have allowed it to transfer from feces into the food. It may be hypothesized that there is a continuous flux of microbes between humans and the surrounding environment, meaning that an indigenous food fermentation may to a large extent reflect the microbiota of the specific geographical region where it is prepared, and also to some extent the intestinal microbiota of the inhabitants of that region.

### 3.3.6 Microorganism viability in fermented foods

Depending on the food processing post fermentation, the viability of the microorganisms that are present can be controlled. For example, in bread production, organism(s) die during the baking process, while, in the case of household milk fermentations, the microorganisms are commonly viable when the product is consumed. In bread, the remaining beneficial effects of the fermentation are those that have taken place before the baking process, e.g. degradation of phytic acid, production of flavoring compounds, production of acids etc. In fermented foods in which microbes are still viable, both the properties of the food product itself and of those of the viable cells that are consumed are of interest. The microbial cells themselves may possess beneficial traits and probiotic properties, which after consumption and entering the intestinal tract of the host may have a beneficial effect on the consumer's health [77].

### 3.3.7 Limitations in microorganism isolation from environmental samples

Several studies have undertaken isolation of microorganisms to address the microbial composition of spontaneous food fermentations [62, 63, 65, 78, 79]. However, isolation of microorganisms from environmental samples is largely dependent on the conditions that we are able to create in our lab environments. Microorganisms can have various demands for growth, for example chemical and physical parameters such as pH, temperatures, medium components etc. We are therefore usually not able to isolate, and enumerate, all microorganisms present in an environmental sample. The combination of conditions in ongoing natural fermentations may be very complex and difficult to mimic in laboratory settings. Isolation of microbes from such a complex environment will therefore be limited by the isolation/cultivation conditions that can be created in the lab.

Further, some features of an ongoing natural fermentation may not be known, i.e. cannot be mimicked in the lab environment. This means that some microbes may only be viable in the specific conditions of an ongoing fermentation, and may not be possible to isolate outside of that fermentation. Hence, isolation of all microbes present in natural fermentations might not be possible [80].

However, there is another method, independent of microbe viability, where the composition of the microbes present in an environmental sample, such as a food fermentation, can be observed. This method is called metagenomics, and it allows determination of the species that are present by means of DNA analysis, i.e. with no need for the microbes to be viable outside of the sample [81]. This method can reveal the presence of microorganisms that will not be detected by cultivation dependent methods [81].

## 3.4 Probiotic potential

Probiotic products are defined as *"products that contain an adequate dose of live microbes that have been documented in target-host studies to confer a health benefit for the host"* [82], while probiotic microorganisms are defined as "*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*" [83]. However, there is no legal definition for the term probiotic, and for this reason the term is improperly used for several commercial products that have not undergone clinical evaluation to prove the microorganism-host effects [82]. Two other terms have been introduced by the US Food and Drug Administration (FDA) that are similar, but not equal, to what is considered probiotics. The first is live biotherapeutic product (LBP), which is described as microbes intended for human use in order to prevent, treat or cure diseases or conditions. To obtain the LBP label, the product has to undergo substantial investigation to meet the criteria [84, 85]. The second term is direct-fed microbial, which is more similar to probiotics, but specifically intended for animal

use [86]. According to a document from the FDA [87], the early direct-fed microbials were marketed to a large extent with labels making health claims or beneficial effects that were not properly scientifically proven. Therefore, many of the direct-fed microbials have been subjected to reevaluation over recent years in order to provide accurate labeling.

In a recent review by Moslehi-Jenabian *et al.* [77] various probiotic effects of yeasts were presented. Some documented probiotic properties included anti-inflammatory effects [88-90], positive effects on diarrheal diseases [91-93], positive effects on immune response [94-96] and antagonistic effects towards bacterial pathogens [97-99]. The study also presents beneficial effects of yeast fermentations, by e.g. folate bio-fortification and biodegradation of phytate [77]. Although several yeasts show positive health effects on human health, *Saccharomyces cerevisiae* var. *boulardii* is today the best studied probiotic-proven yeast strain [100].

For microorganisms to be able to reach the intestinal tract in a viable form, they need to overcome several harsh conditions occurring during the passage from the mouth to the gastrointestinal (GI) tract. To assess the microorganisms' ability to survive through the GI tract, test can be made to assess their viability and growth at various conditions, specifically at low pH, at temperatures of 37°C - 40°C (37°C for humans, and about 39°C - 40°C for pigs) and in the presence of bile [101]. Although non-viable microorganisms may also confer health benefits for the host, e.g. acting as a delivery capsule for enzymes, those are not considered probiotic. The viability during gastrointestinal conditions is therefore an in important prerequisite for probiotic microbes.

It is also worth mentioning something that is sometimes referred to as an "unknown health factor" (UHF) [102]. This unknown health factor, as the name indicates, is used when a beneficial effect from a food is seen, but which cannot be ascribed to any specific *known* microbe or food component [102].

## 3.5 Phytate

Phytate is the salt form of phytic acid (myo-inositol hexa*kis*phosphoric acid, or $InsP_6$). Phytate is found in more or less all cells, but seeds of plants are especially rich in phytate, where it is the main storage form of phosphorus, constituting up to 80% of the seed phosphate [103]. In plants, phytate accumulates mainly in protein storage vacuoles, in grains especially in the aleurone layer (surrounding the endosperm) [104] and in maize in the embryo [103]. Phytate can be degraded to varying extents during the germination process. Germination is the process in which a seed sprouts into a plant and, during this process, endogenous phytase and phosphatase enzymes are activated in order to degrade phytate end release nutrients needed for plant growth [103].

Phytic acid is composed of a myo-inositol ring with six phosphates (Pi) positioned on the carbons (Figure 1).

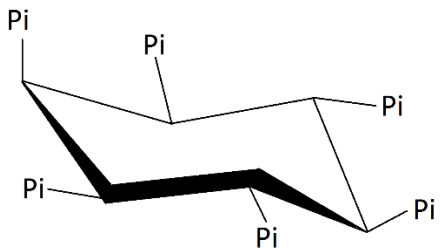


**Figure 1.** *Structure of phytic acid, consisting of a myo-inositol ring with sex phosphate (Pi) groups, one attached to each of the carbons.*

Phytic acid has a negative charge over a wide range of pH, which means that it attracts and chelates positively charged minerals [105, 106], proteins [107] and starches [108]. Different metal ions have a different affinity to phytic acid, according to Tamim and Angel in the following order: Copper (Cu) > Zinc (Zn) > Cobalt (Co) > Manganese (Mn) > Iron (Fe) > Calcium (Ca) [109]. Phytate complexes with copper and zinc are more stable than those with magnesium, calcium and iron (in decreasing order) and there seems to be a relation between stability and atomic mass [106, 110, 111].

When phytate is enzymatically degraded, it occurs in a stepwise manner by the release of one phosphate group at a time, with subsequent formation of $InsP_5$, $InsP_4$, $InsP_3$, $InsP_2$ and finally $InsP_1$. The inhibitory effect on iron and zinc uptake is different from different InsP's, depending on the number of phosphate groups on the inositol ring; both $InsP_6$ and $InsP_5$ inhibit the absorption of iron and zinc, while $InsP_4$ and $InsP_3$ do not [2, 4, 33], but a combination of all four forms has a collective negative impact on iron absorption [2, 33]. In general, the lower the number of phosphate groups on the inositol ring, the lower the amount of minerals and proteins can be bound, and the complexes formed are less stable with a decreasing number of phosphate groups [106].

### 3.5.1 Phytic acid as an inhibitor of metal ion absorption from the diet

The phytate content in cereal-based foods is generally higher in bran and fiber rich products [112], which are also the type of foods that has the highest content of minerals. Phytate, and the chelated metal ions, form complexes and aggregates that cannot be absorbed in the gastrointestinal tract of monogastric animals, due to the lack of phytate-degrading enzymes [113]. This means that the nutrients that are chelated or bound to phytic acid, as well as the phytic acid phosphates, will pass through the gastrointestinal tract without being absorbed [1-3, 33, 34, 103, 114, 115] (Figure 2).

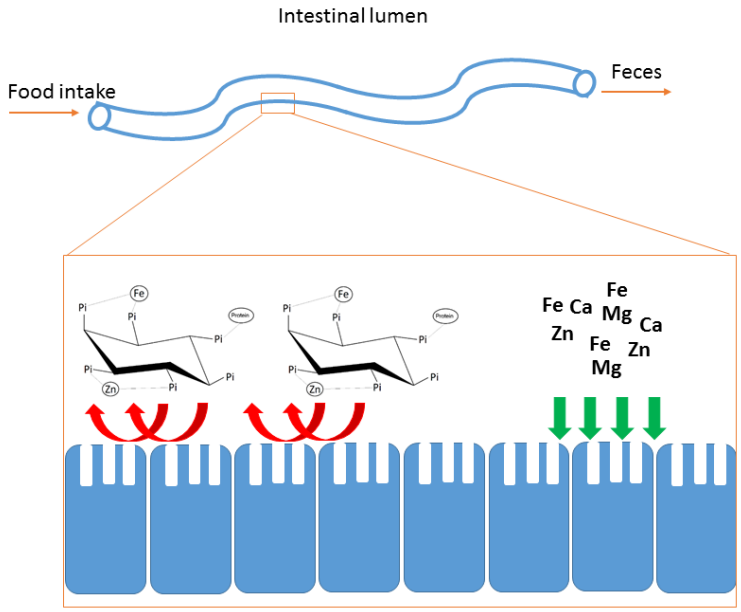


**Figure 2.** *A much simplified explanation of mineral availability from the diet in the intestinal lumen. The availability of metal ions to intestinal cells is dependent on several factors, including the form of the metal (as parts of soluble complexes, as simple salts, or as solid metals in nanoparticles) and the ability to form aggregates with phytate under the intestinal conditions, which inhibits the metal ion absorption.*

In order to achieve degradation of phytate complexes and enable mineral uptake in monogastric animals, the degradation of phytate needs to be mediated by dietary sources of enzymes. Phytate degradation in human foods is attempted through various processing conditions, e.g. by hydrothermal

treatment [116], by activation of endogenous phytases by soaking or malting [117, 118], or by fermentation with phytase-active microorganisms [32, 119]. Booth soaking [120] and thermal treatment [116] may result in mineral losses to the surrounding liquid. Microbial fermentation has shown promising phytate degradation for example in sourdough, and varying phytate degradation can be achieved depending on the microorganisms present as well as on the raw material and the conditions used [32, 119, 121, 122].

In the pig feed industry, phytate is a great obstacle for phosphate uptake in pigs. Since pigs cannot absorb phytate-phosphates from phytate aggregates, an attempt to solve the phosphate malnutrition in pigs has been phosphate supplementation to the feed. This is not a sustainable solution, however, since excess phosphate, both from the supplementation and from the phosphates of the unabsorbed phytate, leaks out to the environment and causes severe eutrophication problems in the surrounding environment [123, 124]. Commercial phytase solutions are being applied to animal feeds, as a more sustainable alternative to phosphate supplementation, by enabling degradation of the feed-phytate and release of the phosphates from the feed-phytate [125-127]. For pig feed applications, a phytase with a low pH optimum (around 3.5 [128]) can be desirable, since this is the approximate pH in the pig stomach. The addition of phytase enzyme solutions is not applied in human food production.

### 3.5.2 The potentially beneficial effects of phytic acid

In contrast to the large number of studies on the anti-nutrient effects of phytate, some studies present potentially beneficial effects of phytic acid. A review by Kumar and coworkers [105] discusses different beneficial effects of phytic acid such as protection against some types of cancer, reduced risk of coronary heart disease, reduced occurrence of fatty liver, reduced risk of diabetes, protection against dental caries and prevention of renal lithiasis. The beneficial effect of phytic acid is ascribed to the "mineral cleansing" effect that phytic acid can potentially have by chelating metal ions, such as iron and calcium, inside our bodies and removing them from our system, thereby reducing for example the risk of iron-induced oxidations [105].

In another review, by Vucenik and Shamsuddin [129], anti-cancer effects were observed based on exogenous administration of phytic acid ($InsP_6$), orally or intravenously. Phytic acid, when administered exogenously, was suggested to be absorbed in the intestinal tract of mice and rats, where the uptake of phytic acid was higher when administered through the drinking water, compared to when mixed into food [129]. However, no phytic acid uptake was seen in humans, concluded from an almost complete recovery of $InsP_6$ and absence of $InsP_6$ degradation products in the small intestine of human ileostomy subjects [113, 130].

The conclusion that consumption of phytic acid through bran- and fiber-rich foods is a suitable way to acquire the positive anti-cancer effects presented by Vucenik and Shamsuddin [129] may be questioned. First, the type of phytate salt(s) (i.e. which metal ions are chelated to phytic acid) in cereals and plants is commonly not known, and may vary greatly. Second, the studies in the review by Vucenik and Shamsuddin [129] uses phytate-magnesium or phytate-calcium salts, which may not be the form of the salt found in plant-based foods. Third, the administration of phytate in the above studies was done intravenously or orally, and not through a complex matrix such as bread or cereal.

## 3.6 Phytase – the phytate degrading enzyme

Phytases (also called myo-inositol hexakisphosphate phosphohydrolase) are enzymes belonging to the phosphatase enzyme family. Phytases can be found in most cells, where they are used in the intracellular metabolism of phytate. Some organisms also produce phytases for degradation of

extracellular phytate in the surrounding environment, mainly reported among different yeasts, bacteria and fungi. Various levels of phytase activity/content have also been found from plants and cereal grains; in the latter the activity was reported to be higher in rye as compared to other cereals [131].

Phytases are large proteins, usually ranging between 40 and 500 kDa in weight. Yeast phytases have also been reported to have varying sizes. For example, the phytase produced by *Schwanniomyces castellii* was 490 kDa [132], the phytase by *Candida krusei* was 330 kDa [133], and the phytase from *Pichia anomala* was 64 kDa [134].

### 3.6.1 Phytase temperature and pH preferences

The optimal temperature and pH at which phytase enzymes show the highest activity varies depending on phytase type and origin. From an application point of view, depending on the intended use of the phytase, various temperature optima and temperature tolerances may be desired. For example, in some processes in the feed industry, a phytase resisting short treatment at elevated temperatures (80-85°C) may be desired, as this temperature is applied in some step of the processes. For yeast phytases, different temperature optima have been reported, from 40 - 80°C [132-136].

With regard to the pH optima, the range for yeast phytases is generally not very broad. A rather low pH optimum, between 4 and 5.5, is often reported for phytase from yeasts [132-136]. Some higher, but still acidic, optima have however been reported e.g. for the yeast *Yarrowia lipolytica* with an optimal pH at 6.2 [137].

### 3.6.2 Phytase classification

Phytases are classified into three main groups called 3-phytases, 6-phytases (sometimes also referred to as 4-phytases) and 5-phytases. The numbers correspond to the phosphoester bond on the phytate, i.e. at which phosphate group that the enzyme initiates the de-phosphorylation. For example, a 3-phytase initiates the de-phosphorylation at the third phosphate group of the $InsP_6$ (D-3 position, figure 3), yielding one free inorganic phosphate (Pi) and one $InsP_5$ where the third phosphate group is removed. To more specifically show the structure of an $InsP_5$ where the 3$^{rd}$ phosphate is removed, one can write D-Ins(1,2,4,5,6)$P_5$, where the numbers in parentheses correspond to the positions where there are still phosphate groups present. Figure 3 shows the numbering of the phosphate groups on phytate. The numbering is generally done based on the D-conformation, but some older studies use the less common L-conformation, which is shown in parentheses in Figure 3.

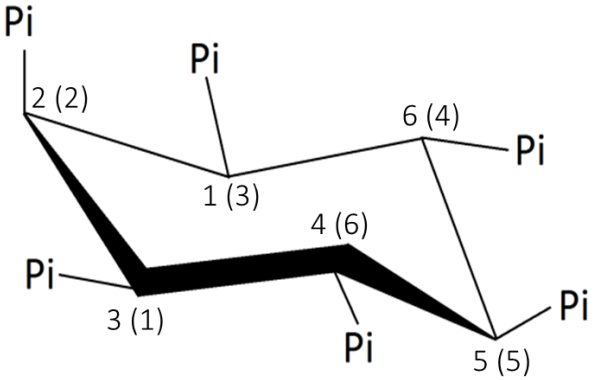


**Figure 3.** *The numbering of phosphate groups (Pi) on the inositol phosphate according to the D-formation, with the L-formation in parentheses.*

The Enzyme Commission (EC) numbers for the three phytase groups, retrieved from NC-IUBMB and UniProt online databases, are EC 3.1.3.8 (3-phytases), EC 3.1.3.26 (6-phytases) and EC 3.1.3.72 (5-phytases). The enzymes in the 3-phytase group are in general of microbiological origin, with pH optima around pH 4-5, while the 6-phytases are generally from plant origins and have pH optima around pH 5 for cereals [138, 139] and from pH 4.8 up to pH 7.5 in legumes [140]. However, exceptions have been found in both groups; some microbial phytases have been found to be 6-phytases and some plant phytases have been found to be 3-phytases. The third and less common phytase group, 5-phytases, has been isolated from lily pollen [141] and from the bacteria *Selenomonas ruminantium* subsp. *lactilytica* [142].

### 3.6.3 Phytase synthesis in presence of inorganic phosphate

It is known that the inorganic phosphate (Pi) concentrations in the surrounding environment affect the yeast phytase expression and the overall phytate degrading capacity [143-145]. As yeast cells sense high surrounding phosphate levels, the need for synthesis of phytases is lower, since the need to release phytate phosphate is lower. At low surrounding phosphate levels, the synthesis can instead be up-regulated, and the yeast cell produces many enzymes in order to release phytate-phosphates in a phosphate-deficit environment. Very simplified, higher phosphate levels impair yeast phytate degradation, while environments with low phosphate levels have the effect of inducing it. However, as phosphate is also a nutrient for the yeast, strictly phosphate-free environments (i.e. also free from $InsP_6$ and other phosphorylated molecules) may strongly impair the yeast growth, and thereby also the phytase expression.

### 3.6.4 Extracellular non-cell-bound phytase

Several yeasts and other microorganisms are able to produce phytase enzymes (for recent reviews see [146, 147]). Yeast phytases can be located inside the yeast cell (inside the yeast plasma membrane), in the periplasmic space (between the plasma membrane and the yeast cell wall), and sometimes also as non-cell-bound extracellular enzymes after being released outside of the yeast cell wall into the surrounding media, see Figure 4. The term *extracellular phytase* is used differently in different studies, sometimes to describe enzymes located outside of the yeast plasma membrane but inside of the yeast cell wall, and sometimes to describe the non-cell-bound enzymes in the surrounding media. In this work, we use the term *non-cell-bound extracellular* for the phytases that fulfill the following two criteria: i) are recovered in the culture supernatant after cells have been removed by filtration (0.22 μm filters), and ii) are not the result of old, lysing cells, but are recovered from young and viable cultures.

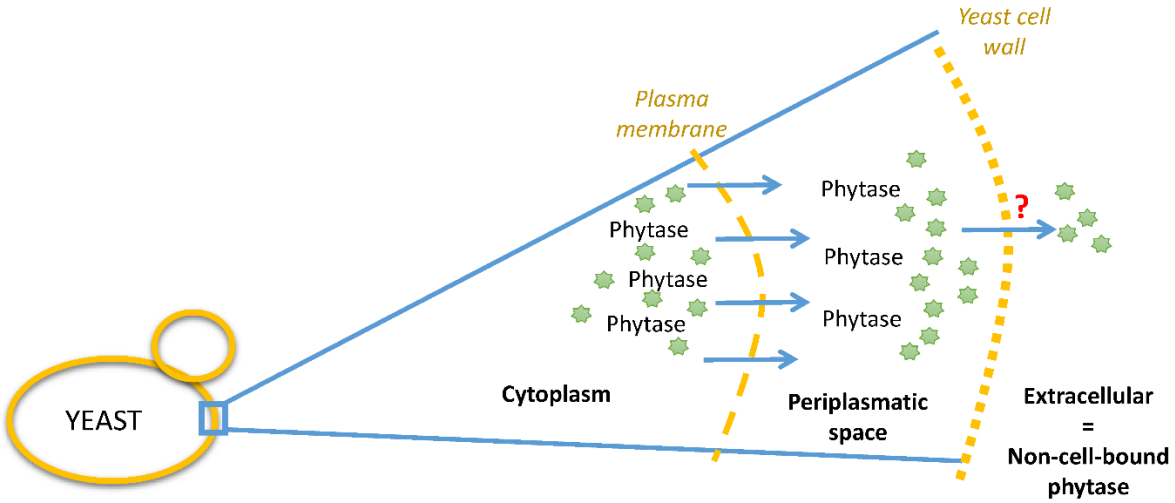


**Figure 4.** *Phytases can be found in the cytoplasm inside of the plasma membrane, in the periplasmatic space between the plasma membrane and the yeast cell wall (still referred to as intracellular in this work), and outside of the yeast cell, as non-cell-bound phytases in the surrounding media.*

There are several benefits with yeasts able to release an enzyme of interest, in this case phytases, through the yeast cell wall and into the surrounding media. In food fermentations, for example, a non-cell-bound extracellular phytase present freely in the culture media will increase chances of interaction with the substrate (phytate), compared to only cell-associated phytases, thereby increasing the rate of phytate degradation. In the feed industry, phytase solutions are often used as additives to the feed in order to aid phytate degradation and phosphate release. A yeast that releases its enzyme(s) to the surrounding media may reduce extraction and down-stream processing costs.

### 3.6.5 Phytase activity definition

Depending on the method used for determination, either analysis of substrate (phytic acid) or product (phosphate), the two following definitions are used:

For ***phytase activity***; based on analysis of phytic acid concentration, one enzyme activity unit (U) is defined as the amount of enzyme needed to release 1 µmol of phosphate from phytic acid per minute (equal to the degradation of 1 µmol phytic acid, $InsP_6$, and formation of 1 µmol $InsP_5$).

For ***total inositol phosphatase activity***; i.e. based on phosphate release, one enzyme activity unit (U) is defined as the amount of enzyme needed to release 1 µmol of phosphate from the whole assay solution per minute (i.e. phosphate release from all present inositol phosphates, by all present phosphatases).

### 3.6.6 Phytase activity assessment

During the work with Paper I [148], it became evident that, in the field of phytase research, there exists a wide variety of analysis methods used for determining phytase activity. Many studies use different versions of phosphate (Pi) analysis to assess the phytase activity, as phosphate is one of the two products in the phytate degradation reaction $InsP_6 \rightarrow InsP_5 + Pi$. In the current work, the direct analysis of the substrate phytate is used to determine phytase activity. Due to the different methodologies used in various studies, it was questioned whether it was possible to accurately compare data between different studies, i.e. between different methodologies. It was hypothesized that the analysis of Pi (product) would yield large overestimations of the phytase activity, compared to the activity

determined by phytate (substrate) analysis, since the former also includes phosphate released from the subsequently produced InsP's $InsP_5$, $InsP_4$, $InsP_3$ and $InsP_2$, ($InsP_1$ is commonly not degraded microbiologically), and not only from phytate ($InsP_6$).

Generally, during enzymatic assays, an aliquot of the enzyme solution (more or less purified) is mixed with a substrate solution containing phytate ($InsP_6$), and incubated during certain amount of time, before samples are analyzed for substrate or product concentration. During the enzymatic assay, phytase enzymes are not only dephosphorylating phytate, but also the subsequently formed $InsP_5$ – $InsP_2$ originating from the phytate degradation (Figure 5). The phosphate released from de-phosphorylation of $InsP_5$ – $InsP_2$ will inevitably also be included in the phosphate analysis after enzymatic assay.

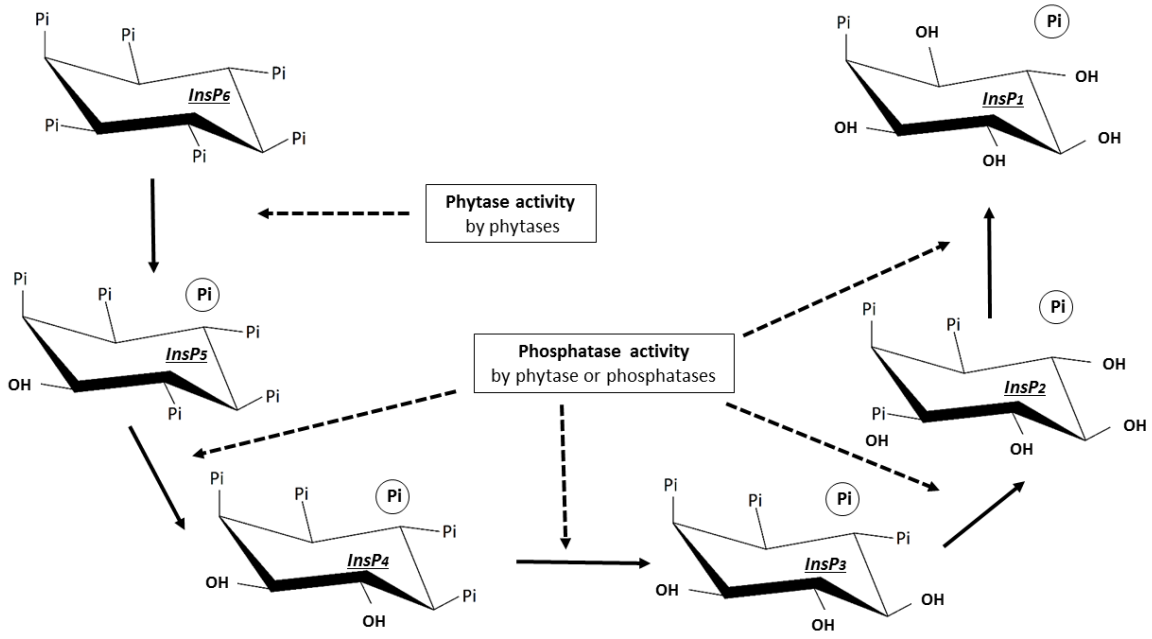


**Figure 5.** *The phytate degradation route, initiated by phytase in the upper left part of the figure by dephosphorylation of $InsP_6$, resulting in one $InsP_5$ and one phosphate (Pi). From $InsP_5$, the stepwise degradation is continued by phytases and/or other phosphatases present, producing $InsP_5$ – $InsP_1$ and releasing more phosphates. The final InsP from dephosphorylation by microbial phytases is usually $InsP_1$, as the axial phosphate group is rarely removed.*

Even in a completely pure phytase solution containing no other phosphatases, where de-phosphorylation of $InsP_5$ – $InsP_2$ is entirely a result of phytase enzymes, this de-phosphorylation is by definition not phytase activity, since phytase activity is defined as *degradation of phytate ($InsP_6$)*. However, the assayed enzyme solutions are in reality often not completely pure, and may contain other phosphatase enzymes that are able to de-phosphorylate several phosphorylated compounds that are present. Hence, the non-phytase phosphatases may carry out de-phosphorylation of $InsP_5$ – $InsP_2$, releasing phosphate that is not a result of the presence of a phytase, but a result of non-phytase phosphatases.

There are also some considerations concerning the phytate substrate used in the enzymatic assays. There are many different commercial suppliers of phytate, using different origins of phytate, various degrees of purity and several versions of the phytate salt (with different counter ions). In a work by

Carlsson and colleagues [149] the contamination of $InsP_5$ in a commercial phytate powder was determined to be 12%. Using a substrate that already contains other InsP's than $InsP_6$, and probably also free Pi, may result in detection of false-positive phytase activity. Therefore, the presence of non-phytate InsP's at the beginning of the assay means that even a phytase free assay may show release of phosphate as a result of non-phytase phosphatases releasing phosphate from the non-phytate InsP's.

## 3.7 Mutagenesis and evolutionary engineering of yeasts

In nature, when an external stress such as lowered pH or elevated temperature is applied to a microbial community, a natural selection of the best adapted individuals in the population will take place, allowing the strongest individuals to survive. This can, after several cultivation cycles where the fittest candidates are allowed to increase, result in microbes able to grow under the applied stress. In nature, such adaptations to various environments can result in, for example, thermotolerant yeast strains found in hot springs [150, 151] or cold-tolerant, i.e. psychrophilic, yeasts found in alps or glaciers [152, 153].

In laboratory environments, there are different approaches to modifying and adapting microorganisms to grow at certain conditions; from directed genetic modifications to evolutionary engineering and random mutagenesis [154]. One example of a genetic modification of a yeast is the construction of a *Saccharomyces cerevisiae* able to metabolize pentoses, to increase the ethanol yield from cellulosic materials that contain both hexose and pentose sugars [155]. Since directed genetic manipulations yields so called genetically modified organisms (GMO), which are generally not accepted for food production and not well received by the consumer, random mutagenesis and evolutionary engineering may be more applicable methods in food research.

In evolutionary engineering, one takes advantage of the natural adaptability of microorganisms. By applying an external pressure, such as elevated temperature, one can select the individual strains that are best adapted, i.e. the ones showing the highest growth rate, as cells with low stress resistance will have slower growth or even die from the applied pressure [156-158]. However, when there is no suitable external pressure to enable evolutionary engineering, a random method such as random mutagenesis can be used [154].

In random mutagenesis, an external factor known to induce genetic mutations, called mutagen, is applied in order to increase the natural mutation rate. The mutagen can be a chemical, in yeast research commonly ethyl methanesulfonate, EMS [159-161], or a physical mutagen such as ultra violet (UV) light [161-163]. EMS is known to produce mainly GC (Guanine Cytosine) to AT (Adenine Thymine) transversions, while UV light has a broader spectrum of mutations, e.g. transversions, transitions and frameshift mutations by single nucleotide deletions (Figure 6) [160, 164]. Chemical mutagens such as EMS are hazardous and usually require labor intense de-activation and waste handling after exposure [165], UV mutagenesis was therefore used in the current work.

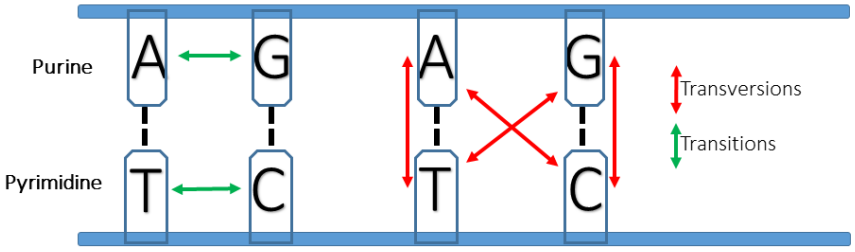


**Figure 6.** *The four nucleotides Adenine and Guanine (being purines), and Cytosine and Thymine (together with Uracil being the pyrimidines), and arrows indicating how transversion (red arrows) and transitions (green arrows) can occur. The transitions can only occur within the purines (A and G) or pyrimidines (C and T), not between those.*

The survival rate of the population must be determined at the beginning of a random mutagenesis experiments. This is done to find a good level of mutagen and exposure time, to balance the cell survival versus the level of mutations; a too high exposure will cause high cell death and induce the number of double mutations, while a too low exposure will result in a low rate of mutation and it may be difficult to identify positive mutants.

After irradiation with UV light, the treated cells should be kept dark in order to prevent induction of the inherent light-dependent repair mechanism, called photo reactivation [164, 166]. After exposure to the mutagen, mutants that have acquired the desired trait are selected. A good selection method is crucial for optimal selection of positive mutants. If, for instance, a toxic-resistant strain is to be selected, a medium containing this toxic compound can be used, since colonies of resistant cells survive and can easily be identified and selected. However, when the aim of the mutagenesis is to improve an already existing trait, as in the case of the present work (improve phytase synthesis/activity), the selection can be more complicated. It can be wise to select a larger number of potentially successful mutants, and to pay extra attention to screening and evaluating the putative mutant strains in order to identify the best mutants.

As the name indicates, random mutagenesis causes random mutations. Therefore, once a set of mutants has been selected, they must be investigated for phenotypic traits in order to ensure that no unwanted mutations have occurred. One common side effect is impaired growth capacity, which can be of varying importance depending on the intended use of the microorganism. In this work, however, the aim is a high overall phytate degrading capacity; thus a maintained growth rate has been an important factor in the selection of mutant strains.

# 4. MATERIALS AND METHODS

This chapter describes in brief the methodology and experimental set-ups used in the Papers included in the thesis. For detailed descriptions, the reader is referred to the corresponding Papers.

## 4.1 Paper I – Release of extracellular, non-cell-bound, phytase

The high phytate degrading strain *Pichia kudriavzevii* TY13, originally isolated from Tanzanian Togwa by Hellström and co-workers [68], was investigated in Paper I.

### 4.1.1 Phosphatase and phytase expression analysis

First, the release of phosphatases into an agar based medium was assessed by preparing agar plates supplemented with 5-Bromo-4-chloro-3-indolyl phosphate (BCIP). BCIP turns blue when degraded by phosphatases, and, as phytases are members of the phosphatase family, this method was used for visualization phytase activity. Phosphatase activity can be seen as cell-bound (the agar in contact with colonies turns blue) and non-cell-bound (agar outside of the colonies turn blue, seen as a blue halo outside of the colony border). A yeast strain known to have cell-bound *phytase* activity was used as reference strain.

To verify that the phosphatase activity seen from the BCIP-agar medium was a result of presence of phytases, and not by presence of non-phytase phosphatases, liquid cultivations were prepared using the common lab media YPD, the complete YNB, and a phosphate-free YNB supplemented with either yeast extract or peptone to the levels used in YPD (10g/l and 20g/l respectively). After incubation, cells were removed by centrifugation and filtration, and the phytase activity in the cell-free supernatant was quantified by a phytase assay.

The phytase assays were prepared by mixing a buffer containing a known amount of the substrate phytate with an aliquot of the culture supernatants, as shown in Figure 7. Each assay mixture was then incubated, at a certain temperature, and samples were withdrawn regularly over a period of 1h. In each sample, the reaction was stopped by an addition of hydrochloric acid immediately after the withdrawal. The samples were then quantified for phytate content using high performance liquid chromatography (HPLC) together with known phytate standards, and a degradation curve could be constructed for each assay reaction.

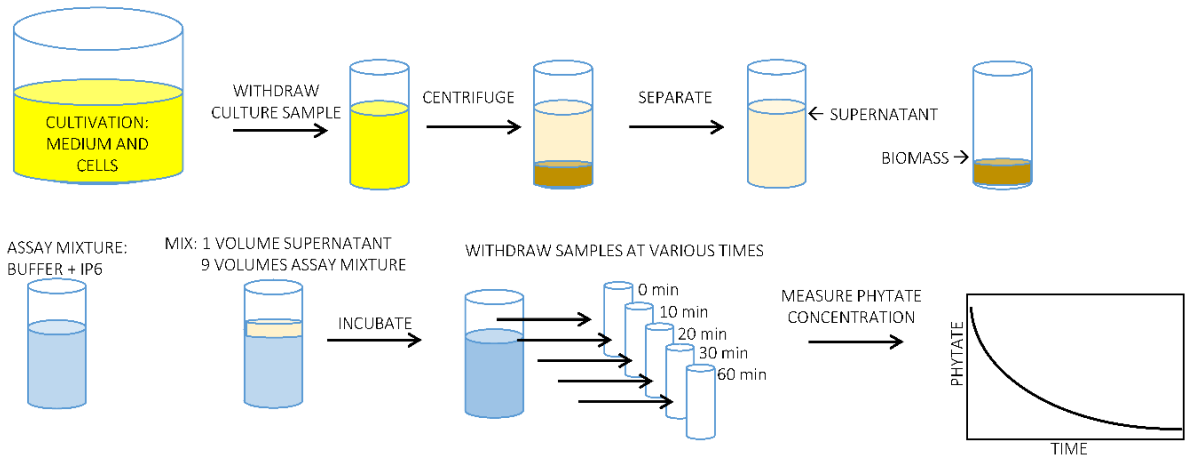


**Figure 7.** *Schematic presentation of the phytase assay method, where the culture supernatant is made cell-free before being mixed with the assay mixture. The assay mixture is incubated and samples are withdraw at regular time intervals. The samples, plus known external standard solutions, are then analyzed by HPLC to determine the phytate concentration at each sampling point.*

### 4.1.2 Investigation of the medium composition's influence on phytase release

As different media showed different effects on the phytase activities in the culture supernatants (see results section 5.1), and since it is known that high levels of phosphate in the surrounding media can repress phytate degradation [143, 144], the phosphate levels in the different cultivation media were analyzed by high performance liquid chromatography (HPLC). The phosphate concentrations of the following media were analyzed; phosphate-free yeast nitrogen base (YNB) supplemented with 10 g/l peptone or 20 g/l yeast extract, complete YNB, and YPD medium without sugar (10 g/l yeast extract, 20 g/l peptone). The same four media were used for the cultivations, but with supplementation of 20 g/l of glucose. The glucose could not be included in the media for the HPLC analysis, as the high sugar content interferes with the analysis. The glucose levels after cultivation is lower as the sugar has been consumed by the cells, and no longer interferes with analysis.

Investigation of whether the difference in released phytase between the yeast extract- and the peptone-supplemented YNB media, respectively, had to do the very low inherent phosphate concentration in the peptone medium was done. A new YNB without phosphate was supplemented with peptone, as before, but additionally supplemented with phosphate ($KH_2PO_4$), to reach the same final phosphate concentration as in the YNB medium supplemented with yeast extract.

As something in yeast extract induced phytase release from the yeast cells, as opposed to peptone addition at the same phosphate concentrations (see results section 5.1), a small study of the potential medium component causing this difference, was done. Based on the manufacturer's protocols for the yeast extract and peptone, ingredients with a 3-10 fold higher concentration in yeast extract as compared to peptone were selected. A set of test media were prepared, to investigate putative effects on the release of phytase, having different levels of amino acids (CSM amino acid mixture, cysteine + glutamine, glutamic acid, alanine + serine + proline) or growth factors (ATP + ADP + AMP + ITP + F6P).

## 4.2 Paper II – Determination of phytase activity by different methods

In Paper II, the potential differences in quantification of phytase activity, based on various methodology, was studied. Principally two methods are used for phytase activity determination; analysis of substrate phytate ($InsP_6$) concentration, or analysis of the product phosphate (Pi) concentration. Various methods are commonly used for phosphate determination, and this Paper compared a selected set of such methods, with substrate concentration analysis using HPLC. The aims were: to i) compare the phytase activity determination of the same phytase assay samples based on different methods, to assess potential differences in the determined phytase activity, and ii) compare the phosphate analysis methods with each other, both on phytase assay samples and on some selected yeast growth media components, in order to assess possible differences in phosphate quantification based on the method.

The phosphate analysis methods in this study were various colorimetric methods, where the free phosphate is quantified based on color development using different reagents. The color development is determined spectrophotometrically and the concentration is determined using external standard samples. The investigated methods and the reagents included in each method were, ***Peterson ANSA*** (Sodium Dodecyl Sulfate (SDS), ammonium molybdate in hydrochloric acid (HCL), 1-amino-2-naphtol-4-sulfonic acid (ANSA)) [167], ***Peterson ascorbic acid*** (SDS, ammonium molybdate in HCL, ascorbic acid), ***Bae et al.*** (Trichloroacetic acid (TCA), ammonium molybdate in $H_2SO_4$, ferrous sulfate) [168], ***Heinonen and Lahti*** (acetone, ammonium molybdate, $H_2SO_4$, citric acid) [169] and ***CPCB/Agilent*** (ammonium molybdate in $H_2SO_4$, glycerol, $SnCl_2$) [170]. As a reference, the phosphate content of each

sample was also analyzed by HPLC, with no addition of reagents. As most of the colorimetric protocols include addition of acids, it was hypothesized that some labile phosphate groups in the samples could be released during the method process, potentially giving higher phosphate results. This potential effect can be avoided by HPLC phosphate analysis. For each colorimetric phosphate analysis, and for HPLC phosphate analysis, standard phosphate solutions were exposed to the same analysis method, to create a standard curve for determination of the sample's phosphate concentration.

From the substrate (phytate) or product (phosphate) analyses of the phytase assay samples, the corresponding phytase activity of each sample was calculated and compared.

## 4.3 Paper III –Improved phytate degradation by classic mutagenesis and selection

The focus of Paper III was to increase the phytase production and/or phytase release and/or phytase activity, by improving the yeast strain. The goal was to further improve the overall phytate degradation, to enhance the release of non-cell-bound phytase to the surrounding medium and to make the yeast less sensitive to repression by high phosphate levels in the surrounding media.

The mutagen was UV radiation, at the commonly used wavelength of 254 nm, corresponding to the UV-C range [159, 161, 163, 171-173]. Initially, the survival rate was determined using a fixed distance to the light source, with various irradiation times. The irradiation time chosen correspond to about a 60% survival rate. Generally a survival rate between 10% - 50% is recommended to achieve a good balance between number of mutants and cell survival without inducing a high level of double mutations [174]. The higher survival rate used in this work was selected in order to allow a larger number of viable cells and decrease the risk of achieving strains with multiple mutations.

Normally, either a liquid suspension of cells is irradiated and then spread on agar plates with selective medium, or, a liquid suspension is first spread on selective agar plates, and the plates are subsequently exposed to irradiation. The first was used in this study since the selective medium contains BCIP, which is sensitive to light and cannot be used during the irradiation. The method setup is presented in Figure 8.

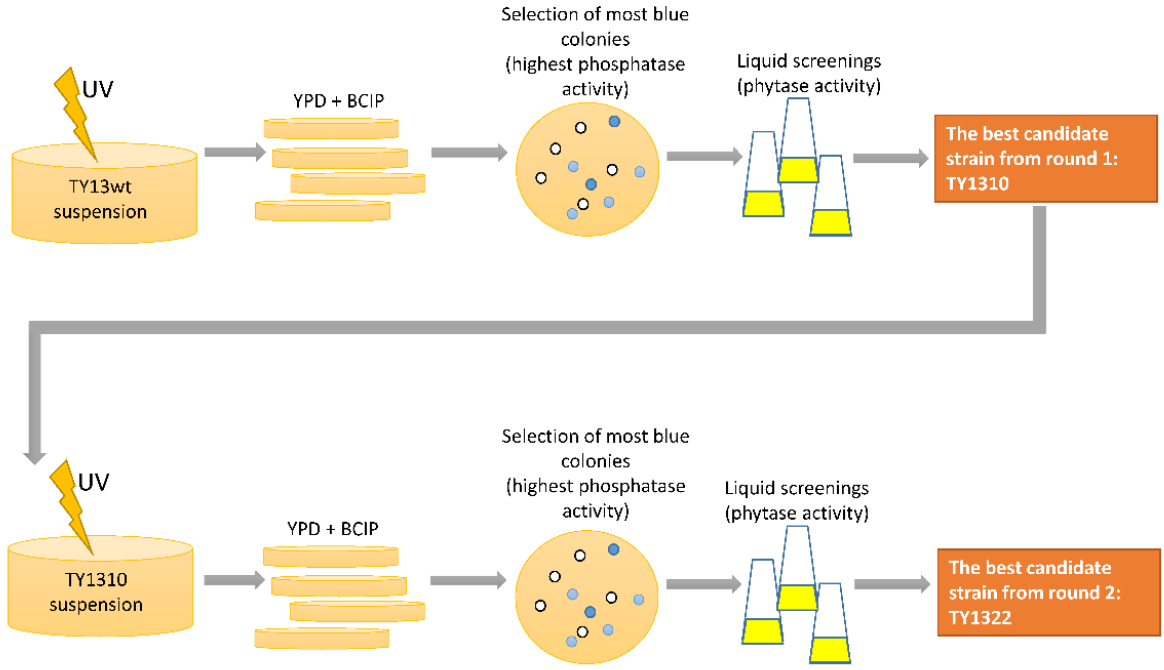


**Figure 8.** *The procedure for the two rounds of ultra violet (UV) mutagenesis with subsequent selection and liquid screenings, resulting in strain TY1310 form round 1, and strain TY1322 from round 2.*

Agar based screening were used to be able to screen a large number of cells, where individual colonies can easily be observed and selected. The selection was done based on phosphatase activity using complete yeast nitrogen base (YNB) agar medium, supplemented with the phosphatase indicator BCIP, which turn blue by the presence of phosphatase activity. Due to the initial selection being done based on phosphatase activity, and not specifically for phytase activity, a large number of potential mutants were selected for the liquid screenings with the aim to increase the chances of finding mutant strains with the right traits.

Under subdued light, aliquots of the irradiated cell suspensions were spread on BCIP agar plates, and subsequently incubated in the dark. After incubation, colonies with a strong blue color formation, or with an indication of a blue halo surrounding the colonies, were selected for further screenings (Figure 8). Several succeeding steps were undertaken to evaluate putative mutant strains (section 4.3.1), and to investigate the phenotypic properties of the final mutant strain TY1322 compared to the wild type strain TY13wt (section 4.3.2), and of the phytase released by strain TY13wt and TY1322 (section 4.3.3). The original strain, TY13wt, and final mutant, TY1322, were subjected to whole genome sequencing to compare their genome differences. The work is still ongoing.

### 4.3.1 Screening methods and evaluation of the developed yeast strain(s)

The screenings were done in single cultures without replicates, due to the large number of isolates. For isolates showing promising results, another round of evaluation was done with higher statistical reliability.

Screenings were done to assess phytate degradation in one high and one low phosphate medium, since it is known that the surrounding phosphate concentrations can influence the overall phytate degradation and the release of phytase [143, 144, 148]. Further, screening for the release of extracellular non-cell-bound phytase were done in a yeast extract supplemented medium, as yeast

extract is known to induce release of phytase (Paper I), and also in one high phosphate medium known to suppress phytase release (Paper I).

After the screenings, an evaluation of the successive development – from the wild type strain *Pichia kudriavzevii* TY13wt, via intermediate mutant strain *Pichia kudriavzevii* TY1310, to the final selected mutant strain *Pichia kudriavzevii* TY1322 – was demonstrated in terms of phytate degradation in one phosphate-free and one high (26 mM $KH_2PO_4$) phosphate medium.

### 4.3.2 Phenotypic characterization of evolved strain

Phenotypic comparison of the final mutant strain, *Pichia kudriavzevii* TY1322, with the parental strain *Pichia kudriavzevii* TY13wt was done. Cultivations were performed to assess growth under stressed conditions such as low pH, presence of ox bile, high temperatures, osmotic stress and growth in the presence of lactic acid or ethanol. The growth on different carbon sources was also determined. The reason for performing these experiments were to: i) ensure maintained growth capacity from the mutant strain compared to the wild type strain, and ii) map the characteristics of those previously not phenotypically characterized strains.

Some of the cultivation conditions tested may resemble environments that can occur in food fermentations. For example, production of ethanol and lactic acid during sourdough or milk fermentations, presence of various carbon sources depending on the raw material, and elevated temperatures or low pH during various food or feed processing steps. Finally, to investigate the potential survival of the strains through the gastrointestinal tract of humans and pigs, viability and growth were assessed at low pH (as occurs in the stomach), in the presence of bile acids (as at the beginning of duodenum), and at elevated temperatures (the human body is about 37°C and a pig is about 39-40°C [175]).

### 4.3.3 Enzyme purification

There are a wide variety of enzyme purification methods available, and their implementation can show varying success depending on several factors, e.g. the concentration of enzyme, enzyme size and the enzyme matrix. The first step is often to precipitate the enzyme using salts or solvents, which is followed by separation of the remaining enzymes using gel filtration or ion exchange [176]. In this work the released extracellular non-cell-bound phytase from the culture supernatants of the yeast strains *Pichia kudriavzevii* TY13wt and TY1322 was purified. The cultivation medium was supplemented with yeast extract, since our previous findings (Paper I) had shown that yeast extract induces phytase release from those strains. To achieve higher levels of non-cell-bound phytase in the culture supernatants, the incubation times were prolonged to 68 h, compared to the 10 h commonly used in this work.

First, all cells were removed from the culture supernatant by sterile filtration through a 0.22 μm pore size membrane. Second, an Amicon filtration equipment with an overpressure of nitrogen gas and a membrane of 10 kDa cut-off was used. This membrane cut-off was selected since it was large enough to allow most smaller components in the supernatant to pass, but small enough to maintain all proteins of 10 kDa or larger in the retentate. As yeast phytases has been reported of varying sizes, ranging from 40 – 500 kDa [176], a 10 kDa cut-off should retain potential phytases in the retentate. During the Amicon filtration, the retentate solution was concentrated and the buffer was exchanged from the spent medium to sterile succinate buffer with pH 5.5.

The protein content and phytase activity was assessed in each of the purification or concentration steps, in order to determine the yield during the work process. The final purified phytase from TY13wt and TY1322, respectively, were assessed for phytase activity during various conditions, including pH values from pH 2 to pH 9, temperatures ranging from 30°C to 80°C, in the presence of some metal ions and presence of high surrounding phosphate levels. The two concentrates were also analyzed at the Proteomics Core Facility, University of Gothenburg, for verification that the enzymes were phytases.

#### *4.3.3.1 Ammonium sulfate precipitation*

After Amicon filtrations were performed, precipitation of the phytases using the ammonium sulfate precipitation method as described by Englard and Seifer [177] was done. The method is based on the fact that proteins become less soluble at higher concentrations of charged molecules (in this case ammonium sulfate) as the added molecules remove the water molecules from the protein, resulting in precipitation of the proteins. Different proteins will precipitate at different ammonium sulfate concentrations. By a stepwise increase in the ammonium sulfate concentration, different proteins will precipitate and the enzyme of interest can be separated from other present enzymes. However, the ammonium precipitation was not successful for the samples in this study (section 5.3).

#### *4.3.3.2 Size exclusion separation*

As the ammonium sulfate precipitation was not successful, further concentration of the Amicon concentrate was made using spin filters (Macrosep, Pall filtration) at 5000 *x*g with a cutoff at 10 kDa. Thereafter, protein separation of the TY13wt and TY1322 concentrates was done using size exclusion chromatography, on a Sephadex G75 gel column. Sephadex is a material consisting of crosslinked dextran and epichlorohydrin, which forms a gel matrix when mixed with a liquid, usually a suitable buffer. Size exclusion chromatography is based on the fact that molecules of different size travels through a gel matrix at different rates. The matrix material exists with different degree of crosslinking, allowing size exclusion in different size ranges. It is also possible to get Sephadex powders with different bead particle sizes, allowing higher or lower flow rates [178]. In this study, the G75 was selected since it is recommended for use with molecules larger than 80 kDa, i.e. it should be suitable for most different reported phytase sizes (40-500 kDa) [178].

During size exclusion chromatography, molecules of smaller size take longer time to transport through the pores of the gel matrix, compared to larger molecules, allowing a separation based on size. The Sephadex gel column was in this work connected to a fraction collector, which allowed for continuous collection of aliquots of the eluent. All fractions were then analyzed for protein content in order to exclude fractions without presence of proteins. The protein containing fractions were then further analyzed for phytase activity. The fractions exhibiting phytase activity, from TY13wt or TY1322 respectively, were pooled and used for further investigations.

## 4.4 Paper IV – Yeast isolation from fermented goat milk from the Yaghnob valley

In 2012, a sample of the traditionally fermented goat milk from the Yaghnob valley was obtained from the ethnical minority population living there, and brought to a lab in Verona, Italy. Yeast and bacteria were isolated immediately from this sample by one of the collaborators in Paper IV, Giovanna E. Felis, and isolates were maintained at -80°C for future studies. The original fermentation sample was then further maintained in-house, according to instructions from the Yaghnob inhabitants, but by adding pasteurized cow milk instead of goat milk. The maintained fermentation sample was subjected to additional yeast isolation in 2015, in this study. Yeasts isolated from the original sample, and in-house maintained culture, were included in this study.

### 4.4.1 Genotypic characterization of Yaghnob yeasts

#### *4.4.1.1 Identification by ITS1-4 region*

For species identification of the isolated yeasts, DNA was extracted from colonies growing on agar plates [179] and subjected to PCR for amplification of the internal transcribed spacer (ITS) region ITS1-4, using ITS1 and ITS4 primers [180]. After sequencing of the PCR products by Sanger sequencing, the sequences could be compared to a library of sequences found in the online BLAST algorithm provided by NCBI, to determine the species identity of each of the isolates. The identities with previously deposited sequences are given as % of coverage and % of similarity between the query sequence and the library sequence. For the isolates in this study, a minimum limit of 95% coverage and 97% sequence similarity was applied for identification.

#### *4.4.1.2 Identification by RFLP fingerprinting of the ITS1-4 sequences*

Restriction fragment length polymorphism (RFLP) of the ITS1-4 sequences [181] was also applied, using the restriction enzymes *Hae*III and *Hin*fI. The RFLP is a method where the PCR product of the amplified ITS1-4 region is enzymatically cleaved using restriction enzymes, which opens the nucleotide chain at specific restriction sites. Depending on the nucleotide sequence, different number of restriction sites may exist on the amplified DNA. Hence, depending on the nucleotide sequence the restriction enzyme will yield various numbers of DNA fragments, of varying length, from the DNA template. The DNA fragments of each yeast isolate can then be separated by gel electrophoresis. The band patterns, or "finger prints", for each isolate can then be used for identification by comparing with patterns previously reported in the literature [181-183].

#### *4.4.1.3 Microsatellite analysis of Saccharomyces cerevisiae*

For the isolates belonging to *Saccharomyces cerevisiae*, the interest in genetic analysis is high; the evolution, ecology and spreading of this species is being investigated by several research groups, and isolation of *S. cerevisiae* from the geographical area of the Yaghnob valley has not been reported previously.

For comparison of the genetic relation between different individuals within a species, the so-called microsatellite analysis can be used, also known as Simple Sequence Repeats (SSR). In this method, the genetic relationship between strains of the same species is studied by so called microsatellite sequences which, as indicated by the SSR name, are repeats of short simple sequences. Those sequences are composed of repetitions of short nucleotide sequences, with up to ten nucleotides (commonly two to six). These repetitive regions are rather prone to mutations as compared to other parts of the genome, mainly by slippage or proofreading errors [184], meaning that the variations in the length of the specific regions (determined by PCR amplification) can be used to differentiate strains within species.

The genetic sequences that flank the repetitive regions are often highly conserved. Due to the conserved nature of those regions, primers can be designed to identify the flanking regions of the repetitive region of interest. Forward and reverse primers for various repetitive regions of the *S. cerevisiae* genome have been presented [185]. The primers can be fluorescent labeled, allowing the amplified sequences to be recognized in pools of different amplifications, and thereafter to be used for the construction of a "finger print" of the fragments.

In this work, the data handling of the microsatellite analysis was performed in collaboration with two of the co-authors of Paper IV, Maddalena Sordo and Irene Stefanini; hence the details of the

methodology of this are not included in this thesis. Ancestry analysis based on the microsatellite data was made by Irene Stefanini using the model-based programs Structure and CLAMPP [186, 187].

### 4.4.2 Phenotypic characterization of Yaghnobi yeasts

Phenotypic characterizations were performed to investigate intra-species variations of strains within species. To investigate the potential of the isolated yeasts to survive through the gastrointestinal tract of humans and pigs, growth and viability at low pH, in presence of ox bile and at temperatures up to 40°C was investigated. The growth of the strains at temperatures up to 48°C was investigated to test the strains thermotolerance. Growth in the presence of ethanol and lactic acid was investigated because those compounds may be formed during mixed fermentation with various yeasts and lactic acid bacteria in, for example, sourdough or milk fermentations. Utilization of various carbon sources (glucose, sucrose, lactose, maltose, mannitol, arabinose, xylose and galactose) was investigated to learn about which substrates the isolated yeasts may be able to utilize. Invasiveness of isolates into an agar based medium was investigated by the method described by Vopalenska and coworkers [188]. Stress tolerance towards oxidative and osmotic stress was studied, and a selected number of isolates from each of the four species was used for determination of minimum inhibitory concentration (MIC) of some common antifungal agents [189]. Determination of MIC is of importance to know whether a potential growth control can be performed by applying antibiotics. In this work, the antifungals fluconazole, clotrimazole and amphotericin B were used. Finally, since one major aim of the thesis was to find phytase active yeasts, the Yaghnob yeast isolates were also tested for phytase activity.

## 4.5 Paper V – Applicability of selected yeasts and bacteria for food fermentations

As a concluding study in this thesis work, selected strains were applied in model food fermentations using different substrates.

### 4.5.1 Soy milk fermentations

Twenty-one yeast strains and ten lactic acid bacteria (LAB), all originally isolated from the Yaghnob yoghurt or Tanzanian Togwa, were used. Soy milk was chosen as the substrate because it is a commonly used vegan milk substitute, which naturally contains several minerals and high levels of protein, as well as the antinutrient phytate. This makes soy milk an interesting substrate to study for potential phytate degradation.

A screening of single mono-cultures was first prepared in order to investigate i) the volatile profile of each soy milk fermentation, ii) survival of fermenting microorganisms during fermentation and subsequent cold storage up to 1 month, and iii) visual assessment of the thickening and stability of the product. Volatile profile analysis is a way to estimate the taste, and subsequent customer acceptance, of the fermented soy milk. It has previously been established which components in e.g. yoghurt that contribute most to its flavor and aroma, and that are most appreciated by consumers. Survival of the inoculated strains is of importance mainly if a product is to be ascribed a probiotic effect, since it needs to contain viable cells according to the probiotic definition: "*products that contain an adequate dose of live microbes that have been documented in target-host studies to confer a health benefit*" [82].

Selection of strains from the mono-cultures was done based on viability of strains during fermentation and storage, and the volatile profiles from fermented products. Flavoring compounds known to contribute to the typical yoghurt taste are acetic acid, lactic acid, ethyl acetate, acetaldehyde, diacetyl, acetone and 2,3-butanone [190]. LAB with low production of acetic acid and high production of 2,3-butanedione, and yeasts with a minimal production of ethyl acetate and carbon dioxide gas, are desirable from a consumer perspective. The formation of carbon dioxide gas is a problem both through

alteration of the texture of the product, but also since it creates post-packaging issues as the gas makes the packages swell during storage in the supermarkets, something that can be judged negatively by the consumer, i.e. this would be detrimental from a business perspective.

Due to the carbon dioxide gas production from the *S. cerevisiae* strains, none of them were included in the co-culture tests. Strain *Pichia kudriavzevii* TY1322 from our previous studies (Paper III) was included in the soy fermentation experiments due to its demonstrated positive effect on the phytate degradation, together with no observation of gas formation. The strains *L. casei* AB2, *K. marxianus* BL8 and AL3, and *Pichia kudriavzevii* TY1322 were selected for co-cultures, and were combined in the following ways in single soy milk fermentations: (**A**) AB2 + AL3, (**B**) AB2 + TY1322, (**C**) AB2 + BL8, (**D**) AB2 + TY1322 + AL3, (**E**) AB2 + TY1322 + BL8 and (**F**) AB2. Fermentations were done at 37°C and 42°C in order to find the optimal combination of strains and fermentation temperature for creating a soy product that is thickened, is not gaseous and has a good phytate degradation.

From the co-culture tests, two final strain combinations were selected to be used for triplicate fermentation to study the volatile profiles, rheological properties, exopolysaccharide (EPS) production, viability of yeasts and bacteria and pH change. The cultures were selected based on thickening and visually observed product stability. The analysis of volatile profiles, rheological properties and EPS production of the final co-cultures is still ongoing. The viability of yeasts and bacteria, pH change and visual appearance of the product were checked during fermentation and subsequent storage at 8°C for one month. Samples were also assessed for phytate content by mixing samples at 1:10 (vol:vol) with 5M HCl to stop ongoing reactions, and then remove solids by centrifuging at 12’000 *x*g for 5 minutes, followed by spin-filtration using Micron YM-30 cellulose filters with a 30’000 molecular weight cut-off at 12’000 *x*g for 10 minutes.

### 4.5.2 Fermentation of soy bean flour and buckwheat flour for decreased phytate content

Duplicate fermentations of one soy flour and one buckwheat flour were performed. Soy flour was selected as it is a common constituent of pig feed formulations. Buckwheat was selected as it is a non-grass plant, that is rich in proteins (containing all essential amino acids) and minerals, especially iron, zinc and selenium [191, 192] and is also rich in the antinutrient phytate [193].

Two LAB strains isolated from the Yaghnob valley, *Lactobacillus plantarum* TJA26 and *Lactobacillus sp* TJA4B, and two yeast strains, *Kluyveromyces marxianus* AL2 and *Pichia kudriavzevii* TY1322, were used. The substrates were one organically marked soy flour (Organic, Saltå kvarn) and one organically marked buckwheat flour (Organic, Saltå kvarn). The fermentations were prepared by mixing the flours with water and biomass from the different strains corresponding to 10^8 CFU/ml of dough. The combinations of the strains were: (**1**) AL2 and TY1322, (**2**) AL2, TY1322 and TJA4B, (**3**) AL2, TY1322 and TJA26, (**4**) AL2, TY1322, TJA4B and TJA26, (**5**) AL2, TJA4B and TJA26, and (**6**) TY1322. The negative controls were samples without inoculation, treated the same way as the other samples. The incubation was done statically at 37°C in tubes with opened lids overnight (18h). The fermentations pH, total titratable acid (TTA), and phytic acid concentrations was analyzed. TTA is a measurement of fermentation that is related to the amount of acids produced during fermentation.

The buckwheat fermentations were further used as starters for bread production. The breads were prepared by mixing wheat flour, rye flour, water, salt and the overnight buckwheat fermentations. The doughs were mixed by hand and left to proof at 37°C for 4h before being baked at 200°C for 10 min. The breads were analyzed for phytic acid concentration and appearance.

Phytic acid analyses of the fermentations were done by freeze drying the samples, followed by extraction of phytic acid and analysis of the concentration by HPLC. The extraction was done by mixing

0.5 g of samples with 10 ml 0.5 M HCl, with stirring at room temperature for 4h. Solids were then removed by centrifugation before analysis.

### 4.5.3 Wheat and rye bread production

For bread production, a mixture of wheat flour (Organic, Saltå Kvarn), rye flour (Organic, Saltå Kvarn), whole meal wheat flour (Organic, Saltå Kvarn), salt, butter and tap water was used. For each 100 g of dough, 2 g of wet weight yeast was used. The doughs were proofed for 2h at 37°C before the doughs were divided into three equally sized buns and proofed for another 1h before baking at 200°C for 10 minutes. Dough samples from 0h and 2h as well as bread samples after baking were withdrawn and used for phytate extraction (described in section 4.5.2) and analysis.

The yeast strains used for bread production were the high phytase strains *Pichia kudriavzevii* TY13wt, isolated from Tanzanian Togwa, the evolved strain TY1322, and the Yaghnobi strains *Saccharomyces cerevisiae* (CL2, CL4, TJY61), *Pichia fermentans* (BL2, CL7, TJY57) and *Kluyveromyces marxianus* (AL3, BL8, BL12). As reference, commercial baker's yeast from Swedish Jästbolaget (annotated JB) was used, and a bread without addition of microorganisms was used as a negative control.

The selected yeast strains were studied in terms of leavening capacity, effects on color, smell and appearance of the breads, as well as the phytate degradation during the process.

# 5. RESULTS AND DISCUSSION

This chapter presents the results in a condensed form for each of the Papers included in this thesis, as well as short discussions of the results and methodologies used. For detailed descriptions of the results, the reader is referred to each of the Papers/manuscripts.

## 5.1 Paper I – Release of extracellular, non-cell-bound, phytase

It had been discovered that a particular strain, *Pichia kudriavzevii* TY13, isolated from Tanzanian Togwa [68], had a high phytate degrading capacity, which led us to further investigate this strain in Paper I . The results showed that the yeast strain possessed high phytate degrading ability depending on the media. Further, it showed a remarkable release of phytase enzymes to the surrounding media both on agar based media (Figure 9), and in liquid media after only 10 h of cultivation.

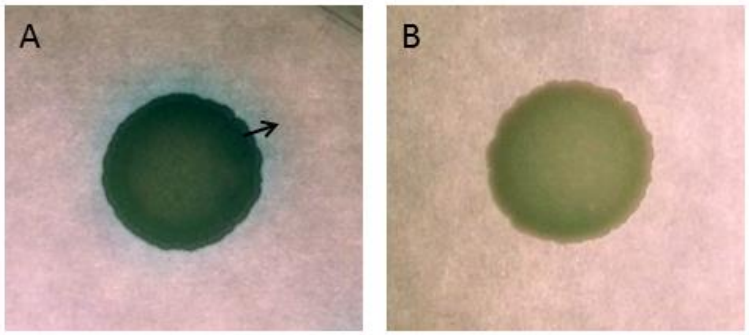


**Figure 9**. *Phytase release on BCIP containing agar plates with strain TY13wt (panel A) versus reference strain S. cerevisiae CBS7764 (panel B).*

From the analysis of viability and concentration of intact cells over time, during cultivation, it was revealed that after 10 h of cultivation 100% of intact cells were observed; after 54 h, the level of intact cells was 98%. A clear decrease in intact cells was seen thereafter. To ensure that the extracellular non-cell-bound activity was a result of secretion/release, rather than the lysis of old cells, 10 h of cultivation was generally used in the continued study. Using cultivation times over 54 h could result in the phytases found in the cell-free supernatant originating from intracellular phytases, released as a result of cell lysis in old cells. However, if the goal is to yield high levels of phytase in the medium supernatant, irrespective of cell viability and origin of phytases, prolonged incubation times generally result in higher phytase levels. The selected cultivation time should be chosen on the basis of what the intention of the study is.

The released phytase from *P. kudriavzevii* TY13 was identified as a 3-phytase, as many other yeast phytases, and initiates the phytate degradation on the 3$^{rd}$ phosphate group of the phytate molecule.

The release of extracellular non-cell-bound phytase in four different liquid media was compared for strain TY13 and for a reference strain, *Saccharomyces cerevisiae* 7764. The media were YPD, complete YNB and YNB without phosphate but supplemented with either yeast extract or peptone (Figure 10). The extracellular phytase levels were highest in the yeast extract medium, closely followed by the peptone medium, the latter which also had a very low phosphate level. Extracellular phytase levels were moderately high in YPD medium, but no detectable phytate degradation was found from the complete YNB medium (which also had the highest phosphate content). The reference strain showed no extracellular phytase content/activity in any of the media (Figure 10).

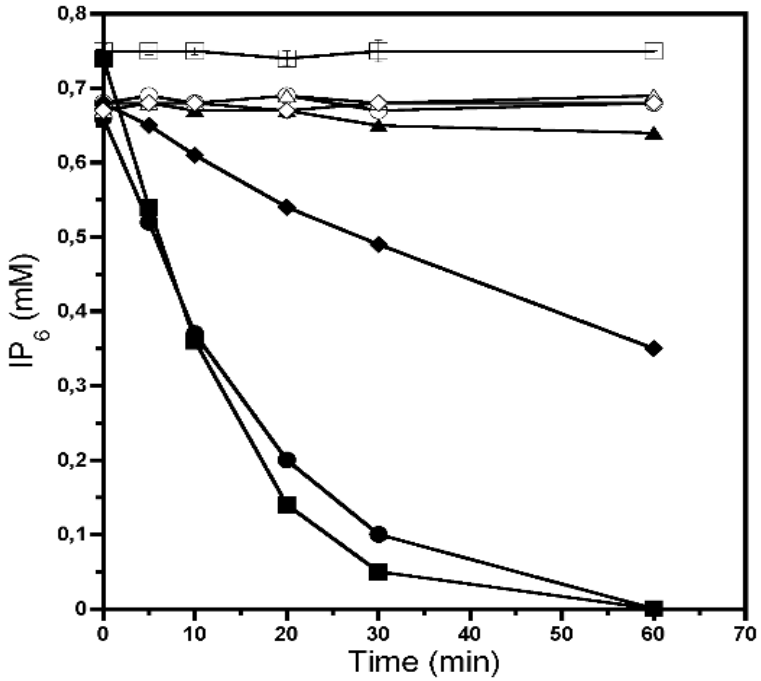


**Figure 10**. *Phytate degradation by the cell-free supernatant of strain Saccharomyces cerevisiae CBS7764 (open symbols) versus strain Pichia kudriavzevii TY13 (closed symbols), after cultivation in complete YNB (Δ), YPD (◊), YNB+YE (□) and YNB+Pep (○).*

Something in the yeast extract induced release of phytase to the surrounding media of strain *P. kudriavzevii* TY13. Further, addition of inorganic phosphate to the peptone YNB medium and to YNB without phosphate, to the same level as found in the yeast extract YNB medium, had a negative effect on the extracellular phytase levels in the peptone YNB medium (Figure 11), confirming that the low level of phosphate in peptone was a contributing reason behind the high levels of phytase found in this medium. The observation that low levels of surrounding phosphate induced phytase release was confirmed and is in accordance with previous studies [143, 144].

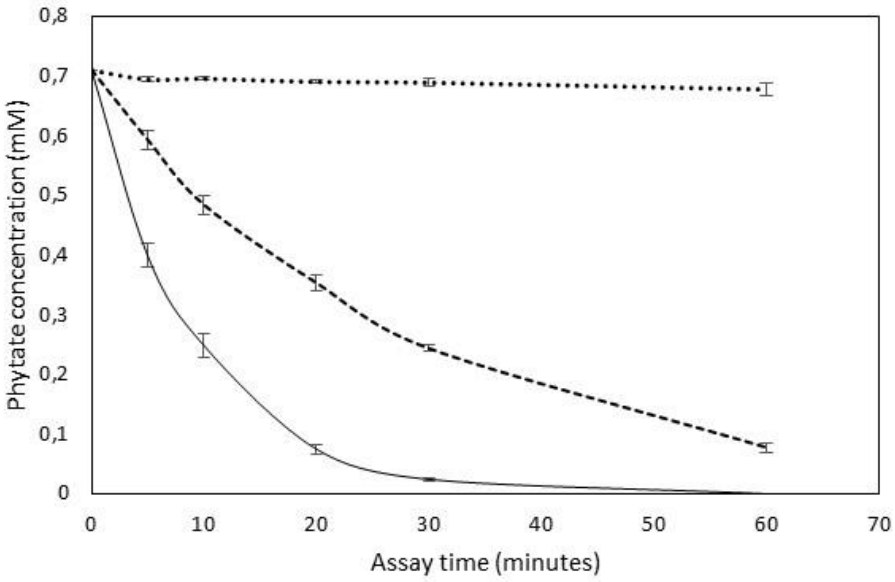


**Figure 11**. *Phytate degradation assay using cell free supernatants from strain Pichia kudriavzevii TY13 after cultivation in two phosphate-adjusted media, YNB+PO4 (dotted line) and YNB+Pep+PO4 (dashed line), and the phytase release inducing medium YNB+YE (solid line). All three media had the same phosphate concentration to allow study of the phytase activity depending on media, irrespective of the differences in inherent phosphate concentrations. Data was obtained through the MSc thesis work of Jauquline Nordqvist [194].*

A set of media components was selected from the manufacturer's ingredient declarations of yeast extract and peptone, and used for formulation of some modified media, with the aim to identify components that induced phytase release. However, the investigation of phytase release in the different modified media revealed that none of the tested components induced the phytase release, under the implemented cultivation conditions. The complexity of peptone and yeast extract makes the investigation of single components very complicated and time consuming. The inducing effect observed from yeast extract may be due to some specific component, to a combinatory effect of several components, or to the presence of some other unknown factor not included in the manufacturer's protocols. The investigation of media components was not included in Paper I, but is presented in the Master thesis work performed by Jauquline Nordqvist [194].

### 5.1.1 Analytical method considerations Paper I

Since peptone and yeast extract are both complex media, it was challenging to distinguish differences between the two that could be responsible for the observed differences in phytase release. A simple setup was used; based on the manufacturers list of contents, a set of components varying three-fold or more between the two media ingredients was identified, and media with altered ingredient concentrations were composed. There are some issues to be considered in this approach; first of all, only the components described by the manufacturers were considered, but there might be other components present in the media that have not been analyzed, or that are unknown. Second, there can be a synergistic effect of several components in the yeast extract causing the induced phytase release, which can be difficult to elucidate and problematic to mimic in a synthetic medium. Further investigations are needed to identify the reason behind the induced phytase release from yeast extract medium.

In Paper I the phytase activity analyses were done using young and viable cultures, to minimize the risk of detecting "false" extracellular phytases resulting from old lysing yeast cells. The tradeoff using younger cultures is that shorter cultivation times allows less total phytase release, i.e. the phytase activity results obtained in Paper I could probably be higher if longer cultivation times had been applied. We analyzed phytase activity by direct measurement of phytate (substrate) concentration, while in several other studies the release of phosphate (product) is used as a measurement of phytase activity. This means that the phytase activity detected in our samples is probably several fold lower compared to results based on analyzing phosphate concentration (for more details, see Paper II). For accurate comparisons, the results in Paper I should be compared with data from cultures of similar age, determined with a similar methodology.

### 5.1.2 Conclusions from Paper I

The phytase active yeast *P. kudriavzevii* TY13 can extensively degrade phytate. The origin of the yeast, from Tanzanian Togwa fermentation, indicates that the strain can probably be considered as safe for food consumption, as it has been repeatedly consumed in a viable state in Togwa fermentation. Strain TY13 is also able to release a large proportion of its phytase enzyme to the surrounding environment in young cultures, and not as a result of old and lysing cells. This can be useful both in food fermentation applications and in the production of crude enzyme solutions. Enzymes present in a free (i.e. non-cell-bound) state in the food matrix during fermentation, the interaction between the enzyme and substrate can induced, as opposed to when there are only cell-bound enzymes. In the case of production of enzyme solutions, cells able to release enzymes can reduce the extraction and production costs. In addition, the results reported in Paper I imply that including yeast extract in the production of phytase solutions, and possibly also in food fermentations, with strain TY13 can improve the phytase synthesis.

## 5.2 Paper II – Determination of phytase activity by different methods

We were able to show in Paper II that, depending on the methodology used, determination of phytase activity of the same sample gave different results. The phosphate concentrations detected in the assay samples, analyzed by the various phosphatase analysis methods, varied depending on the method (Figure 12).

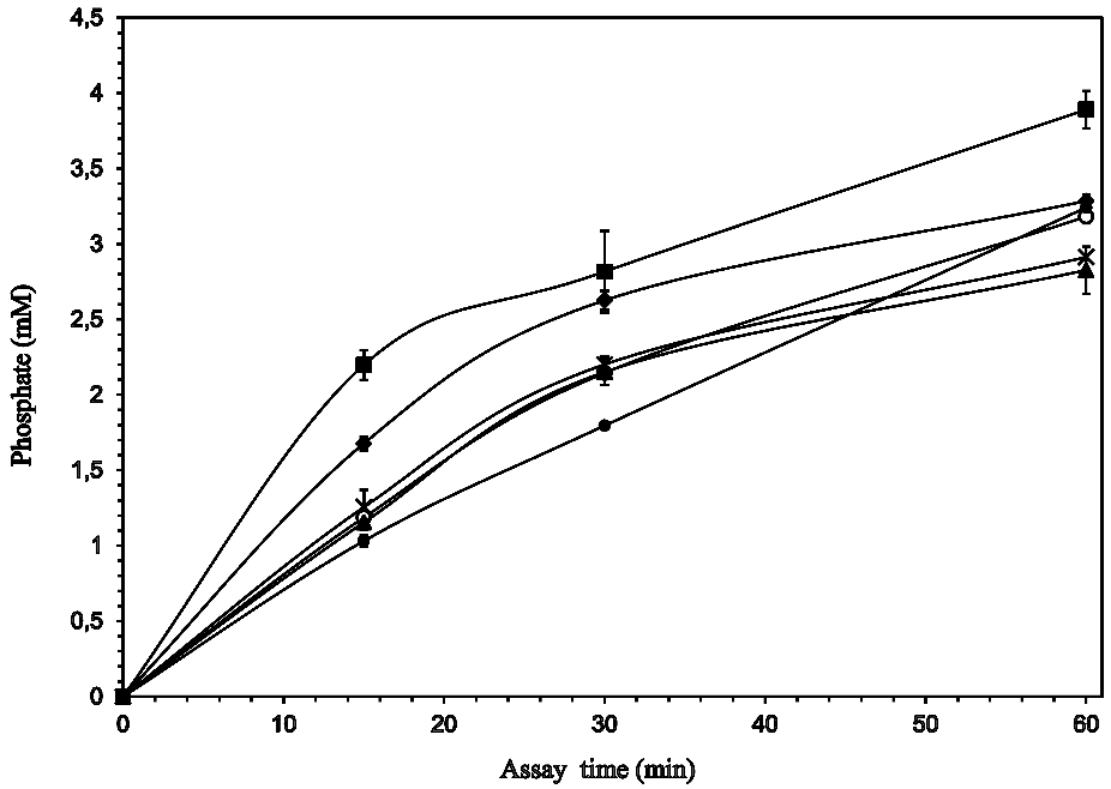


**Figure 12**. P*hosphate concentration detected from the same assay mixture, at time 0 min, 15 min, 30 min and 60 min, analyzed by different methods: Pi-HPIC (*o*), Peterson (▲), Ascorbic acid version of Peterson (×), Bae et al. (♦), Heinonen and Lahti (■) and CPCB / Agilent (●).*

It was further revealed that the phytase activity based on phosphate (product) analysis versus phytate (substrate) analysis, differed up to nearly four times in magnitude in the same sample, from 152 mU/ml based on analysis of the substrate phytate, to 275-586 mU/ml based on different phosphate analysis methods. The reason for the diverse results is that phytase activity determined by phosphate analysis also includes phosphates released from non-phytate substrates, such as the non-phytate inositol phosphates that are continuously formed during the degradation of phytate (Figure 13), or actions by non-phytase phosphatases. This inevitably yields overestimations in phytase activity, compared to quantifying the substrate (phytate) degradation directly. Phytase activity, by definition being the degradation of phytate ($InsP_6$) to $InsP_5$, is more accurately determined by substrate ($InsP_6$) analysis; this method directly addresses the substrate of the reaction and does not include the risks observed for the product (Pi) analysis-based methods.

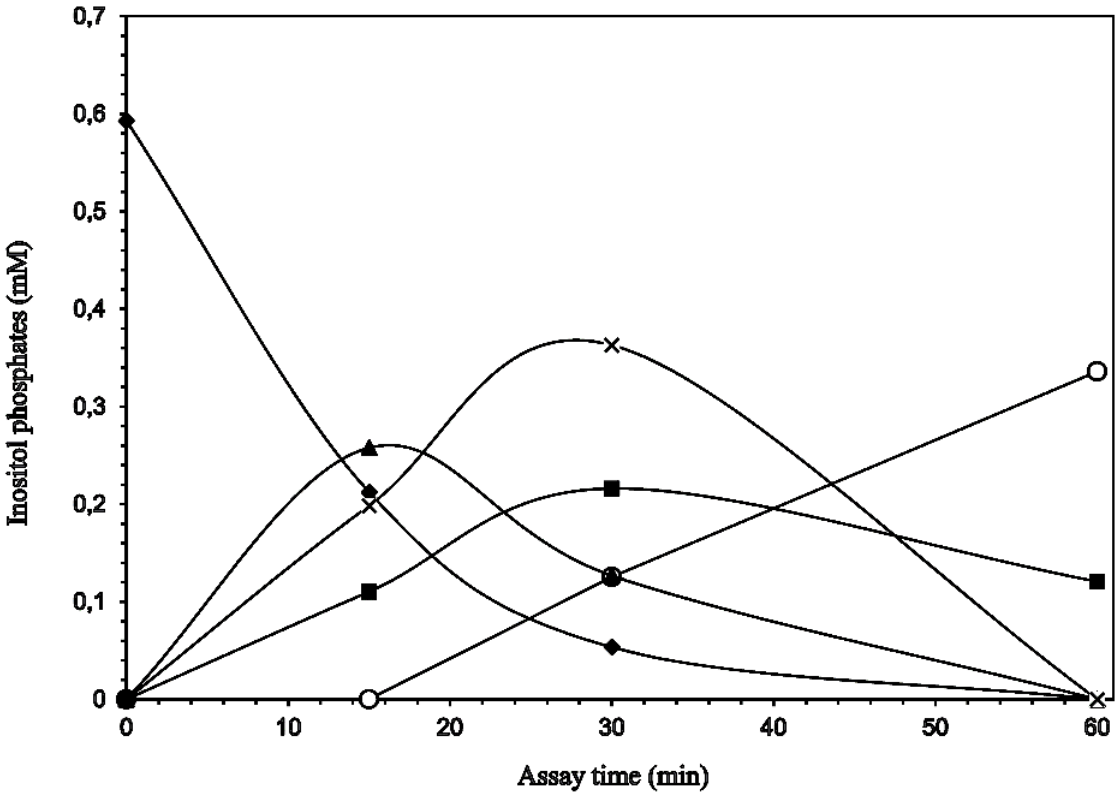


**Figure 13**. *Phytate (InsP$_6$, ♦) degradation with simultaneous formation – and degradation – of InsP$_5$ (▲), InsP$_4$ (×), InsP$_3$ (■) and InsP$_2$ (○). InsP$_1$ was not detectable using this system. As early as at 15 minutes of assay, InsP$_5$, InsP$_4$ and InsP$_3$ had been formed, meaning that, at this point, phosphates had been released from InsP$_6$, InsP$_5$ and InsP$_4$, i.e. phosphate analysis will include phosphates from all those InsP's.*

It was concluded in this work that the term *phytase activity*, i.e. defined as the activity by which the phytase enzyme catalyzes the degradation of phytate, should be separated from the *total inositol phosphatase activity*. *Total inositol phosphatase activity* is the activity determined by analyzing released product phosphate (Pi), i.e. including all phosphates' released from all present phosphorylated compounds by all present phosphatase enzymes. It is important to specify what is actually being analyzed in order to be able to draw accurate conclusions and make reliable comparisons. Analyzing substrate (Pi) release is useful for describing the overall phosphate releasing capacity of an enzyme solution, which is often the case for enzymes intended for use in the feed industry.

The phosphate analysis methods were also compared with each other for determination of the phosphate content of a few media ingredient components. The results were varying, for example, in one yeast extract the variations were as much as three times in magnitude between the highest and lowest detected phosphate value. The chemicals added in the colorimetric methods may release labile phosphates from other phosphorylated compounds present in the samples, and the samples may potentially contain compounds that interfere with the colorimetric methods, causing the varying results. The results reported in Paper II underline that a comparison of phytase activity results between different studies must be done with caution, as the choice of method can have such a great effect on the phosphate levels detected and the subsequently determined phytase activity.

### 5.2.1 Analytical method considerations Paper II

The analysis of phosphate content in different media components and assay samples revealed that the different methods gave a variety of results. Additional substrates and analysis methods should be included to assess the reason for the higher/lower results of the colorimetric methods, and more thorough investigations of the chemistry behind the results should be made. We do not know the reason for the variations in the results observed, but we hypothesized that the use of various acids in the colorimetric protocols induced the release of labile phosphate groups. However, more in-depth investigations of the reason for the variations in phosphate detection were beyond the scope of this work. The methods and samples included in this study were considered adequate to prove our hypothesis about the risks of using product concentration as a measure of phytase activity.

### 5.2.2 Conclusions from Paper II

The findings of Paper II are highly relevant, first in being able to interpret the data in this thesis, since the phytate degradations here are determined by analyzing substrate (phytate) and not product (phosphate), as in most other studies. Further, to the best of our knowledge, this is the first comparison of phytase activity determination based on substrate versus product analysis. The difference between phytase activities that were detected, in assessments using different methods, was surprisingly large, and the results of this study can hopefully aid in accurate comparison of phytase activity versus total phosphatase activity in future studies. In addition, it became evident that the phosphate analysis methods themselves yielded very varying results when applied to aliquots of the same samples, indicating the need of careful method evaluation and verification in phosphate analysis using colorimetric assays.

## 5.3 Paper III – Improved phytate degradation through random mutagenesis of a yeast

In this Paper, strain development of the wild type strain *Pichia kudriavzevii* TY13wt (Paper I) was done by UV mutagenesis. The first round of mutagenesis resulted in a very promising mutant, *Pichia kudriavzevii* TY1310, showing improved phytate degradation both in the phosphate-free and high-phosphate medium. However, no improvement in phytase release was seen. To further improve this strain a second round of mutagenesis was done, using *Pichia kudriavzevii* TY1310 as the parental strain. The second mutagenesis resulted in the identification of a further improved strain, *Pichia kudriavzevii* TY1322. This strain showed enhanced phytate degradation, especially in a high phosphate medium, as compared to *Pichia kudriavzevii* TY1310. However, the release of phytase to the surrounding environment was not found to be improved; the reason for this is not known. It raised the question of whether the release of phytase from those strains occurs by active or passive mechanisms.

Mutants TY1310 and TY1322 showed clearly improved phytate degrading capacity compared to the wild type strain TY13wt, especially in the high phosphate medium where there was no phytate degradation seen by TY13wt during the first hours of incubation (Figure 14).

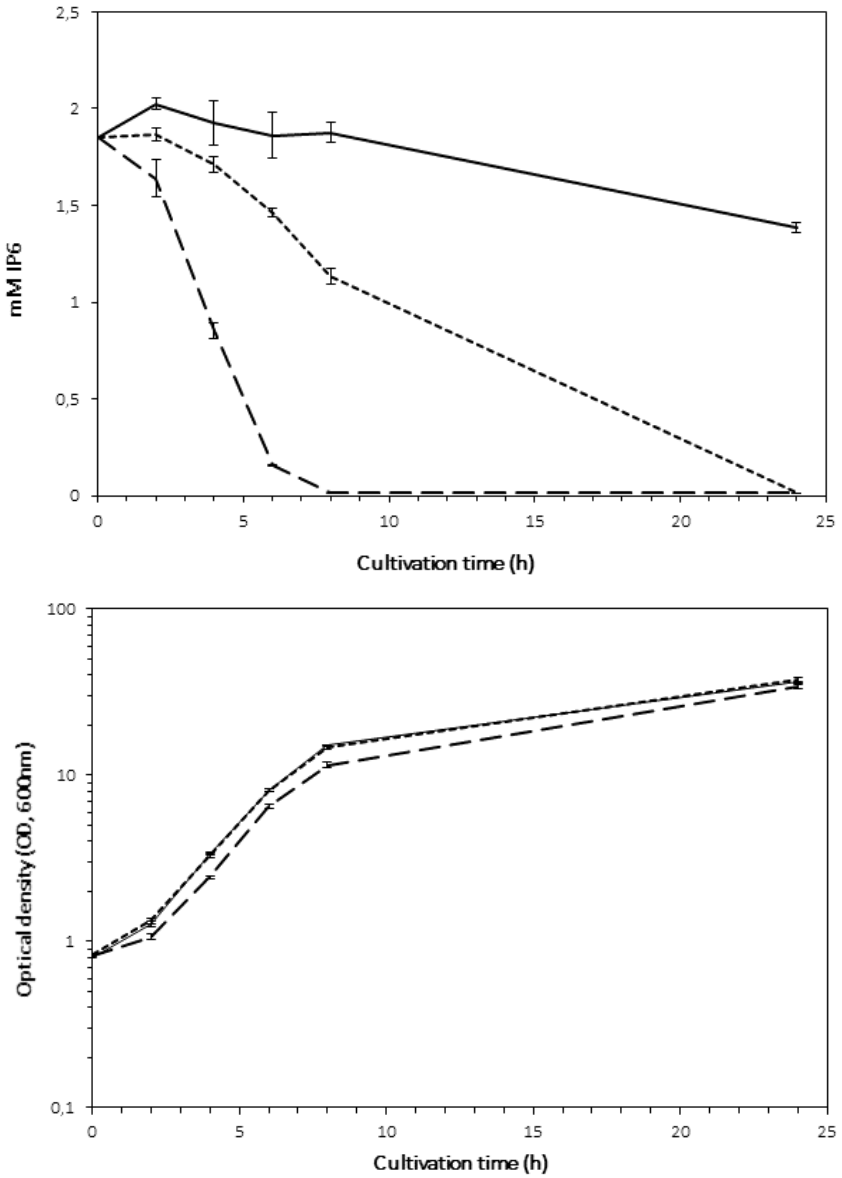


**Figure 14.** *Phytate degradation (top panel) and growth as optical density (bottom panel) during cultivation in a high (26 mM) phosphate medium by the three Pichia kudriavzevii strains TY13wt (wild type strain, solid line), TY1310 (intermediate mutant strain, dotted line) and TY1322 (final mutant strain, dashed line) for a total cultivation time of 24h.*

A minor impairment in growth was observed for strain *Pichia kudriavzevii* TY1322 (Figure 14). This was not unexpected, however, as this strain directs a large part of its metabolic energy towards phytase synthesis, hence leaving less energy for cell division and growth, i.e. impairing the growth rate. Growth rate reduction has also been observed in a genetically modified high phytase producing strain of *Saccharomyces cerevisiae* [38]. Further, analysis of biomass associated phytase activity (i.e. phytate degradation by washed biomass in enzymatic assay) revealed that strain TY1322 has a close to eight times higher activity than the wild type strain TY13wt after cultivation in a high-phosphate medium. This is a remarkable improvement, and it is surprising that the growth was not particularly impaired.

Potential phenotypic differences between the wild type strain TY13wt and mutant strain TY1322 were investigated. As TY13wt had not been phenotypically characterized previously, and this was therefore an important step also in mapping the two strains' properties and their potential in future applications.

The investigations revealed the same response for TY13wt and TY1322 in all tests, indicating that no unwanted mutations had influenced the TY1322 performance. The two strains showed ability to grow at i) low pH (pH 2), ii) high temperatures (46°C) and iii) in presence of ox bile (2%), and showed resistance to osmotic stress (60% glucose medium) and ability to grow in presence of ethanol (6% v/v) and lactic acid (1% v/v). Several of the investigated growth conditions are interesting from an application point of view, for example mixed fermentations containing lactic acid bacteria creating low pH environments, or media with high initial sugar concentrations causing osmotic stress, and some processes applies high temperatures.

The strains are able to grow at low pH, high temperatures and in the presence of ox bile. Those are conditions resembling the conditions that may occur in the gastro-intestinal (GI) tract of humans and pigs. Those results may indicate that, if the strains are consumed in a viable state, they could survive passage through the GI tract. This is an important characteristic for probiotic strains, as they need to be administered alive and be able to survive through the GI tract [82]. The potential ability of strains to degrade phytate not only *during* food preparation, but also continuously in the GI tract, may be classified as a probiotic trait; in humans with mineral deficiencies that consume a cereal-based diet with a simultaneous consumption of phytate degrading microbes, enhanced mineral availability from the food may be allowed also through release inside the GI tract. Strain TY1322 show very promising phytate degrading ability and interesting stress tolerances, but obviously, the phytate degradation and mineral release from a model food during fermentation and digestion must be assessed in order to know more about the full potential of TY1322.

Initially, released non-cell-bound phytases of TY1322 and TY13wt were subjected to precipitation using ammonium sulfate solution. However, after attempting to precipitate the phytase at various ammonium sulfate concentrations, the enzyme was still found in solution, at 80% ammonium sulfate saturation. The work continued using other methods.

Concentration and purification of the released phytases was continued by fractionation and separation on a sixe exclusion gel column. The Amicon concentration allowed for removal of compounds smaller than 10 kDa. The size exclusion chromatography then allowed separation of the remaining compounds based on size. However, it is possible that two same-sized compounds are eluted in the same fraction. Hence, other non-phytase proteins of similar size as the phytase may be retrieved in the fraction where the phytase activity was found. This means that 100% purification of the phytase samples cannot be guaranteed by this method.

To evaluate the outcome of the purification, the phytase samples of TY13wt and TY1322 were denaturated and separated on polyacrylamide gel, including the i) original supernatant samples, ii) concentrated samples, and iii) pooled phytase active fractions. This revealed a resilient purification, comparing the purified samples with the original culture supernatant samples. Proteomics analysis of the pooled phytase-active fractions from TY13wt and TY1322, respectively, revealed that phytases are plausibly the main components of both samples, having the common histidine-acid phosphatase (HAP) motif RHGXRXP [195]. These results taken together made us confident in having obtained a highly purified sample. Since 100% purity cannot be guaranteed, the samples are not referred to as “pure”, but as “purified”, in this work.

The purified phytase samples were assessed for pH and temperature optima, revealing an optimal temperature at 55°C and two pH optima, at pH 3.5 and 5.5. A phytase with a pH optimum at 3.5 is relevant for the pig feed industry, as phytases used in pig feed production should preferably have high

activity around pH 3.5, since that corresponds to the pH of the pig stomach [196], and can allow continuous phytate degradation in the gastrointestinal tract. In addition, the low but rather broad optimal pH range (pH 3.5 to 5.5) is suitable for implementation in various food fermentations, such as sourdough preparation or milk fermentations, where presence of lactic acid bacteria lower the pH during the fermentation.

The phytate degradation caused by the released phytase, i.e. phytate degradation by the cell-free supernatant of TY1322 after cultivation in the YNB+YE (phytase release inducing medium), was not higher than that from strain TY13wt under the same conditions. That means that although the phytase activity was improved in TY1322 both at high and low phosphate conditions, no increase in phytase release was seen. Further, biomass-associated activity from TY1322 cultivated in YPD resulted in 66% phytate degradation, while the biomass cultivated in YNB+YE showed complete degradation under the same conditions. This means that the phytate degradation from both the biomass and the cell-free supernatant of TY1322, respectively, is higher after cultivation in the YNB+YE medium compared to cultivation in the YPD medium. It is not known from this study whether the phytase release to the surrounding medium from strain TY13wt and TY1322 is due to active or passive export.

### 5.3.2 Analytical method considerations Paper III

Genetically modified organisms (GMO's), resulting from directed genetic modifications are not well received by consumers and are therefore not commonly used in the food industry. A random mutagenesis approach was instead used in this work as the aim was to find a yeast strain for use in the food and feed industries. However, the risk of using a random approach is the potential induction of unwanted genetic modifications, affecting for example stress tolerance and viability. Thorough phenotypic characterization was undertaken for the evolved strain TY1322 to assess this risk. Furthermore, not knowing the type or location of the mutation in the genome means that the stability of the mutation is unknown. In this work we evaluated the stability of the mutation in strain TY1322 by repetitive cultivations for several generations and with continuous analysis of the phytate degrading ability. No observation of any alteration in phytate degradation capacity was seen, indicating a stable mutation.

The selection method used in this study is based on phosphatase activity, not specifically phytase activity. Hence, the selection of mutants was done on the assumption that increased phytase activity correlates to increased phosphatase activity, which is not necessarily true. There are likely other phosphatases than phytase present in the yeast; i.e. an increased phosphatase activity may come without increased phytase activity. Furthermore, in the unfortunate case that the mutagenesis results in increased phytase activity accompanied with simultaneous decrease in a non-phytase phosphatase activity, the increased phytase activity may be masked by overall unchanged phosphatase activity seen from BCIP screening. Further, the actual selection was done by visual estimation of the formation of a blue color, which may in itself be quite tricky. Knowing about the potential risks of the method choice, a large number of potential mutants was selected for the initial screenings in this work, increasing the chances of finding positive mutants. Since the work resulted in the identification of strongly improved phytase active strains, no further development of the selection method was done.

The enzyme purification resulted in a concentrated phytase solution from each TY13wt and TY1322. As the size exclusion using Sephadex G75 eluted the phytase in the first protein peak, proteins of similar size as the phytase may be mixed in this fraction. Ideally, further purifications could have been done, but considering the yields from the purifications the high prevalence of phytases according to

the proteomics analysis, the results are regarded satisfactory. Further, with the overall aim of this thesis work in mind, a perfectly purified phytase is not necessary, as detailed protein studies were beyond the scope of this work. The commercially available phytase solutions on the market today are also not completely pure, i.e. a perfectly pure sample may not be necessary for application purposes.

### 5.3.3 Conclusion from Paper III

The work in Paper III resulted in the yeast strain *Pichia kudriavzevii* TY1322, with a highly improved phytate degrading ability and strongly improved resistance to the commonly inhibitory effects of high phosphate concentrations. This, together with the broad pH and temperature tolerance in this strain, indicates a good potential for various applications in both the food and feed industries, either as a fermentation organism or as production organism for phytase solutions. In addition, this work demonstrated the usability of phosphatase activity as a selection method for phytase activity in the strains investigated.

## 5.4 Paper IV – Yeast isolation from fermented goat milk of the Yaghnob valley

The isolation of yeast from the Yaghnob fermented goat milk resulted in a total of 52 isolates, which were identified as *Kluyveromyces marxianus* (29 isolates), *Pichia fermentans* (12), *Saccharomyces cerevisiae* (10) and *Kazachstania unispora* (1). The small species variation was accompanied by a large phenotypic intra-species variation, revealed by assessing growth on different carbon sources, at various pH and temperatures, strain invasiveness, hyphae formation, and tolerance towards different stress conditions. As several other studies have observed a larger number of species from similar substrates [62, 63, 197-199], it was unexpected to find this low number of species from the fermented milk. However, in the study of Bai and colleagues [63], *Kluyveromyces marxianus*, *Saccharomyces cerevisiae* and *Pichia fermentans* were identified as the most dominant species in traditional fermented milk of the Tibetan plateau in China. Since the same three main species were isolated both from the original Yaghnob sample, and from the in-house maintained sample, it is hypothesized that the species composition in this fermented milk has evolved to become stable and resistant to external contamination. Even after three years of in-house maintenance by using pasteurized cow milk, without applying any specific hygienic protocols, the species that have been isolated are still the same as in the original sample (apart from one isolate of *Kazachstania unispora*), and the sample's physical appearance properties are maintained.

### 5.4.1 Genetic alterations in *Kluyveromyces marxianus*

It became evident after restriction fragment length polymorphism (RFLP) analysis that two different band patterns were observed for the isolates belonging to the species *Kluyveromyces marxianus* (Figure 15), now referred to as group I (where the third band corresponds to the commonly reported size of 120 bp) and group II (where the third band corresponds a size of 140 bp).

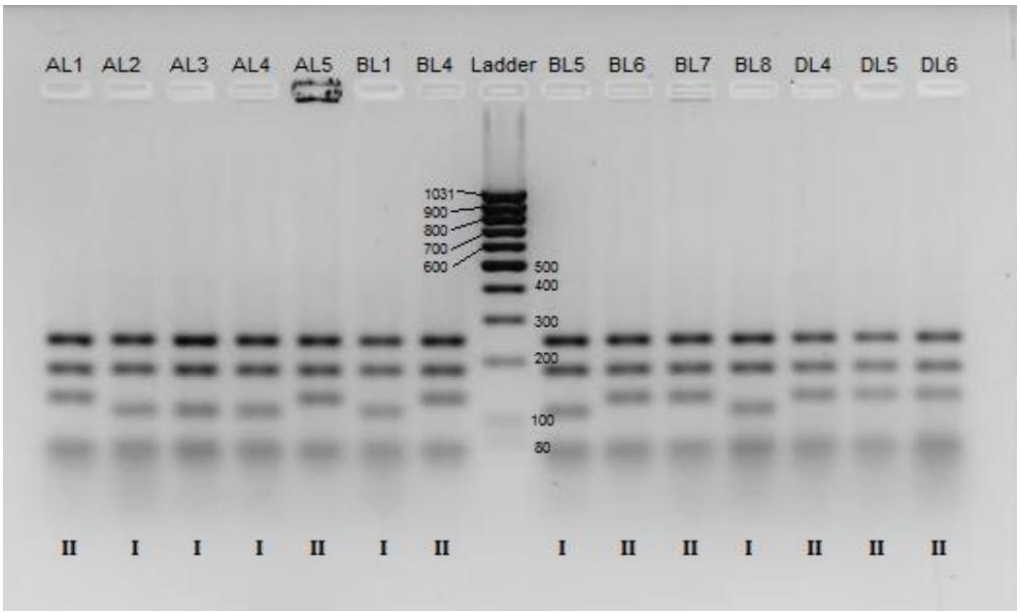


**Figure 15**. *Band patterns after restriction fragment length polymorphism of some Kluyveromyces marxianus isolates, showing the two genetically different groups I and II, seen by a different size of the third largest band, being either 120 bp (group I) or 140 bp (group II).*

Multiple sequence aligning was done using the ClustalW2 tool, which allows several sequences to be compared, and the output indicates positions where the submitted sequences are similar (*) or not similar. It was found that there was a single nucleotide difference (marked with a rectangular box in the Figure 16) in the ITS1-4 region of the *K. marxianus* strains, where a G-nucleotide was seen for group I isolates, and an A-nucleotide for group II isolates.

```
AL3_fw_A10.ab1      TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
TJY60_fw_B11.ab1    TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
TJY59_fw_B10.ab1    TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
BL5_fw_D07.ab1      TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
CL5_fw_A05.ab1      TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
CL6_fw_A06.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 118
AL5_fw_A12.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 118
AL1_fw_A08.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
AL2_fw_A09.ab1      TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
AL4_fw_A11.ab1      TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
BL8_fw_D10.ab1      TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 118
DL10A_fw_C11.ab1    TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 119
DL10B_fw_C12.ab1    TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
BL6_fw_D08.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 116
BL4_fw_D06.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 118
BL7_fw_D09.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 118
DL6_fw_C06.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 119
DL5_fw_C05.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 118
BL14_fw_E04.ab1     TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 116
TJY54_fw_B05.ab1    TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 119
DL11_fw_D01.ab1     TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 114
DL12_fw_D02.ab1     TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 113
DL4_fw_C04.ab1      TGCGCGGC-AATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 112
TJY52_fw_B03.ab1    TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 113
BL1_fw_D03.ab1      TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 115
BL3_fw_D05.ab1      TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
DL2_fw_C02.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 112
BL13_fw_E03.ab1     TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 114
BL12_fw_E02.ab1     TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 115
                    ******** * **************************************************
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**Figure 16**. *ClustalW2 alignment of the ITS1-4 region of the Kluyveromyces marxianus isolates, revealing a one-nucleotide difference (marked by the rectangular box) where group I (in bold text) had a G nucleotide, and group II (in grey text) had an A nucleotide.*

Since the ITS1-4 region constitutes a well conserved region, a genetic polymorphism found there may indicate that there are further genetic variations in the less conserved parts of the DNA of those strains. As significant phenotypic variations were also found between the two genetically different groups, we have reason to believe that there could be additional genetic variations in other parts of the genome. Considering the genetic and phenotypic variations observed between the two groups of *K. marxianus* strains, it may perhaps indicate the isolation of a new species.

### 5.4.2 Microsatellite analysis of *Saccharomyces cerevisiae*

From a phylogenetic tree constructed with the Yaghnobi *S. cerevisiae* and another circa 350 previously isolated *S. cerevisiae* strains of various origin, it became evident that the strains isolated from the Yaghnob fermentation form a separate cluster, apart from other isolates. This indicates that these strains have evolved separately and may also explain why several of the strains show some non-typical *S. cerevisiae* phenotypic characteristics, such as growth at high temperatures. This raises further questions about the genetic variation between the Yaghnob *S. cerevisiae* isolates as compared to previous isolates and about the evolutionary path of those strains. It may be hypothesized that the human gut of the people living in the Yaghnob valley functions as a reservoir for *Saccharomyces cerevisiae* strains, similar to what has recently been presented for the wasp intestine [46]. It could then be speculated that the temperature of the human body (37°C) has enabled a positive selection of strains able to survive at this temperature, resulting in the *S. cerevisiae* strains from the Yaghnob valley being able to grow at elevated temperatures. The high temperature tolerance among several of the *S. cerevisiae* isolates led us to hypothesize that some of these strains may potentially belong to the well-studied probiotic strain *S. cerevisiae boulardii*, showing higher temperature tolerance than other *S. cerevisiae* strains [101]. However, assessment of the actual probiotic potential of the strains, in strain-host studies, is needed to ascribe any of the strains' any probiotic potential.

### 5.4.3 Genome sequencing of selected strains

To investigate whether further genetic variations exist among the *K. marxianus* strains, one strain from each of the two genetic *K. marxianus* groups was subjected to whole genome sequencing. For the *S. cerevisiae* strains the whole genome of all the ten isolates was subjected to sequencing. The sequencing is done in collaboration with the Edmund Mach Foundation, Trentino, Italy, and data elaboration is still ongoing.

### 5.4.4 Analytical method considerations Paper IV

The selection method and conditions are important for the isolation of microorganisms from fermented foods. The microorganisms isolated from an environmental sample are to some extent a reflection of the isolation methods used. As described in the background, some microbes may be very difficult to cultivate in a lab environment, since we do not have all methods or knowledge necessary for creating a suitable environment. Several different isolation media were used in this work, covering varying pH values and nutrient compositions. Generally, relatively rich media were used, suitable for enrichment of a broad range of microorganisms. Furthermore, both aerobic and anaerobic isolations were done, based on the assumption that the lower sediments in the goat milk fermentation may be more or less free of oxygen and may host anaerobic microorganisms. Irrespective of the efforts spent on achieving adequate isolation methods, it must be assumed that several microbes from the fermentations were not isolated, as is often the case when environmental samples are investigated by both cultivation-based methods and genetic methods such as metagenomics [200]. If there will be additional sampling of the Yaghnob milk fermentation in the future, it would be interesting to perform metagenomics studies to get a more complete picture of the microorganisms that are present.

Nevertheless, as the aim of this work was to find strong candidate strains for future food fermentation purposes, it is important that they can grow well in common lab media environments, and, for that reason, no major attempts were made to develop the isolation process further.

As mentioned, a low number of species were found in this work. The low species variation was considered to be a reflection of an actual low species variation in the Yaghnobi fermented goat milk, rather than being a result of our choice of method, since the isolation and cultivation conditions used in this study are known to be effective for isolation of several other species in other studies.

The genetic identification of isolates was done using well documented traditional methods, by sequencing the ITS1-4 region and additionally performing RFLP digestion of the ITS1-4 sequences with well-known restriction enzymes. The genetic variations found in the ITS1-4 sequences of some of the *Kluyveromyces marxianus* isolates was not explained by previous literature using those methods for identification [181-183]. Since the ITS1-4 region is known to be well conserved, this peculiar and specific alteration may indicate further genetic variations in the other parts of the genome. However, since the main focus of the present thesis is the study of microbial strains for application in food, the genetic overview of microbial strains has not been investigated in detail in this work.

### 5.4.5 Conclusions from Paper IV

From an evolutionary and ecological point of view, the genetic variations within the *K. marxianus* species and the phylogenetic results for the *S. cerevisiae* strains are intriguing and open further questions about microbial flow in the environment and how species evolve in remote areas. From that point of view, Paper IV may be the initial step towards further microbiological research on the microbiota in the geographical region of the Yaghnob valley.

The broad intra-species strain variation in each species led to the hypothesis that the high strain variation could compensate for the low species variation, allowing a phenotypically broad microbial composition in the fermentation, despite the fact that it hosts a low number of species.

From an application point of view, Paper IV revealed several strains with phenotypic traits indicating a potential suitability for food fermentation applications, including tolerance to several stress conditions. Whether those traits are necessary for an application, depends on which process is considered. For example, several strains degrade lactose and could potentially be used in milk fermentations, to achieve products with lowered lactose content for lactose-sensitive consumers. Some strains are also able to grow at high temperatures and at low pH, which can be applied in processes in order to minimize the risk of external contamination. Although it can be postulated that several of the strains identified in Paper IV could be promising for implementation in various food fermentation processes, application trials are needed for accurate conclusions.

## 5.5 Paper V – Utility of selected yeasts and bacteria in small scale food fermentations

### 5.5.1 Soy milk fermentations

The selection of strains from the mono-cultivations was done on the basis of volatile profile and viability during fermentation and incubation. The results in terms of viability for the LAB revealed that only the strains of *L. casei* were able to survive and grow readily in the soy milk substrate and able to thicken the product during fermentation. Among the yeasts, gas producing strains, including all *S. cerevisiae*, were excluded since gas production induced phase separation of the product (Figure 17) and if commercializing, this would cause post-packaging overpressure. The *P. fermentans* strains initially showed a decrease in viable cell count during fermentation and increased again after one week

of cold storage, reaching the initial inoculum level again at 192h. Due to this, those strains were not used in further studies. The *K. marxianus* strains showed the highest viability and increased by one log factor during the incubation, then slightly increasing or stabilizing the number of viable cells from the start of cold storage until the end, corresponding to one month. The pH of the cultures never reached below pH 4 for any of the tested strains, and the initial pH of the soy milk was pH 6.2 ± 0.1.

**Figure 17**. *Production of gas in the soy milk fermentations, depending on inoculum strains, resulting in mild to severe phase separation.*

The selection among the *K. marxianus* strains was done based on the mono-cultures' volatile profiles, focusing on a selection of strain with minimal ethyl acetate and carbon dioxide gas production, resulting in strains BL8 and AL3. Among the three *L. casei* strains, selection was based on their respective volatile profiles. A LAB with a low production of acetic acid and high production of 2,3-butanedione is desirable from a consumer perspective, which led to the selection of strain *L. casei* AB2. Further, the strain *Pichia kudriavzevii* TY1322 from our previous studies (Paper III) was included for its previously demonstrated positive effect on phytate degradation, together with absence of gas production and maintained viability during fermentation and cold storage in soy milk.

Various combinations of the selected strains were used for single co-cultures. The cultures were analyzed for viability of strains, physical appearance and phytate degradation. The LAB count increased over time in all cultures, reaching 8 log CFU/ml. The yeasts also increased in all cultures, but generally to a lower extent than the LAB, and reaching a slightly lower final level. The lowest yeast load was found in the culture combination **B**. Two yeasts were present n culture **D** and **E**, TY1322 and AL3 (in **D**) and TY1322 and BL8 (in **E**), and it was seen in both cultures that the relative number of TY1322 versus AL3 or BL8 decreased over time, indicating that TY1322 might need to be inoculated to a higher level than the *K. marxianus* strains in order to be able to compete for substrates and maintain a high viable count. The final pH of all LAB containing cultures fell between 4.0 and 4.6. No phase separation was seen after fermentation, but after one month of cold storage, slight gas production and phase separation was seen from cultures **A** and **D** (containing strain AL3). Similar results in viability, pH and stability were seen both at 37°C and 42°C.

The phytate analysis of the single co-cultures revealed between 0.4% and 17.9% phytate degradation in combinations **A**, **C** and **F** (cultures without strain TY1322) irrespective of incubation temperature. The highest phytate degradation for culture **B** (TY1322 and AB2) was found from the fermentations done for 4h at 42°C + 20h in refrigeration, reaching 52% phytate degradation. The combination **D**

(TY1322, AB2, AL3) reached 72.6% and combination **E** (TY1322, AB2, BL8) reached 71.2% degradation, both after fermentation for 5h at 37°C + 19h in refrigeration.

*L. casei* was able to thicken the product in all fermentations, but something related to strain AL3 seemed to induce an instability of the product after cold storage, seen by the product separating into a more solid phase and a clear liquid phase (similar to that in Figure 17). For this reason, the two final cultures were proposed to be **B** (*L. casei* AB2 and *P. kudriavzevii* TY1322), and **C** (*L. casei* AB2 and *K. marxianus* BL8). Those two combinations were used for triplicate fermentations, with adjusted inoculum levels, basically to improve the viable count of strain TY1322. The pH after 4h of fermentation at 37°C and 20h cold storage was in the AB2+TY1322 culture 4.01 ± 0.02, while in AB2+BL8 it was 4.50 ± 0.1. The CFU/ml at this time was for AB2 $7x10^8$ and $4x10^8$ in the TY1322 and BL8 combinations respectively. The yeasts showed a corresponding CFU/ml of $1x10^7$ for TY1322 and $2.5x10^7$ for BL8. There was basically no phytate degradation seen in culture **C**, and 63 ± 2% degradation was seen in culture **B**. The increase compared to the previous fermentation using the same strain combination is probably a reflection of the higher inoculum level of strain TY1322.

### 5.5.2 Fermentation of soy bean flour and buckwheat flour for decreased phytate content

One soy flour and one buckwheat flour were fermented using different LAB and yeasts for 18h at 37°C. The samples were subsequently analyzed for pH, total titratable acid and phytate content.

The soy flour fermentations showed 8.4 ± 0.06% phytate degradation in the un-inoculated negative control fermentation, 16.0 ± 0.06% from the fermentation containing AL2, TJA4B and TJA26, and 53.4 ± 0.03% – 77.6 ± 0.01% in the other fermentations. This clearly show the importance of including strain TY1322 in fermentations for reduced phytate content, as the culture without TY1322 only showed 16 ± 0.06% phytate degradation. It is also seen that the phytate degrading effect is improved in fermentations where TY1322 is combined with strain AL2 compared to fermenting alone. The final pH values in the soy flour fermentations were between 5.75 and 6.20, and the TTA was between 2 and 3.5 ml (after dilution adjustment) for all samples. The initial pH of soy flour + water was 6.35 ± 0.05, and the initial TTA was 2ml.

The buckwheat flour fermentations showed very high phytate degradation in all samples, 90.9 – 92.2%. Surprisingly, 91.1 ± 0.001% degradation was found in the negative control in which no microbial inoculation had been done. The final pH values in the buckwheat fermentations, including the negative control were between 4.7 and 5.9. The TTA was highest in the negative control. The initial pH of the buckwheat flour + water was 6.3 ± 0.1, and the initial TTA was 2ml.

In this study, soy flour without addition of microorganisms showed no degradation after incubation overnight at 37°C, while the buckwheat showed the opposite, with very high degradation in the negative control. This indicates that endogenous phytases and/or inherent microorganisms in the buckwheat flour have been activated after the addition of water and incubation at 37°C, while there is no such effect seen in soy flour. As in all flours (perhaps particularly in organically marked ones, where treatment with e.g. pesticides is prohibited), there is a natural presence of microorganisms. The presence of microorganisms, yeasts and bacteria, was also confirmed for both the soy and buckwheat flours by streaking the negative controls onto YPD and MRS agar plates. However, in the soy flour, endogenous phytases and/or inherent microorganisms with phytase activity were not present, or were not able to degrade the phytate.

The buckwheat fermentations were further used as a starter for bread production containing a mix of wheat and rye flour, water and salt. The phytate levels in all breads, including the negative control, were below the detection level, estimated to be between 0.1 – 0.15 µmol phytate / g (dw) bread. This shows that the phytate degrading effect seen from buckwheat flour, was also seen when the buckwheat fermentations were used as inoculum for bread doughs.

### 5.5.3 Wheat and rye bread production

For bread production, organically produced wheat, rye and whole wheat flours were used, together with salt, butter and tap water. The phytate analysis of doughs and breads revealed phytate degradation in all samples during the total 3h of proofing. The negative control showed the lowest phytate degradation, about 40%, while the yeasts showed between 43% and 59%. The commercial yeast JB had a degradation about 54%, while the best strain, being *Kluyveromyces marxianus* BL8, had 59% degradation. Surprisingly, the high phytase active yeast *Pichia kudriavzevii* TY1322 showed only about 48% phytate degradation. The reason for the unexpectedly low phytate degradation from strain TY1322 can potentially be ascribed to its poor performance as a single fermenting organism in this matrix. Strain TY1322 has shown immensely higher phytate degrading capacity compared to the commercial strain JB and the *K. marxianus* strains in lab media (data not shown). To improve the phytate degrading ability of TY1322, it was combined with a stronger leavening yeast, *K. marxianus* AL3. This resulted in 60% phytate degradation. For strain TY1322 to exhibit its full capacity, as seen in synthetic media, the conditions of the dough fermentation must be optimized further.

The leavening ability and the bread appearance were assessed by visual observation of the final breads, looking at the height, gas bubble formation inside the breads, and color of the breads. As seen in Figure 18, which show the single inoculum breads of TY1322 and JB, the mix of TY1322 and AL3 and the negative (not-inoculated) control. The leavening ability of TY1322 appears comparable to that of the commercial yeast under those conditions, although the gas bubble distribution from single TY1322 breads was less homogeneous than the JB and the mix (TY1322 + AL3) breads.


**Figure 18.** *Cross section of breads baked with no yeast (top left), commercial baker's yeast (top right), P. kudriavzevii TY1322 (bottom left) and a mix of P. kudriavzevii TY1322 and K. marxianus AL3 (bottom right).*

Further, there was no noteworthy difference in the smell or appearance of the breads, although there is room for improvement to achieve more appealing breads.

### 5.5.4 Analytical considerations and conclusion from Paper V

The results of this study are promising, but more work is needed for optimization of fermentation conditions and inoculum levels and, finally, evaluation in large scale set-ups will be needed to gain information about the strains' usability in full scale production. The promising effect of strain TY1322 seen in our previous work (Paper III) is also demonstrated in the soy milk and soy flour fermentations, indicating the potential application of the strain, and/or its phytase, as a phytate-degrading tool in food and feed production.

A greater pH lowering effect is often seen from lactobacilli fermentations, as they usually lower the pH to about 3.5 before they inhibit their own growth [201]. For buckwheat, the pH was lowered more in the negative control than in the fermented samples, indicating the presence of pH-lowering microorganism(s) in this sample. The growth of these possibly inherent microorganism(s) may be repressed in samples 1-6 as a result of the competition of substrates with the added strains in these samples. The results from the buckwheat fermentations cannot be used to draw any conclusions about the applied strains abilities, as a prominent effect was seen also in the negative control, i.e. neither of the microorganisms applied can be ascribed the phytate-degrading effect in those fermentations. However, from a nutritional point of view, the inherent ability of buckwheat flour to degrade phytate is interesting, and further studies should be done on the mechanisms behind this to learn whether it is an endogenous phytase or an inherent microorganism causing the phytate degradation.

The use of various microorganisms in soy milk clearly showed that different strains are differently suitable for this application. Gas producing strains that may be very useful in breads, were directly inappropriate for soy milk fermentation.

# 6. CONCLUSIONS

This work resulted in the isolation, identification and selection of yeast strains with a potential for future use as starters in food and feed fermentations.

Isolation of yeasts from a traditional goat milk fermentation from the Yaghnob valley, Tajikistan, resulted in the identification of several genetically and phenotypically varying strains belonging to four species. First, several strains showed resistance to various stress conditions, such as high temperatures and low pH. Second, the genetic variations seen in some of the strains, together with accompanying phenotypic variations, indicate that strains isolated from the Yaghnob fermented milk may have evolved separately from other strains of the same species. This is an opening for future studies of the microbiota in this geographical region, both from a genetic and a phenotypic point of view.

Mutagenesis of the already promising strain *Pichia kudriavzevii* TY13wt resulted in a considerably improved strain, *Pichia kudriavzevii* TY1322, with overall better phytate degrading ability, especially at high surrounding inorganic phosphate levels. Both TY13wt and TY1322 show release of phytase to the surrounding media. Using strain TY1322 in food and/or feed fermentations may aid phytate degradation, and potentially give an increase in the availability of minerals from the food/feed matrix.

Evaluation and comparison of methods used for phytase activity determination led to the suggestion that phytase activity should be determined on the basis of phytate (substrate) concentration, while total phosphatase activity is better determined by phosphate (product) concentration.

Selected yeasts were investigated for their applicability as starters in cereal-based fermentations together with selected lactic acid bacteria. The substrates used were soy milk and flours of soy, buckwheat and whole wheat, and analysis of volatile profiles, strain viability, phytate degradation and product appearance resulted in the suggestion of a few strains as potential future starter cultures. Considering application in food/feed products where the microorganisms are consumed alive, several strains presented in this work also show the ability to survive conditions mimicking those occurring in the gastrointestinal tract of humans and pigs.

The specific conclusions from this work are:

- Yeast strains *Pichia kudriavzevii* TY13wt and TY1322 are able to readily degrade phytate and release phytase enzymes to the surrounding medium (Papers I and III).

- The released phytase is a 3-phytase (Paper I), having pH optima at pH 3.5 and 5.5, and temperature optimum at 55°C (Paper III).

- Phosphatase activity indicator BCIP was a useful tool for visualization of phytase activity (Papers I and III).

- Mutagenesis yielded the yeast strain *Pichia kudriavzevii* TY1322, with improved phytate-degrading ability at high surrounding phosphate levels, compared to wild type strain TY13wt (Paper III).

- Yeast extract induces the release of non-cell-bound phytase from strains *P. kudriavzevii* TY13wt and TY1322 to the surrounding media (Paper I and III).

- Determining phytase activity by analysis of phosphate (product) concentration results in up to four times higher values compared to determination based on substrate (phytate) concentration analysis (Paper II).

- Isolation of yeasts from a traditional goat milk fermentation from the previously not explored geographical area of the Yaghnob valley revealed broad phenotypic intra-species strain variations, potentially indicating the isolation of some new species (Paper IV).

- According to the findings in Paper IV, a potential co-evolution is proposed between *S. cerevisiae* yeast strains and the humans living in the Yaghnob valley, based on the theory of a flux of strains between the food fermentation and the humans and their intestinal tract. The human intestinal tract is proposed as a reservoir of strains, allowing selection of strains able to survive through the intestinal conditions, supported by the isolation of temperature-tolerant (37°C and up) *S. cerevisiae* isolates and several strains tolerating low pH (pH 3) and the presence of bile (Paper IV).

- Soy milk fermentations showed that the three *Lactobacillus casei* strains AB1, AB2 and AB3 were the only bacterial strains able to thicken the product and maintain high viable counts. The yeast strains belonging to *K. marxianus, S. cerevisiae* and the strain *P. kudriavzevii* TY1322 maintained a high viable count throughout fermentation and storage (Paper V).

- Fermentation of soy milk and soy flour showed that cultures including the strain *P. kudriavzevii* TY1322 resulted in 53.4 – 77.6% phytate degradation, and the phytate degradation was improved by co-cultivation with *K. marxianus* strain AL2 or BL8 (Paper V).

- Buckwheat flour fermentation resulted in equally high phytate degradation in inoculated samples as in the not-inoculated negative control. It seems that the buckwheat contained native enzymes, or phytase active microorganisms, that were activated during the incubation and mediated the phytate degradation (Paper V).

# 7. FUTURE PERSPECTIVES

In this work, we observed a high phytate-degrading ability by strain *Pichia kudriavzevii* TY13wt, and even higher in the strain TY1322 achieved by mutagenesis of TY13wt. The effect was seen both in lab media and in fermentation trials in soy milk and soy flours. However, in following work, it would be interesting to study: i) whether the decrease in phytate gives an increased availability of the food minerals, and ii) whether the mineral metals from a food fermented with TY1322 are absorbed in the gastrointestinal tract to a higher extent than metals from food fermented with other strains.

A more detailed understanding of the mechanisms behind the release of phytase seen from strains TY13wt and TY1322 would be of great interest in terms of trying to further improve the release.

Further optimization of the fermentation conditions, and strain combinations, is needed to yield improved availability of minerals from plant-based foods and a food product that is appealing to the consumer.

It would be very interesting to again isolate yeasts from a new sample of the goat milk fermentation in the Yaghnob valley, to see whether the species composition is still the same and to isolate a larger number of strains to acquire a greater number of strains to study. A deeper understanding of the microbial evolutions and ecology, and of the interactions between the microorganisms and the human population in the Yaghnob valley would be interesting.

# ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisors Thomas Andlid, Paola Mattarelli and Jenny Veide Vilg for our time together. I am very grateful for all interesting discussions we have had and for your support during my time as a PhD student. Thank you to my LiFT mentor, Ulla Svensson, for giving me another perspective on things, for interesting discussions about research, career possibilities and life in general.

Thank you to my amazing office companions Lillie and Niklas for all discussions and jokes we have shared, and for always letting me know when you have found left-over fika in the coffee room! Thank you to Annette and Nisse for having such big hearts, for always caring, and for always having time to talk. Thank you to Hanna H and Cecilia T for being such fantastic friends, both caring about my wellbeing, and complementing my outfits! To my fellow Food Science PhDs in the LiFT graduate school; Cecilia T, Karin L, Nikul, Malin, Andy, Karin J, Niklas, thank you for making the courses and travels so fun. Thank you to everyone who has come and gone in the micro-lab over the last years, making the time in the lab more fun. Thank you Jauquline, I could not have wished for a better master student and friend in the lab. Thank you to Mi-Lo, Helena and Anna for keeping track of everything that I have not kept track of, and for the constant storage of cookies for "night work emergencies". Thank you Assi for your support and help. An extra big thank you to Jenny Veide Vilg, your support has meant so much to me during this time, and I am glad to have had you as my friend, mentor and colleague since I started as a project assistant at IndBio. Thank you to all other, new and old, colleagues at Food and Nutrition Science for making my PhD time such a great experience.

Thank you to the people at IndBio for opening my eyes for the PhD life, and for the nice company at all the yeast conferences.

Thank you to everyone who has been part of my research experience in Italy, people at the University of Bologna (in Bologna and Cesena), at the Fondazione Edmund Mach in San Michele, and at the University of Verona. Special thanks to Paola and Monica for always having the door open for my questions, and to Paola and her family in Verona for welcoming me in their home and making me feel as part of their family.

Thank you to Christer Larsson and Nathalie Scheers for reviewing my thesis and coming with important and interesting questions.

Finally, I would like to thank my family and friends for supporting me and being part of my life. Although I love the research life, I also highly value my time outside of the office and lab, and this time has been fantastic since I have spent it with you! Thank you to my sister Malin, mom Cattis and dad Leif for always being there for me and loving me. Thank you to my fantastic friends for filling my life with happiness and laughter; Henny with family, Sofie with family, Jonna, Fia, Johanna A, Hanna H, Cecilia T, Helena, Johanna G, Rebecca, Matilda and Sara. Especially thank you to Hanna N, Luca and Hilda for making my time in Italy the best it could ever be! Thank you to Alkistis and Andrea for my unique English accent, and for our fun reunions over the last years.

Thank you to everyone else for having been part of this journey!

Without funding this research could not have been done. I therefor thank the Swedish Research Council Formas (project nr 222-2012-1064) for funding this research. I also gratefully thank Stiftelsen Olle Engkvist Byggmästare for the grant covering my travel and housing expenses during my research visit in Italy.

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