FOOD and HEALTH

FOOD and HEALTH
E-ISSN 2602-2834

Food Health 6(2), 117-127 (2020) • https://doi.org/10.3153/FH20013 Research Article

Extracellular phytase activites of lactic acid bacteria in sourdough mix prepared from traditionally produced boza as starter culture

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Cite this article as:
Doğan, M., Tekiner İ.H. (2020). Extracellular phytase activites of lactic acid bakteria in sourdough mix prepared from traditionally produced Boza as starter culture. *Food and Health,* 6(2), 117-127. https://doi.org/10.3153/FH20013

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Submitted: 01.11.2019
Revision requested:
no revision requested
Last revision received: -
Accepted: 23.01.2020
Published online: 27.02.2020

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ABSTRACT

Fermentation using Lactic Acid Bacteria (LAB) and LAB species can exhibit extracellular activities such as decreasing of antinutritional factors, in particular phytic acid (PA) or phytate. The objective of this study was to assess extracellular phytase activities of LAB in sourdough mix prepared from traditionally produced boza as starter culture. To do this, thirthy-five boza samples were collected from Central Anatolia, Marmara and Eastern Anatolia regions in Turkey to be used as starter culture for preparing sourdough mix. In each mixture, LAB strains and phytase (+) ones were screened by culture-based examination, characterized by VITEK® MS, and extracellular phytase activity of each LAB strain was determined by spectrophotometry. Overall, 29 presumptive strains of LAB were isolated. Of them, 21 were found to be phytase (+). The average extracellular phytase activity was 656.8±188.1 U//mL, and a *Pediococcus pentosaceus* EK1 isolate showed the highest activity as 1285.5 U/mL. In conclusion, the traditionally produced bozas have been found as potential starter culture reservoirs for sourdough fermentation with significantly higher extracellular phytase activities, thus challenging opportunitites to lower antinutritional factors, in particular phytic acid (PA) or phytate in the foods for the consumers.

Keywords: Boza, Fermentation, Health, Lactic Acid Bacteria, Phytic acid, Phytase, Sourdough

Introduction

Cereals and cereal-based products are a good source of phenolic compounds, lignans, phytosterols, phytic acid, fiber, vitamins, minerals and other biologically active compounds. However, they are rich in phytic acid (myo-Inositol (1, 2, 3, 4, 5, 6)-hexakisphosphate, InsP6) or salts, also known as phytates. Phytic acid (PA) is a naturally occurring compound found in all seeds and cells of plants. It accumulates up to seed ripening during development, and phosphorus is its main form of storage accounting for 60% of total phosphorus content in cereals, legumes, nuts and oil seeds (Lott et al., 2000; Grases et al., 2017).

Many studies show that a diet based on foods with high phytate content may cause anemia and deficiencies in mineral absorption. Phytate levels can be reduced by phytases, which are the valuable enzymes by phtate hydrolysis. Phytate hydrolysis produces low myo-inositol phosphates by enzymatic degradation. This enzymatic degredation can be achieved by increasing activity of phytase, or adding phytase active microorganisms (Hurrell et al., 2003; Shi et al., 2004; Nuobariene et al., 2015; Moll & Davis, 2017).

Traditional cereal-fermented products are widely consumed all over the world, in particular in Asia and Africa. For instance, boza is one of the well-known fermented cereal-based beverages. To make boza, a ground amount of different cereals such as millet, corn, rice, rye, oats, and wheat is cooked with water, and the mixture is allowed for fermentation by adding sugar. There exist diverse microorganisms in the boza occurring from raw materials, production process and storage conditions. On the other hand, the dominant microflora mainly include LAB (Osimani et al., 2015; Petrova & Petrov, 2017).

The food industries and scientific related areas are emphasizing the capacity of fermentation using LAB species to improve the nutritive quality of cereals and cereal-based foods by decreasing of some antinutritional factors such as PA or phytate, tannins and enzyme inhibitors. The activities of LAB sepcies during cereal fermentation produce a broad range of metabolites and compounds, including organic acids, exopolysaccharides, antimicrobial compounds, and useful enzymes LAB species encoding phytases may be utilized as starter culture suitable for legume and cereal fermentations (Sumengen et al., 2013; Rollán, Gerez, & LeBlanc, 2019).

Only a few strains of LAB have been reported to show intracellular phytase activity (Lopez et al., 2000; De angelis et al., 2003; Reale et al., 2004), whereas there have been other studies reporting that LAB involved in sourdough fermentation exhibits extracellular phytase activities (Cizeikiene et al., 2015; Karaman et al., 2018; Yildirim and Arici, 2019).

In this study, we aimed to assess extracellular phytase activities of LAB in sourdough mix prepared from traditionally produced boza as starter culture.

Materials and Methods

Collection of Boza Samples

During the year 2019, thirty-five traditionally produced boza samples were collected from the boza producers located in the Regions of Marmara (n=15), Central Anatolia (n=10) and Eastern Anatolia (n=10) in Turkey. All the collected samples were taken to the laboratory under sterile conditions at 4°C until further analysis.

Chemicals and Reagents

The chemicals and reagents used in this study were DeMan, Rogosa and Sharpe (MRS) agar (Merck 1.10660, Germany), MRS Broth (Merck 1.10661), M17 agar (1.15108 Merck) and M17 broth (Merck 1.15029) for cultural examination, preidentification and storage of LAB strains from sourdoughs; crystal violet, safranin and lugol dyes for biochemical and morphological tests; physiological saline solution (PSS) (8.5 g NaCl dissolved in water, autoclaved 15 minutes at 121°C, and cooled to room temperature) for dilution, and 20% glycerol (Merck 10494) for store of culture, respectively. 0.1% sodium phytate (Sigma Aldrich 68388, Germany) and 0.2% glucose to MRS/M17 Broth medium (52.2 g/L) was used for phytase (+) LAB strains (Media with a pH of 6.2 sterilized at 121°C for 15 minutes at 1.2 atm. In the identification of phytase (+) LAB strains, *Escherichia* (*E.*) *coli* ATCC® 25922™ for positive testing control, and 1 µL alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix solution for crystallization of the strain to be tested according to the instructions by VITEK® MS (bioMerieux, Marcy I'Etoile, France). Finally, 100 mM sodium acetate (Sigma Aldrich W302406) - acetic acid (Sigma Aldrich W200603) buffer, and 500 µl of 10% (w/v) trichloroacetic acid solution (TCA) (Sigma Aldrich T3399) for determination of extracellular phytase activities of the phytase (+) LAB. All the chemicals and reagents were prepared according to the Instructions by ISO 11133 (2014), Songré-Ouattara et al. (2008), Raghavendra & Halami (2009), and Dubois et al. (2012).

Preparation of Sourdough

Ten grams of boza sample were initially mixed with 150 g of whole-wheat flour, 2 g of table salt and 350 mL of drinking water in a mixer for 5 minutes. Subsequently, the blend was allowed for fermentation at 35°C for 24 hours. At the end of the duration, 50 g of whole-wheat flour and 25 mL of drinking water more were added to the dough, the dough was kneaded for 1 minute, refreshed, and left to ferment again at

35°C during 10 days. At every 24 hours, 50 g whole-wheat flour and 25 mL of drinking water were added to the dough, and the dough was kneaded for 1 minute as previously suggested by Menteş al. (2007).

Culture-Based Analysis and Isolation of LAB Strains

The cultural examination of the suspected LAB strains were made according to the Instructions by ISO 11133 (2014) and ISO 6887-6 (2013). Ninety mL of PSS was added to 10 grams of the homogenized and fermented sample to prepare serial dilutions of 10^{-2} and 10^{-3}, respectively. After that, 1 mL of the diluted suspension was transferred to MRS agar or M17 agar, allowed for incubation at 37°C for 24-48 hours (NÜVE EN-500, Ankara, Turkey). At the end of the incubation, suspected LAB colonies were examined morphologically under microscope. To ensure the purity of the suspected colonies, MRS and/orM17 were inoculated into broth tubes, and activated at 37°C for 24 hours under aerobic/anaerobic conditions. Then, the matte-cream colored colonies were evaluated as LAB strains. Dyeing was performed for pure cultures; Gram (+), cocci and rods were determined under the light microscope, and followed by the catalase test. Those negative for catalase test were selected.

Detection and Enumeration of Phytase (+) LAB Strains

To detect phytase (+) LAB strains, sodium phytate MRS/M17 broths were prepared to inoculate the suspected LAB strains with 200 µl active cultures. Then, the suspensions were allowed for incubation at 37°C for 24 hours (NÜVE EN-500, Ankara, Turkey). After incubation, 100 µl of the incubated culture were inoculated into MRS/M17 agar containing sodium phytate, and left for incubation at 37°C for 24 hours, 48 hours, and 96 hours. Phytase production of the strain was determined by production of clear zones (in millimeters) around the colonies on the sodium phytate containing medium as previously described by Bae et al. (1999) and Songré-Ouattara et al. (2008). For enumeration of phytase (+) LAB strains, 100 µl of the MRS/M17 broth suspension containing sodium phytate were pipetted, and transferred to an Eppendorf tube containing 900 µl of PSS to obtain a diluted culture of 10^{-1}. Following that, 100 µl of the homogenized sample were taken, and serial dilutions from 10^{-1} to 10^{-7} were prepared. Among these dilutions, 100 µl of each dilution from 10^{-4} to 10^{-7} were spreaded on MRS/M17 agar. After incubation at 37°C for 24 hours, the viable bacterial were counted in 30-300 colony-containing petri dishes (Songré-Ouattara et al., 2008; Tharmaraj and Shah, 2003).

Characterization of Phytase (+) LAB Strains Using MS

The phytase (+) LAB strains were characterized using VITEK® MS according to the manufacturer's instructions. A reference strain of *E. coli* ATCC® 25922™ was used for the positive test control (Dubois et al., 2012).

Determination of Extracellular Phytase Activity

One unit of phytase activity (U) is defined as the amount of enzyme producing one nmol of inorganic phosphorus per minute at 50°C. Phytase enzyme activity was calculated by incubating the sourdough mix prepared with 250 µl cell suspensions and 250 µl of 2 mM substrate in 100 mM sodium acetate-acetic acid buffer for 15 minutes at 50°C (NÜVE EN-500, Ankara, Turkey). A blind tube was prepared by adding 10% TCA solution before adding the substrate. Then, reaction was stopped by adding 500 µl of 10% (w/v) TCA. Finally, inorganic phosphate was calculated at 700 nm using iron sulfate-ammonium molybdate method by a UV-VIS spectrophotometer (Shimadzu UV-1280, Kyoto, Japan) (Raghavendra & Halami, 2009).

Results and Discussion

In this study, the extracellular phytase activity of the LAB strains isolated from the sourdough mix prepared from the traditionally produced boza samples as starter culture were assessed. Our study showed that 29 presumptive strains of LAB were isolated. Of them, 21 (1 *Enterococcus faecium,* 5 *Lactobacillus casei*, 1 *Lactobacillus fermentum*, 4 *Lactobacillus pentosus*, 3 *Leuconostoc lactis*, and 7 *Pediococcus pentosaceus*) were found to be phytase (+). The average extracellular phytase activity was 468.2 U/mL and 1285.5 U/mL, and a *Pediococcus* (*P.*) *pentosaceus* EK1 strain showed the highest activity as 1285.5 U/mL.

Sourdough has been produced since 3.000 BC by fermentation method. Since the 19th century, its use has decreased due to faster production and faster consumption habits, and replaced with commercial baker's yeasts, i.e., *Saccharomyces* (*S.*) *cerevisiae*. However, the use of sourdough has started increasing in the recent years due to public interest in healthy eating and artisanal products. Sourdough is a specific ecosystem inhabited by mainly heterofermentative LAB species such as *L. fermentum, L. paralimentarius, L. plantarum*, and *L. sanfranciscensis* and yeasts. The diverse compositions of sourdough microbiota is affected by the diversity of fermentation processes. Sourdough has diverse contributions to the foods, such as improvement of nutritional properties, extension of shelf life, and enhancement of sensory characteristics (De Vuyst et al., 2014; Gänzle & Ripari, 2016; De Vuyst et al., 2017; Kourkouta et al., 2017; Papadimitriou et al., 2019; Catzeddu, 2019). In this study, we prepared the sourdough

mix using the traditionally produced boza as starter culture, instead of utilizing a starter culture such as *S. cerevisiae*, or other sourdough food. This way of fermentation is one of the most widely preferred approaches to making fermented food. The distiributions of the collected boza samples based on the geographical region in Turkey were 28.6% Central Anatolia, 42.9% Marmara, and 28.6% Eastern Anatolia.

Boza is one of the most well known cereal-based fermented drinks. Its pleasant taste, flavor, and nutritional value have made it a very popular beverage among the people of all ages. It is normally produced by fermentation involving mixed cultures of LAB and yeasts. However, LAB is always the basic microflora in the boza with an average LAB/yeasts ratio of 2.4 (Erkmen & Bozoğlu, 2016). Differences between the microflora of boza are related to production processesse, storage temperature and period, and raw materials. The lactic acid fermentation is one of the two different simultanesously occurring types of fermentation in the boza production, which produces lactic acid, and determines the acidic character of this traditional beverage. Vast majority (96.3%) of the strains common in boza were the multiple LAB species (25.6% *Leuconostoc* (*L.*) *paramesenteroides*, 21.9% *L. sanfrancisco* and 18.6% *L. mesenteroides*) (Hancioglu & Karapinar, 1997; Petrova & Petrov, 2017; Irkin, 2019). On the other hand, *L. plant*arum (24%), *L. acidophilus* (23) and *L. fermentum* (19%) were dominant in the Bulgarian boza, whereas *L. plantarum* was the major species isolated from Turkish boza samples (Gotcheva et al., 2001; Kivanc et al., 2011; Lokumcu Altay et al., 2013). A recent study in Turkey by Borcaklı et al. (2018) showed that various LAB involving *Lactococcus lactis*, *leuconostocs* (*L. pseudomesenteroides*, *Lc. lactis*, *Lc. citreum*), and *Lactobacillus* spp. (*L. plantarum*, *L. paracasei*, *L. brevis*, *L. delbrueckii* subsp. *delbrueckii*) were identified as the common members of the microbial community in the boza samples (Borcaklı, Öztürk, & Yeşilada, 2018). In our study, initial cultural examination revealed 29 presumptive LAB strains (1 *E. faecium*, 11 *L. casei*, 1 *L. fermentum*, 6 *L. pentosus*, 3 *L. lactis* and 7 *P. pentosaceus*) from the fermented sourdoughs. Of them, 21 (1 *E. faecium*, 5 *L. casei*, 1 *L. fermentum*, 4 *L. pentosus*, 3 *L. lactis* and 7 *P. pentosaceus*) were found to be phytase (+), whereas 8 (6 *L. casei* and 2 *L. pentosus*) were phytase (-). Our results showed that multiple LAB strains were common in the sourdoughs, and similar to those previously conducted nationall and international works (Table 1 & Figure 1).

Table 1. Results of phytase screening in culturally isolated presumptive LAB strains

No	Name of strain	Result of phtase screening (n)		Origin of boza *
		Phytase (+)	Phytase (–)	
1	*E. faecium*	1	0	CA
2	*L. lactis*	3	0	CA
3	*P. pentosaceus*	7	0	CA, M, EA
4	*L. casei*	5	6	M, EA
5	*L. fermentum*	1	0	M
6	*L. pentosus*	4	2	M, EA
	Total	**21**	**8**	

**CA: Central Anatolia, M: Marmara, EA: Eastern Anatolia*

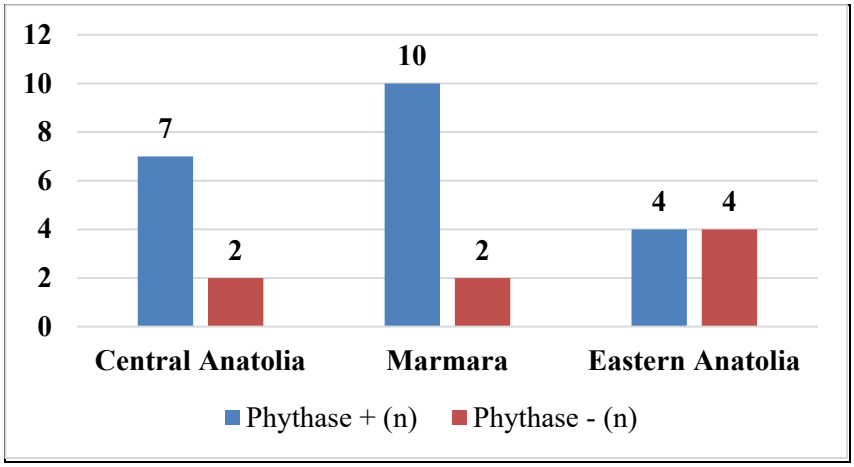

Figure 1. Distribution of phythase (+) and phythase (-) LAB strains based on the origins of the collected boza samples

Phytic acid is an antinutrient because of its ability to bind nutrients such as minerals and proteins, either directly or indirectly, and thus adversely affect their solubility, functionality, absorption, and digestibility (Damayanti et al., 2017). The organisms, including plants, microorganisms, and animal cells have the ability to synthesize phytases. Generally, fungi produce extracellular phytases, whereas bacteria produce the cell-associated enzymes mostly. In the literature, only bacteria exhibiting extracellular phytase activity are those of the genera *Bacillus* and *Enterobacter.* LAB were within the first bacteria to be evaluated because of their involvement in food fermentations and in the human health. However, not all LAB are linked to food fermentations (Papadimitriou et al., 2016). Phytases have gained great interest for biotechnological applications, in particular for the reduction of phytate content in feed and food (Konietzny & Greiner, 2004). Sumengen et al. (2013) studied phytase produced from *L. plantarum* isolated from a fermented food (Shalgam), and determined extracellular and intracellular enzyme activities of *L. plantarum* to be 984.50 U/mL and 494 U/g, respectively (Sumengen et al., 2013). Metabolism of sourdough microbiota and the activity of cereal enzymes are interdependent (Gänzle, 2014). According to Reale et al. (2007), the extent of phytate degradation is mostly independent from LAB strain used for fermentation, and phytate degradation during cereal dough fermentation is positively correlated with endogenous plant phytase activity. Lactic acid fermentation significantly decreases phytate content in plant-based foods. It is widely believed that this reduction is because of the activity of the intrinsic plant phytases, and LAB strains provide suitable conditions for the endogenus cereal phytases by lowering pH value in the medium. So far, only *L. amylovorus* and *L. plantarum* were reported to produce significant extracellular phytase activities. On the other hand, Reale et al. (2007) claims that if a wild-type LAB strain produces extracellular phytase activity, its production can be sufficient for the phytate dephosphorylation during fermentation (Reale et al., 2007). Similarly, Leenhardt et al. (2005) reported that a moderate drop of the dough pH (around 5.5) was sufficient to lower significantly the phytate content of a wholemeal flour (Leenhardt et al., 2005). However, a few strains of LAB have shown consistent phytase activity to degrade phytate by producing extracellular phytases (Anastasio et al., 2010). Therefore, there has been a growing interest in deriving alternate strategies of phytate utilization by probiotics in the human, as they are capable of producing phytase to combat mineral deficiency of zinc and iron (Priyodip, Prakash, & Balaji, 2017). During boza fermentation, phytic acid is catalyzed by the activation of phytase enzyme in LAB, resulting in cause and upsurge of mineral absorption (Borcaklı, Öztürk, & Yeşilada, 2018). Zamudio et al. (2001) investigated the intracellular and extracellular phytase activities of six LAB (*Ped. pentosaceus*, *Leuc. mesenteroides*, *Lact. casei*, *Lact. fermentum*, *Lact. delbrueckii* and *Lact. plantarum*). There was no intracellular phytase activity, whereas *L. plantarum* showed the highest extracellular phytase activity (6.3 mU/mL) (Zamudio et al., 2001). Khodaii et al (2013) reported that *L. casei* from dairy products exhibited higher phytase activity (> 0.004 U) than those isolates from pharmaceutical products (40% versus 27%) (Khodaii et al., 2013). Cizeikiene et al. (2015) showed that the highest extracellular phytase activity produces *Pediococcus pentosaceus* strains from rye sourdough with 32 to 54 U/mL, respectively, under conditions similar to leavening of bread dough (Cizeikiene et al., 2015). On the other hand, a study by Goswami et al. (2017) did not show phytases activity of the LAB strains in the extracellular medium. The specific activities of the studied lactobacilli against phytate varied from 0.03 U/mg to 0.43 U/mg proteins, being the lowest in *L. fermentum* and the highest in *L. Plantarum* (Goswami et al., 2017). In this study, we detected 21 phytase isolates out of 29 presumptive LAB strains in the prepared sourdoughs. At the end of 24 hours, the vitabilities of the phytase (+) isolates varied between 8.52 log cfu/g (*P. pentosaceus* EK1 from Marmara Region) and 3.60 log cfu/g (*P. pentosaceus* NB32 from Central Anatolia region). Phytase production of each strain was mainly determined by production of clear zones around the colonies on the sodium phytate containing medium (Sümengen, Dinçer, & Kaya, 2012). Phytase activity of each strain at the end of 24 hours were changed from 6 mm (*P. pentosaceus* EK1 from Marmara) down to 3 mm (*L. casei* strains from Marmara and Eastern Anatolia, and *P. pentosaceus* from Central Anatolia), respectively. Accordingly, the average extracellular phytase activity was found to be 656.8±188.1 U//mL, and a *P. pentosaceus* EK1 isolated from the sourdough prepared using the boza from Marmara region as starter culture showed the highest activity as 1285.5 U/mL among them, as similar to that reported by Cizeikiene et al. (2015) (Table 2 & Figure 2). Our results showed that the a LAB strain, *P. pentosaceus* EK1, isolated from sorudough mix prepared using traditionally produced boza from Marmara Region as starter culture yieleded a performance of extracellular phytase activity better than the previously identical strains isolated from different sources of foods.

Table 2. Viability and phytase activities of LAB strains

	Isolate type / code	Origin*	V(log cfu/g)**		PA (mm)***			EPA (U/mL)****
			0 h	24 h	24 h	48 h	96 h	24 h
1	*E. faecium* NB32A	CA	4.30	5.93	3.5	4	4	548.2
2	*L. casei* B21	M	3.54	5.69	3	4	4	594.6
3	*L. casei* B31A	M	3.99	5.99	4	5	5	682.7
4	*L. casei* K11	EA	4.41	5.51	3	3	4	487.3
5	*L. casei K22*	EA	3.92	5.11	3	4	4	506.4
6	*L. casei* K32	EA	3.72	5.97	3	4	4	635.3
7	*L. fermentum* B1A	M	3.57	6.93	5	5.5	6	743.7
8	*L. pentosus* B1	M	4.68	5.54	4.5	5	5	678.5
9	*L. pentosus* B31	M	4.30	7.69	4	5	5	763.0
10	*L. pentosus* B33	M	3.96	6.62	5	5.5	7	634.4
11	*L. pentosus* B33A	M	4.53	6.86	5	7	9	714.7
12	*L. lactis* B11	CA	4.96	5.46	4	5	5	463.6
13	*L. lactis* B12	CA	4.93	5.48	5	6	6	943.1
14	*L. lactis* B32	CA	4.46	6.92	5.5	6	8	810.5
15	*P. pentosaceus* EK1	M	3.80	8.52	6	7.5	11	1285.5
16	*P. pentosaceus* EK2	M	3.94	5.40	3	3.5	4	559.4
17	*P. pentosaceus* EK3	M	4.36	5.71	3	4	4	576.3
18	*P. pentosaceus* K33	EA	3.89	4.90	4	4	5	603.6
19	*P. pentosaceus* NB1	CA	4.46	5.98	3.5	4	4	497.6
20	*P. pentosaceus* NB32	CA	3.56	3.60	3	3	4	521.7
21	*P. pentosaceus* NB34	CA	4.81	6.00	4	4	4	532.8

**CA:Central Anatolia, M: Marmara, EA: Eastern Anatolia, **V: Viability, ***PA: Phytase Activity, ****EPA: Extracellular Phytase Activity*

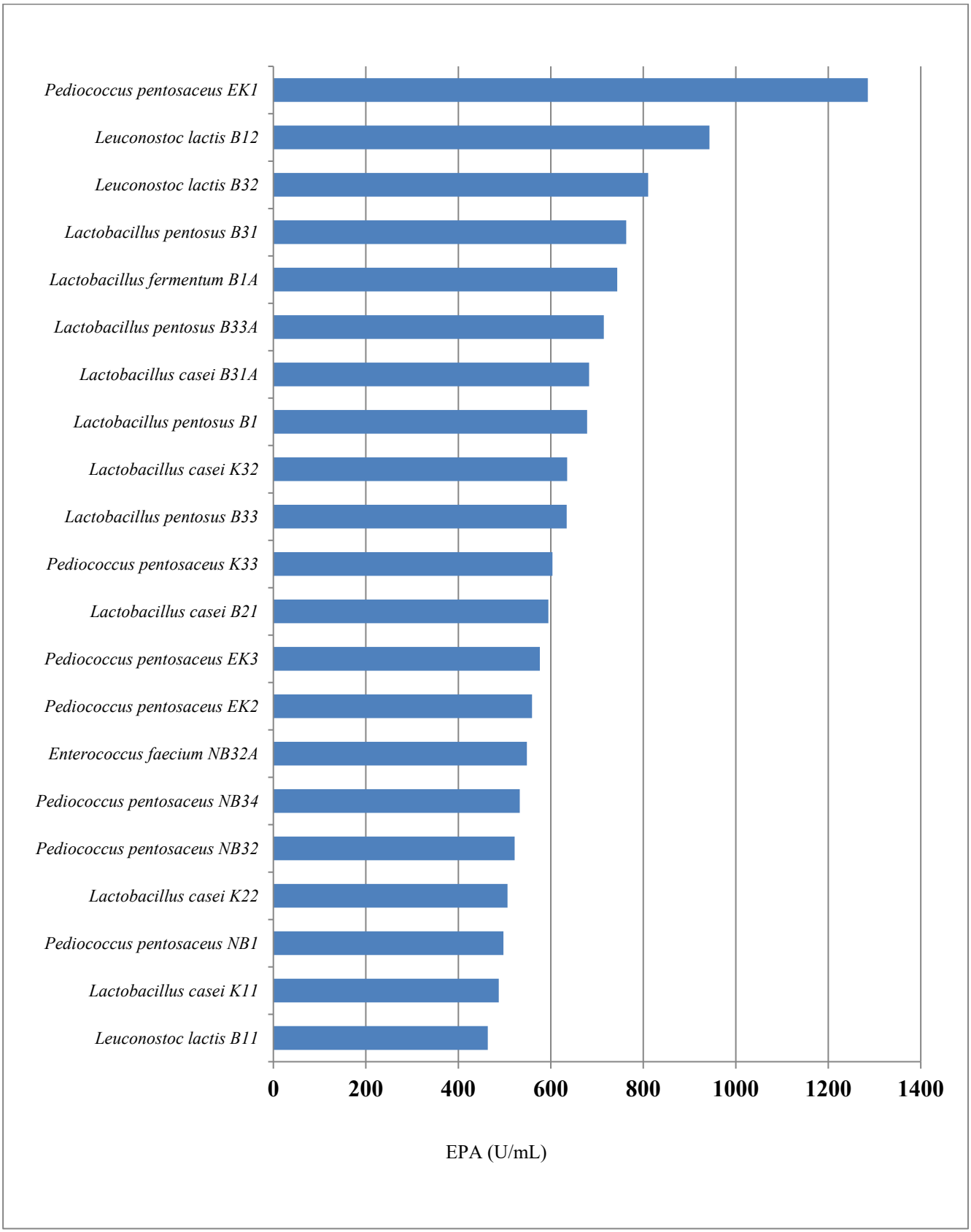

Figure 2. Extracellular phytase activity (EPA) of phytase (+) LAB isolates

Conclusion

In conclusion, the traditionally produced bozas have been found as potential starter culture reservoirs for sourdough fermentation with significantly higher extracellular phytase activities, thus challenging opportunitites to lower antinutritional factors, in particular phytic acid (PA) or phytate in the foods for the consumers.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived the conflict of interests.

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