Phytase active yeasts isolated from bakery sourdoughs

Nuobariene, Lina; Arneborg, Nils; Hansen, Åse Solvej

Published in:
9th Baltic Conference on Food Science and Technology "Food for Consumer Well-Being"

Publication date:
2014

Document version
Publisher's PDF, also known as Version of record

PHYTASE ACTIVE YEASTS ISOLATED FROM BAKERY SOURDOUGHS

Lina Nuobariene, Nils Arneborg, Åse Solvej Hansen

Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg, Denmark, e-mail: aiva@food.ku.dk

Abstract

Nowadays, consumption of whole-grain bread is gaining popularity across the world due to its many health promoting effects. Despite that whole grains contain 20–70% of the daily requirements of the minerals, their absorption in human gut are very low due to antinutritional phytate. To increase the bioavailability of minerals, enzymatic degradation of phytate is needed. The aim of this study was to isolate and identify phytase active yeasts from sourdoughs. The use of selected microorganisms with high phytase activity could find use as starter cultures to improve mineral bioavailability in whole-grain bread fermented in a short time as alternative to sourdough bread. In addition, purification and characterization of phytase produced by *Saccharomyces cerevisiae* was done. The screening of phytase-positive strains was carried out at temperature of 30 °C and pH 5.5, conditions optimal for leavening of bread dough. To investigate whether the yeasts produce phytases selective defined minimal medium was developed. Phytase activity was determined colorimetrically by measuring the inorganic phosphate released by enzyme. Specific extracellular phytase activities of yeasts were at least 20-fold higher than the intracellular activities. The highest activities were observed in two *Saccharomyces cerevisiae* isolates, i.e. L1.12 (10.6 U $(10^{10}$ CFU$)^{-1}$) and L6.06 (8.2 U $(10^{10}$ CFU$)^{-1}$). The purified phytase produced by *S. cerevisiae* was most active at pH 4.0 and 35°C. This phytase is quite stable at pH range from 3.5 to 5.5 and temperature range from 25 °C to 40 °C.

Keywords: phytase, sourdough, yeast, *Saccharomyces cerevisiae.*

Introduction

Major epidemiological studies have shown that consumption of whole-grain foods is protective against the development of several chronic diseases. Whole-grains have been found to help regulation of blood glucose and protect against type 2 diabetes (de Munter et al., 2007) as well as reduce the risk of obesity (van de Vijver et al., 2009). The importance of whole-grain foods intake on blood glucose and weight regulation is considered as a result of the content of dietary fibre and magnesium in whole grains. Other studies concern the contribution of dietary fibre, folate and, to a certain extent, vitamin B_6 and magnesium from whole-grain foods as protective components against cardiovascular diseases and certain type of cancer (Slavin, 2004).

Besides dietary fibre, the whole-grain foods are also important source of minerals. It was shown that 100 g of whole grains contain 20–70% of the daily requirements of the minerals, such as iron, zinc, magnesium, potassium, phosphorus and manganese (Cordain, 1999). However, most of the minerals in cereals are complexly bound to phytic acid as phytate, consequently reducing their bioavailability (Kumar et al., 2010).

Iron deficiency is the most common nutrient deficiency, widespread both in western and developing countries (WHO, 2002). Zinc represents the other nutritionally significant mineral that has been associated with phytate inhibition effect.

To increase the bioavailability of minerals from a given food, enzymatic degradation of phytate and its dephosphorylated isomer IP5 (inositol pentaphosphate) is needed (Sandberg et al., 1999). Efficient reduction of phytate can be achieved by enzymatic degradation during food processing, either by increasing the activity of endogenous phytase, or by addition of phytase-active yeast, lactic acid bacteria, or other microorganisms.

Cereals exhibit high phytase activities with values ranging from approx. 900 to 2900 U kg^{-1} dry matter in wheat grain, and from 4100 to 6100 U kg^{-1} dry matter in rye grain (Eeckhout, Depaepe, 1994; Greiner, Egli, 2003). However, the activities in wheat were considered insufficient to notably improve the mineral bioavailability in whole-grain wheat bread (Türk, Sandberg, 1992). Several studies were carried out on phytase activity from baker's yeast (Andlid et al., 2004; Türk, Sandberg, 1992). They showed that only marginal amount of phytate was degraded during 3 h fermentation.

From an industrial point of view, the extracellular phytase activity would be more important for bread making than the intracellular phytase activity, because cells of the yeast should be intact in dough in order to ensure a good fermentation. In this case, the intracellular phytase will not have access to phytate in the dough. So far, no high phytase-active yeasts were identified for bread industry in order to increase mineral bioavailability.

The aim of this study was therefore to isolate and identify yeasts from different commercial sourdoughs and to study their phytase activity under conditions optimal for leavening of wheat bread dough (30 °C and pH 5.5) in order to identify species and/or strains with high phytase activities that might be used in baking industry. Further, purification and characterization of high-active extracellular phytase produced by generally recognized as safe (GRAS) yeast *Saccharomyces cerevisiae* was done.

Materials and Methods

Microorganisms. Yeasts tested for phytase activity were isolated from seven Lithuanian sourdoughs, further purified and identified (Nuobariene et al., 2012). *Saccharomyces cerevisiae* L1.12 isolated from rye sourdough and identified as a phytase-positive

yeast strain was used for further phytase production and purification.

Growth test for phytase-active yeasts. To check the ability of yeasts to synthesize phytase, yeast strains were cultivated on Defined Minimal Medium (Delft) plates supplemented with phytic acid dipotassium salt (P5681, Sigma-Aldrich, Broendby, Denmark) as the only phosphorus source (Delft+Phy). As control, yeast strains were cultivated on phosphate-containing (Delft+P, positive control) and phosphate-free (Delft-P, negative control) medium (Nuobariene et al., 2012). Growth tests were performed both on solid and in liquid medium.

Phytase assay. Extracellular phytase extracts were prepared according to Nuobariene et al. (2011). Phytase activities were determined as described previously (Nuobariene et al., 2012). Briefly, 10 µl of enzyme extract was added into pre-incubated 40 µl 3 mM phytic acid dipotassium salt solution, gently mixed by inverting tube and incubated at 30 °C. Samples were taken every 15 min (0, 15, 30, and 45 min) during assaying. The reaction was stopped by adding 50 µl 10% TCA (trichloroacetic acid solution). A separate enzyme blank was prepared from sodium acetate/HCl buffer mixed with enzyme extract and TCA. Substrate blank was prepared from substrate solution mixed with TCA. To determinate the content of liberated inorganic phosphate during phytase activity freshly prepared acid molybdate solution was used in colorimetric analysis.

One unit of phytase activity was defined as the amount of phytase that liberates 1 µmol inorganic phosphate per minute from 3 mM phytic acid dipotassium salt at pH 5.5 and at 30 °C.

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

After cultivation, the yeast cells from the fermented broth were removed by centrifugation (5000 *g* for 10 min, 4 °C); culture supernatant with extracellular protein fractions was collected and filtered (0.2 µm Minisart filters; Bie & Berntsen, Herlev, Denmark). 100 mL of prepared extracellular protein extract, as described above, was desalted and concentrated to 40 mL using Centriprep 3K centrifugal filter device with Ultracel YM membrane (the nominal molecular weight limit 3000).

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

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

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

The effect of pH on phytase stability was studied by incubating the purified enzyme at 30°C for 1, 2 and 4 h at various pH values ranging from 3.0 to 7.0 followed by measuring the residual activity under standard assay conditions.

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

Thermostability of the phytase was tested by incubating enzyme extract without substrate addition at a temperature range of 25 °C to 65 °C for 2 h and samples were taken every 10 min. Residual activities where then measured as indicated above, at a fixed temperature of 30 °C and pH 5.5.

Results and Discussion

Phytase-active yeasts from sourdough. The number of yeasts in all tested sourdoughs was about the same and ranged from 6.7 to 7.9 log CFU (colony forming units) g^{-1} sample. In total, 140 yeast colonies were isolated, purified and subjected to the rep-PCR analysis. Based on results from cluster analysis, isolates were grouped according to their fingerprint pattern (Nuobariene et al., 2012). For a representative number of isolates within each group; i.e. 11 isolates in total, the identification to species level were further done by sequencing of the D1/D2-region of the 26S rRNA gene. Based on results from sequencing, two yeast species isolated from seven different sourdoughs were identified, i.e. *S. cerevisiae* and *Pichia kudriavzevii* (formerly named as *Issatchenkia orientalis*, anamorph *Candida krusei*). A pure culture of *S. cerevisiae* was found in five out of seven tested sourdoughs with fermentation temperature of 30–35 °C, while *P. kudriavzevii* was the dominant

species in other two sourdoughs with fermentation temperature of 24–26 °C.

To check the ability of identified 11 yeast strains to produce phytase they were cultivated on/in solid/liquid minimal Delft medium supplemented with phytic acid dipotassium salt as the only phosphor source. All tested strains grew very well on/in Delft+Phy medium which indicates that tested strains produce phytase (Figure 1).

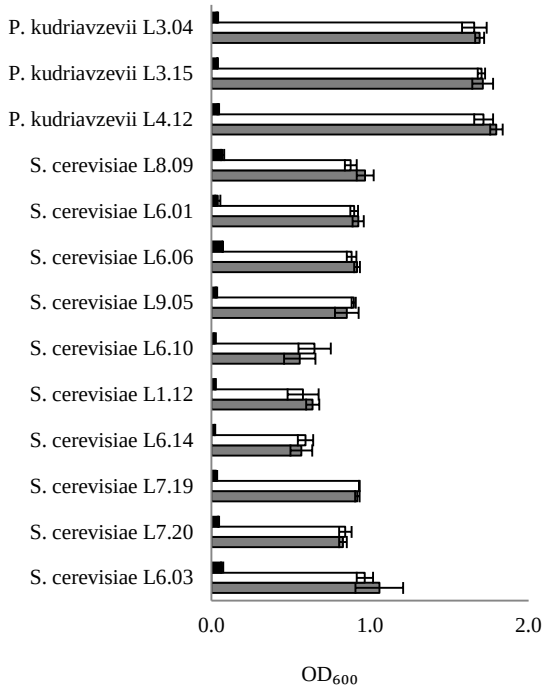

Figure 1. Optical density values of yeast cell growth at 30 °C in experimental media after 48 h incubation

Black bars-Delft-P medium, white bars – Delft+P medium, grey bars – Delft+Phy medium

However, from growth test, based on monitoring of cell growth in phytate rich environment, it is impossible to predict which yeast strains will have higher or lower activities (Nuobariene et al., 2011). Therefore, it is essential also to perform phytase activity assay on each strain.

The highest specific extracellular activity of all isolates was observed in *S. cerevisiae* L1.12 with a value of 10.6 U $(10^{10}$ CFU$)^{-1}$, followed by *S. cerevisiae* L 6.06 with a value of 8.2 U $(10^{10}$ CFU$)^{-1}$ (Figure 2). Extracellular specific activities among other *S. cerevisiae* strains differed dramatically and ranged from 0.9 to 7.3 U $(10^{10}$ CFU$)^{-1}$. The specific extracellular phytase activity values within three *P. kudriavzevii* strains varied between 1.1 and 4.6 U $(10^{10}$ CFU$)^{-1}$.

Cultivation of *S. cerevisiae* L1.12 for phytase production. The activity of phytase in response to phytic acid dipotassium salt and the growth of *S. cerevisiae* L1.12 during 72 h of cultivation are shown in Figure 3. The yeast cells reached their stationary growth phase after 42 h of cultivation. Increase in phytase activity during the exponential growth phase was very slow.

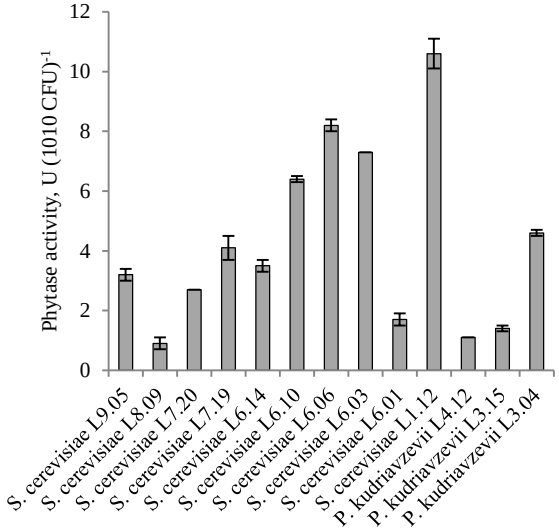

Figure 2. Specific extracellular phytase activity from yeast strains of *S. cerevisiae* and *P. kudriavzevii*

However, rapid increase in phytase activity was observed in the late exponential growth phase (36–42 h of cultivation) and was continuously increasing during early stationary growth phase. It can be seen from Figure 3 that the maximum extracellular volumetric phytase activity (0.062±0.005 U mL^{-1}) was obtained when yeast cells reached the stationary phase and remained almost constant for the next 12 h of cultivation. Afterwards, slight decrease in phytase activity was observed in late stationary growth phase. This finding is in agreement with Quan et al. (2001) findings which showed that phytase production by *Candida krusei* occurred in the late stage of exponential growth phase and that phytase activity increased gradually with increasing incubation time.

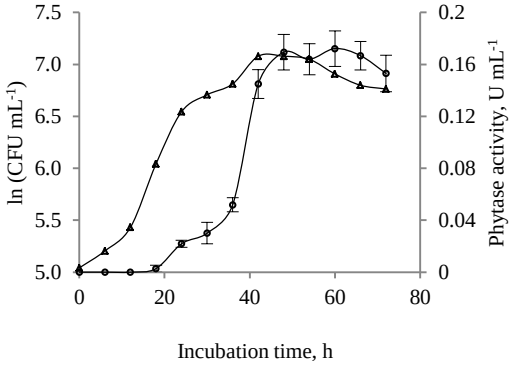

Figure 3. Phytase activity and yeast growth at different time points

Circles – phytase activity, U mL^{-1}; triangles – yeast growth, ln (CFU mL^{-1})

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

However, phytase produced by yeast species, mentioned above mostly are attributed as potential for commercial application in the feed industry. Therefore, purification and characterization of *S. cerevisiae* L1.12 isolated from sourdough which may be an ideal candidate for improving mineral bioavailability in whole grain is of importance.

Table 1

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

Purification steps	Total activity	Total protein	Specific activity	Yield
	U	mg	U mg^{-1}	%
Culture supernatant	24.6	4.62	5.3	100
Ultrafiltration	24.5	4.12	6.0	99.7
Con-A Sepharose	0.9	0.02	42.9	3.7

The results of phytase produced by *S. cerevisiae* L1.12 purification are summarized in table 1. The first purification step, ultrafiltration, leads to an increase in purity of only 1.1-fold but recover nearly all the protein from the original extract. Second purification step, a Con-A affinity chromatography, took advantage of the presence of high glucose and mannose side chains in phytase. This resulted in separation of phytase from major non-glycosylated proteins. The phytase was purified 8.1-fold, with a yield of 3.7% to a maximum specific activity of 42.9 U mg^{-1}.

Effect of pH and temperature on phytase activity. Figure 4 demonstrate the effect of pH variation on phytase activity produced by *S. cerevisiae* L1.12 at 30 °C (relative activities).

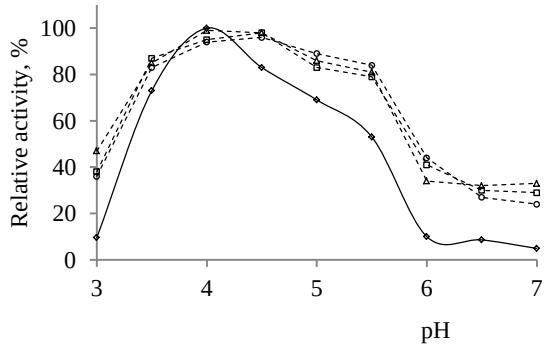

Figure 4. Effect of pH on the relative activity (solid line) and stability (dashed lines) of extracellular phytase from *S. cerevisiae* L1.12

The optimum pH at 4.0 for purified phytase is in agreement with Vohra and Satyanarayana (2001). In et al. (2009) reported that yeast phytase had sharp decline in activity as the pH value moved towards the neutral range 0.5-units from optimum. In contrast, we observed less than 15% decrease in relative phytase activity when the pH value was moved 0.5-units from optimum towards the neutral or acidic range. This can be explained by the fact that the pH of rye dough is about 4.3–4.6, except for the sourdough where pH is around 3.7 (Nielsen et al., 2007) and that phytase purified from rye sourdough yeast is well adapted for current conditions. However, in the whole wheat sourdough where pH varies between 5.3 and 5.5 the activity of yeast phytase seems to be only about 50–70% of optimum activity.

The effect of pH on the enzyme stability indicates that the extracellular phytase produced by *S. cerevisiae* isolated from rye sourdough is active in the pH range 3.5–5.5, with more than 80% of the initial activity remaining. However, sharp decline in activity was observed when the enzyme was incubated at pH 6.0 as well as in more neutral pH.

Figure 5 shows the effect of temperature variation on purified phytase activity at pH 5.5 (relative activities). Maximum purified extracellular phytase activity was exhibited at 35 °C with 98% and 78% of residual activity at temperature of 30 °C and 40°C, respectively. The residual activity at 55 °C was found to be only 7%. Comparison of the optimal temperature of phytase suggested that the phytase produced by *S. cerevisiae* L1.12 isolated from rye sourdough had slightly lower optimal temperature than those of *S. cerevisiae* isolated from the mash of traditional Korean yakju (40 °C) (In et al., 2009).

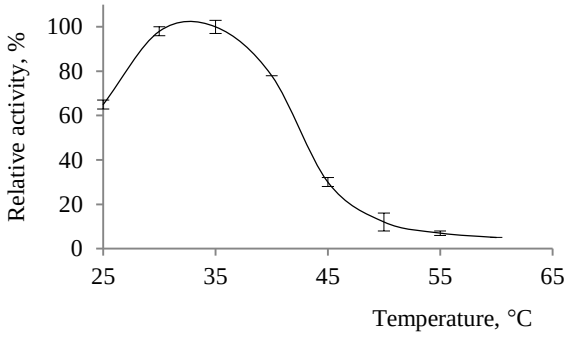

Figure 5. Effect of temperature on the relative activity of extracellular phytase from *S. cerevisiae* L1.12

Effect of temperature on the stability of purified phytase during different incubation time is summarized in table 2. During an incubation period of 2 h, no loss of phytase activity was observed at temperature below 40 °C, while about 8% of the activity was maintained after 15 min incubation at 55 °C. No phytase activity was detected when enzyme was incubated at 65 °C (Table 2). Although phytase produced by *S. castellii* and *Pichia anomala* have been reported to be

thermostable at temperature above 70 °C (Segueilhaet al., 1992; Vohra, Satyanarayana, 2001), our findings are in agreement with those of Quan et al. (2002). They observed that phytase produced by *C. krusei* is easily inactivated at temperature above 50 °C. Further, our tested phytase at 45 °C displays an increasing trend in activity after 2 h of incubation. Even though the phytase of *S. cerevisiae* L1.12 has a low activity at 45 °C, seems that it is very stable at this temperature.

Table 2

Effect of temperature on the stability of extracellular phytase from *S. cerevisiae* L1.12

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

*nd – not detected

Conclusions

The highest extracellular phytase activities were observed in two *S. cerevisiae* strains; i.e. L1.12 and L6.06 and therefore they may be the best candidates for improving bioavailability in whole-grain bread.

Concanavalin A chromatography process was successfully implemented to purify phytase from *S. cerevisiae* L1.12 which resulted in 8.1-fold purification with a yield of 3.6% to a maximum activity of 42.9 U mg^{-1}. The optimum pH and temperature values for purified phytase were found to be 4.0 and 35 °C, respectively.

Acknowledgment

This project was financed be a PhD scholarship from faculty of LIFE Science, University of Copenhagen, Denmark. The technical assistance of Basheer Yousef Aideh is deeply appreciated.

References

1. Andlid T. A., Veide J., Sandberg A. S. (2004) Metabolism of extracellular inositol hexaphosphate (phytate) by Saccharomyces cerevisiae. *International Journal of Food Microbiology*, Vol. 97(2), p. 157–169.
2. Cordain L. (1999) Cereal grains: humanity's double edged sword. *World Review of Nutrition and Dietetics*, Vol. 84 p. 19–73.
3. de Munter J. S. L., Hu F. B., Spiegelman D., Franz M., Van Dam R. M. (2007) Whole grain, bran, and germ intake and risk of type 2 diabetes: A prospective cohort study and systematic review. *Plos Medicine*, Vol. 4(8), p. 1385–1395.
4. Eeckhout W., Depaepe M. (1994) Total Phosphorus, Phytate-Phosphorus and Phytase Activity in Plant Feedstuffs. *Animal Feed Science and Technology*, Vol. 47 (1–2), p. 19–29.
5. Greiner R., Egli I. (2003) Determination of the activity of acidic phytate-degrading enzymes in cereal seeds. *Journal of Agricultural and Food Chemistry*, Vol. 51(4), p. 847–850.
6. In M. J., Seo S. W., Kim D. C., Oh N. S. (2009) Purification and biochemical properties of an extracellular acid phytase produced by the Saccharomyces cerevisiae CY strain. *Process Biochemistry*, Vol. 44(1), p. 122–126.
7. Kumar V., Sinha A. K., Makkar H. P. S., Becker K. (2010) Dietary roles of phytate and phytase in human nutrition: A review. *Food Chemistry*, Vol. 120(4), p. 945–959.
8. Li X. Y., Chi Z. M., Liu Z. A., Li J., Wang X. H., Hirimuthugoda N. Y. (2008) Purification and characterization of extracellular phytase from a marine yeast Kodamaea ohmeri BG3. *Marine Biotechnology*, Vol. 10(2), p. 190–197.
9. Nielsen M. M., Damstrup M. L., Dal Thomsen A., Rasmussen S. K., Hansen A. (2007) Phytase activity and degradation of phytic acid during rye bread making. *European Food Research and Technology*, Vol. 225(2), p. 173–181.
10. Nuobariene L., Hansen A. S., Arneborg N. (2012) Isolation and identification of phytase-active yeasts from sourdoughs. *Lwt-Food Science and Technology*, Vol. 48(2), p. 19–196.
11. Nuobariene L., Hansen A. S., Jespersen L., Arneborg N. (2011) Phytase-active yeasts from grain-based food and beer. *Journal of Applied Microbiology*, Vol. 110(6), p. 137–1380.
12. Quan C. S., Fan S. D., Mang L. H., Wang Y. J., Ohta Y. (2002) Purification and properties of a phytase from Candida krusei WZ-001. *Journal of Bioscience and Bioengineering*, Vol. 94(5), p. 41–425.
13. Quan C. S., Zhang L. H., Wang Y. J., Ohta Y. Y. (2001) Production of Phytase in a Low Phosphate Medium by a Novel Yeast Candida Krusei. *Journal of Bioscience and Bioengineering*, Vol. 92(2), p. 15–160.
14. Sandberg A. S., Brune M., Carlsson N. G., Hallberg L., Skoglund E., Rossander-Hulthen L. (1999) Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans. *American Journal of Clinical Nutrition*, Vol. 70(2), p. 24–246.
15. Segueilha L., Lambrechts C., Boze H., Moulin G., Galzy P. (1992) Purification and Properties of the Phytase from Schwanniomyces-Castellii. *Journal of Fermentation and Bioengineering*, Vol. 74(1), p. 7–11.
16. Slavin J. (2004) Whole grains and human health. *Nutrition Research Reviews*, Vol. 17(1), p. 9–110.
17. Türk M., Sandberg A. S. (1992) Phytate Degradation During Breadmaking - Effect of Phytase Addition. *Journal of Cereal Science*, Vol. 15(3), p. 28–294.
18. van de Vijver L. P. L., van den Bosch L. M. C., van den Brandt P. A., Goldbohm R. A. (2009) Whole-grain consumption, dietary fibre intake and body mass index in the Netherlands cohort study. *European Journal of Clinical Nutrition*, Vol. 63(1), p. 31–38.
19. Vohra A., Satyanarayana T. (2001) Phytase production by the yeast, Pichia anomala. *Biotechnology Letters*, Vol. 23(7), p. 551–554.
20. WHO (2002) The World Health Report. Reducing Risks, promoting Health. [accessed on 27.02.2014]. Available: http://www.who.int/whr/2002/en/whr02_en.pdf?ua=1

ISSN 2255-9809

LATVIA UNIVERSITY OF AGRICULTURE

9th Baltic Conference on Food Science and Technology
"Food for consumer well-being"

FOODBALT 2014

Conference Proceedings

Jelgava, May 8–9, 2014