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RESEARCH ARTICLE

Screening, Characterization and Physicochemical Optimization of Phosphorus Solubilization Activity of Potential Probiotic *Lactobacillus spp.*

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ABSTRACT

Present study was designed to develop indigenous phytate hydrolyzing probiotic Lactobacilli to increase phosphorous availability in poultry gut with ultimate goal of efficient and enhanced poultry production. A total of 90 Lactobacilli were isolated from indigenous poultry droppings and homemade fermented milk products. Lactobacilli were screened for phytate hydrolyzing ability and enzyme activities were measured calorimetrically by ammonium molybdate assay. Out of 62 isolates showing phytate hydrolysis, 16 were confirmed by cobalt chloride staining. Out of 16, three isolates (PDP10, PDP24 and FYP38) were identified as *Lactobacillus gallinarum*, *Lactobacillus reutri* and *Lactobacillus fermentum* respectively, and further selected for physico-chemical optimization of phytase production. PDP10, PDP24 and FYP38 showed highest phytase activity at 35°C (6.86±0.15, 5.12±0.12 and 5.65±0.13 IU/ml, respectively), pH 5.0 (6.86±0.15, 5.12±0.11 and 5.50±0.13 IU/ml respectively), 1% NaCl (4.78±0.14, 4.18±0.13 and 5.58±0.02 IU/ml respectively) and 0.3% bile salts (3.76±0.12, 1.12±0.11 and 2.21±0.15 IU/ml respectively). Glucose was optimum carbon source for phytate hydrolysis. Optimum nitrogen source was peptone for PDP10 and PDP24 while tryptone for FYP38. It is concluded that phytate hydrolyzing Lactobacilli reported in this study might be used as probiotics for increased phosphorous availability in poultry after further investigations.

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INTRODUCTION

Poultry industry is one of the major sectors strengthening the economy of countries. In Pakistan, commercial poultry farming started in early 1960s, showed tremendous growth in few decades and became a 2nd largest industry. Due to large scale rearing, the poultry industry is facing serious economic losses due to infectious diseases and low phosphorus availability (Cegielska-Radziejewska *et al.*, 2013). Phosphorus being a main constituent of energy rich compounds such as ATP, ADP and GTP is a vital nutrient to biological systems. Phosphorous is found widely in nature in unavailable forms (organic phosphorus compounds) i.e. phytic acid (myo-inositol 1,2,3,4,5,6-hexakisdihydrogen phosphate) and mixed cation salts of phytic acid, designated as phytate (Awad *et al.*, 2014).

Poultry feed is primarily based on cereals, seed products and legumes containing 60-90% of the organic phosphorus as phytate which have anti-nutritional effect due to its ability to chelate divalent cations such as calcium, magnesium, iron and zinc. Phytases can cleave phospho-monoester bonds in phytic acid, increasing phosphorous availability (Gaind and Singh, 2015). Monogastric animals (poultry, human, fish and swine) lack phytases and cannot utilize phytate (Awad *et al.*, 2014). Phytases have been widely used in monogastric diet to improve nutritional value, feed conversion and environmental protection against phosphorus-rich manures (Jain *et al.*, 2016).

Sources of phytases may include plants, animals and microorganism (Azeem *et al.*, 2015). However, microbial extracellular phytases are more promising due to easy isolation. There are considerable populations of phosphate

solubilizing microorganisms (PSM) in soil particularly in plant rhizospheres which play an important role in biogeochemical phosphorus cycling in terrestrial and aquatic environments (Paul and Sinha, 2017). Different strains of fungi, bacteria and yeast have been used for large scale phytase production (Naves *et al.*, 2012). Low stability of phytases at high temperatures (>85°C), a limited pH range, and poor storage stability are major bottleneck in the commercialization of phytases. At 50-55 °C, phytase undergoes an irreversible conformational rearrangement resulting in 70-80% loss in enzymatic activity and get inactivated at feed pelleting temperature (70-90°C for 5-10 min) (Ushasree *et al.*, 2014). This has led to the development of great interest in identification and characterization of microbial phytases from diversified habitats, focusing on their activity and stability in different food matrices (Roopashri and Varadaraj, 2015).

According to Food and Agriculture Organization (FAO) and World Health Organization (WHO), probiotics are 'the living microorganisms which when administered in adequate amount confer a health benefit to the host. Probiotics like Lactobacillus *spp.*, *Lactobacillus amylovorus*, *Lactobacillus brevis* and *Lactobacillus fructivorans* with phytase activity may be used in poultry feed to increase phosphorus availability (Sümengen *et al.*, 2012). The effectiveness of probiotic phytase producing Lactobacilli as feed additive is dependent on their stability at stomach pH, body temperature and resistance to the action of proteases (Jain *et al.*, 2016). Present study is designed as a first step in a multi-step project to develop indigenous phytase producing probiotic Lactobacilli from different sources, the optimization of phytate hydrolysing activity and the effect of physical and chemical factors on phytate hydrolysing activity.

MATERIALS AND METHODS

Isolation of Lactobacilli: Indigenous poultry droppings (n=10) and yogurt samples (n=10) were collected in sterile containers and transported to Microbiology Department, University of Veterinary and Animal Sciences. Samples were stored at 4°C. Lactobacilli were isolated by plating serially diluted samples on de Man Rogosa and Sharpe (MRS) agar, followed by incubation at 37°C for 48 hours. Distinguished colonies were selected, purified and stored in MRS broth supplemented with glycerol (15%) at -20°C.

Identification of Lactobacilli: Selected isolates were preliminary identified as Lactobacilli by Gram's staining and catalase test. DNA was extracted by using WizPrep gDNA, Mini Kit according to manufacturer's requirements. Lactobacillus genus was confirmed by genus specific primers XB5-F (GCC TTG TAC ACA CCG CCC GT) and LbLMA1-R (CTC AAA ACT AAA CAA AGT TTC). For specie identification,16SrDNA was amplified using universal primers 8FLP-F (AGT TTG ATC CTG GCT CAG) and XB4-R (GTG TGT ACA AGG CCC GGG AAC) and sequenced, as described earlier (Nawaz *et al.*, 2011). Sequences were submitted to NCBI GenBank.

Screening of phytate hydrolyzing Lactobacilli: Lactobacilli isolates were screened for their sodium phytate hydrolyzing ability by spotting on Phytase screening medium (PSM) agar (containing 0.3% glucose, 0.1% tryptone, 0.1% sodium phytate, 0.0004% $FeSO_4$, 0.03% $CaCl_2$. $2H_2O$, 0.05% $MgSO_4$. $7H_2O$, 0.004% $MnCl_2$ and 1.5% agar) and incubating at 37°C for 24-96 hours. Colonies surrounded by zone of phytate hydrolysis were considered as phytate solubilizing Lactobacilli.

Confirmation of phytate hydrolysis: False positive results by microbial acid production were eliminated by cobalt chloride staining as described elsewhere (Bae *et al.*, 1999). Briefly, 2% cobalt chloride solution was poured on plate containing hydrolysis zone and incubated for 5 minutes at room temperature. After incubation solution was removed and plate was flooded with freshly prepared solution containing equal volumes of 6.25% ammonium molybdate and 0.42% ammonium vanadate solution. Plates were again incubated for 5 minutes at room temperature and stain was removed. Isolates showing clear zones were considered phytate hydrolyzing Lactobacilli.

Preparation of cell free Supernatants: Isolates were inoculated in MRS broth modified by the addition of 0.2%sodium phytate and incubated at 37°C for 24hours. Cells were extracted by centrifugation at 6000rpm for 10 minutes. Carefully pipetted out supernatant (crude enzyme source) was filtered through 0.45µm syringe filter into a new sterile tube.

Phytase activity assay: Phosphomolybdate colorimetric method was used to measure enzyme activity following the previously reported method (Jain *et al.*, 2016). Briefly, cell free supernatants (120µl) were mixed with 0.1M acetate buffer pH 5.0 containing 0.2% sodium phytate (480µl). Reaction mixture was incubated at 37°C for 30 minutes. Stop solution (Trichloroacetic acid, 600µl) was added followed by addition of colouring reagent (600µl) for the formation of blue coloured complex. Colouring reagent was prepared by mixing 4 parts of 1.5% ammonium molybdate with 5.5% sulphuric acid and 1 part of 2.7% ferrous sulphate solution. Phosphorus concentration was measured by taking absorbance at 0 min and 30 min at 630 nm. Results obtained were compared with the standard curve prepared by using KH_2PO_4 as inorganic phosphate source. One unit of the enzyme activity was defined as the amount of phytase required to liberate 1µmol of inorganic phosphate per minute under defined assay conditions (Roopashri and Varadaraj, 2009).

Optimizing physical conditions for phytate hydrolysis: Phytate hydrolyzing activity of Lactobacilli was optimized at different physical parameters (temperature, pH, osmotic pressure and aerobic/anaerobic conditions). Isolates were grown in modified MRS broth containing 0.2% sodium phytate with different pH (4, 5, 6, 7 and 8), NaCl concentration (1, 2 and 4%) incubated in aerobic and anaerobic conditions at different temperatures 30°C, 35°C and 42°C for 24 hours followed by enzyme activity assay.

Optimizing chemical conditions for phytate hydrolysis: Different chemical conditions (carbon, nitrogen sources and bile salts) affecting phytate hydrolysis were optimized by growing isolates in modified MRS broth supplemented with different carbon sources (glucose, sucrose and lactose), nitrogen sources (peptone, tryptone and urea) and different concentrations of bile salt (0.3, 1 and 2%) at respective optimum temperature for 24hours. Enzyme activities were determined using standard curve.

Statistical analysis: Data of enzyme activities was expressed as Mean±S.D and compared by one-way ANOVA followed by Turkey's Multiple comparison test at $P<0.05$.

RESULTS

All tested isolates were Gram positive rods with no catalase activity. Genus specific amplification (≈250bp) confirmed that all isolates were Lactobacilli while amplification and sequencing of 16Sr DNA (≈1400bp) of selected isolates revealed that PDP10, PDP24 and FYP38 were *Lactobacillus gallinarum*, *Lactobacillus reutri* and *Lactobacillus fermentum* respectively. GenBank accession numbers of FYP38, PDP 10 and PDP24 are MF980923, MF980923, MF980925, respectively.

Out of total of 90, 62 isolates showed phytate hydrolysis while 16 isolates retained phytate hydrolysis after cobalt chloride staining. Out of 16 selected isolates, eight isolates PDP05, PDP09, PDP10, PDP16, PDP23, PDP24, PDP30 and PDP35 were of poultry origin and eight FYP12, FYP15, FYP17, FYP21, FYP26, FYP31, FYP38 and FYP42 were of fermented foods origins.

Phytase activities of cell free supernatants of selected isolates were determined by ammonium molybdate assay. Enzyme activities of poultry origin Lactobacilli PDP05, PDP09, PDP10, PDP16, PDP23, PDP24, PDP30 and PDP35 were 3.77±0.35, 1.68±0.27, 4.76±0.15, 0.44±0.06, 3.93±0.09, 4.12±0.1, 1.86±0.13 and 0.10±0.08 IU/mL, respectively. Enzyme activities of fermented food origin Lactobacilli FYP12, FYP15, FYP17, FYP21, FYP26, FYP31, FYP38 and FYP42 were 3.28±0.17, 2.89±0.22, 2.71±0.21, 2.95±0.20, 2.11±0.08, 0.43±0.04, 5.51±0.13 and 3.70±0.09, IU/mL respectively. On the basis of highest phytase enzyme activities in this study and fulfilment of other probiotic prerequisites (data not shown), three isolate PDP10, PDP24 and FYP38 were selected as potential phytate hydrolyzing probiotics.

Enzyme activity of selected isolates PDP10 and FYP38 was significantly higher at 35°C as compared to 30°C and 42°C. Whereas enzyme activity of PDP24 was significantly higher at 35°C and 42°C as compared to 30°C, as illustrated in Table 1. Highest activity of selected isolates (PDP10, PDP24 and FYP38) was obtained at pH 5.0, as given in Table 2.

Enzyme activities of PDP10 and FYP38 in MRS broth without NaCl and with 1% NaCl were significantly higher ($P<0.05$) as compared to that at 2 and 4% NaCl. Enzyme activity of PDP24 in MRS broth without NaCl, with 1 and 2% NaCl was significantly higher as compared with 4% NaCl, as given in Table 3.

Phytase activity of PDP 10 and PDP24 was significantly higher in anaerobic condition as compared to aerobic condition while FYP38 had no significant difference in activity in aerobic and anaerobic conditions. PDP10, PDP24 and FYP 38 enzyme activities were significantly higher in presence of glucose as compared to lactose and sucrose, as illustrated in Table 4.

Table 1: Phytase activity of cell free supernatants of Lactobacilli isolates grown at different temperatures

Isolates	Phytase activity (Mean±S.D IU/ml) at different temperatures		
	30°C	35°C	42°C
PDP10	4.56±0.17[a]	6.86±0.15[b]	4.76±0.15[a]
PDP24	4.02±0.09[a]	5.12±0.12[b]	5.09±0.11[b]
FYP38	5.31±0.17[a]	5.65±0.13[b]	4.43±0.09[c]

S.D: Standard deviation, IU/ml: international Units of enzyme per ml. [a, b, c]Mean in same row with different superscripts differs significantly.

Table 2: Phytase activity of cell free supernatants of Lactobacilli isolates grown at different pH values

Isolates	Phytase activity (Mean±S.D IU/ml) at different pH values				
	4	5	6	7	8
PDP10	3.62±0.12[a]	6.86±0.15[b]	4.56±0.17[c]	4.71±0.15[d]	4.86±0.15[d]
PDP24	3.18±0.13[a]	5.12±0.11[b]	4.02±0.09[c]	4.10±0.12[c]	4.33±0.10[d]
FYP38	3.94±0.12[a]	5.50±0.13[b]	5.31±0.17[b]	4.01±0.15[c]	4.43±0.09[d]

S.D: Standard deviation, IU/ml: international Units of enzyme per ml. [a, b, c]Mean in same row with different superscripts differs significantly.

Table 3: Phytase activity of cell free supernatants of Lactobacilli isolates grown in MRS supplemented with different NaCl concentrations

Isolates	Phytase activity (Mean±S.D IU/ml) at different NaCl concentrations			
	MRS	1%	2%	4%
PDP10	4.76±0.15[a]	4.78±0.14[a]	4.22±0.12[b]	3.12±0.11[c]
PDP24	4.12±0.11[a]	4.18±0.13[a]	4.10±0.10[a]	3.91±0.10[b]
FYP38	5.51±0.13[a]	5.58±0.12[a]	4.92±0.15[b]	4.13±0.17[c]

S.D: Standard deviation, IU/ml: international Units of enzyme per ml, [a, b, c]Mean in same row with different superscripts differs significantly.

Table 4: Phytase activity of cell free supernatants of Lactobacilli isolates grown in different carbon sources

Isolates	Phytase activity (Mean±S.D IU/ml) in different carbon sources		
	Glucose	Lactose	Sucrose
PDP10	4.36±0.11[a]	4.11±0.15[b]	4.15±0.17[b]
PDP24	4.38±0.18[a]	4.02±0.16[b]	4.01±0.15[b]
FYP38	5.01±0.15[a]	4.21±0.11[b]	3.45±0.13[c]

S.D: Standard deviation, IU/ml: international Units of enzyme per ml. [a, b, c]Mean in same row with different superscripts differs significantly.

Table 5: Phytase activity of cell free supernatants of Lactobacilli isolates grown in different nitrogen sources

Isolates	Phytase activity (Mean±S.D IU/ml) in different nitrogen sources		
	Peptone	Tryptone	Urea
PDP10	4.54±0.13[a]	4.32±0.15[b]	4.30±0.10[b]
PDP24	4.23±0.19[a]	4.10±0.11[b]	3.98±0.12[b]
FYP38	4.56±0.14[a]	5.29±0.17[b]	4.82±0.15[c]

S.D: Standard deviation, IU/ml: international Units of enzyme per ml. [a, b, c]Mean in same row with different superscripts differs significantly.

Table 6: Phytase activity of cell free supernatants of Lactobacilli isolates grown in different bile salt concentrations

Isolates	Phytase activity (Mean±S.D IU/ml) in different bile salt concentrations		
	0.3%	1%	2%
PDP10	3.76±0.12[a]	1.75±0.12[b]	1.95±0.18[c]
PDP24	1.12±0.11[a]	0.5± 0.04[b]	0.9±0.06[c]
FYP38	2.21±0.15[a]	1.12±0.13[b]	1.10±0.16[c]

S.D: Standard deviation, IU/ml: international Units of enzyme per ml. [a, b, c]Mean in same row with different superscripts differs significantly.

Influence of different nitrogen sources on phytase production showed that phytase activity of PDP10 and PDP24 was significantly higher in presence of peptone as compared to tryptone and urea while enzyme activity of FYP38 was significantly higher in presence of tryptone as compared to peptone and urea, as given in Table 5. PDP10, PDP24 and FYP38 showed significantly higher activity in 0.3% bile salt concentration as compared to1 and 2% bile salt presented in Table 6.

DISCUSSION

Organic phosphorus hydrolysis by microbial phytases has extensively been considered in diverse biotechnological applications, including environmental protection and agricultural, animal, and human nutrition (Menezes-Blackburn *et al.*, 2013). Phytases added to poultry feed increase phosphorus and mineral ions uptake and reduces phosphorus excretion in droppings (Ocampo Betancur *et al.*, 2012). In view of increasing demand of phytases, it is essential to produce and characterize cost-effective phytases from new microbial sources.

In present study phytase hydrolysis was checked on phytase screening medium as described previously (Aziz *et al.*, 2015) while other studies have also used modified MRS agar with sodium phytate and wheat bran extract agar (Saraniya and Jeevaratnam, 2015). Approximately 68% isolates showed phytase like activity. These results are in accordance with a previous study (Sharma and Trivedi, 2015). This high percentage may be a result of hydrolysis by lactic acid, produced by Lactobacilli. This ambiguity was eliminated by counterstaining method, as adopted previously (Bae *et al.*, 1999). Retention of hydrolysis zones confirmed that phytate hydrolysis is due to phytate solubilizing enzyme.

After counterstaining only 16 isolates were selected, similar results were reported earlier (Sharma and Trivedi, 2015). In present study, cell free supernatant was used as a source of crude enzyme extract as previously used for determination of extracellular phytase activity by *Lactobacillus amylovorus* (Sreeramulu *et al.*, 1996). In contrast, various studies reported only intracellular phytase activity (Raghavendra and Halami, 2009). Previous studies have also reported *L. sanfranciscensis* and *Lactobacillus plantarum* as good phytase producers (Sreeramulu *et al.*, 1996).

Different calorimetric methods have been used for the measurement of enzyme activity, in terms of units, while current study made use of posphomolybdate colorimetric method as described elsewhere (Bae *et al.*, 1999). Out of sixteen isolates PDP10, PDP24 and FYP38 were selected as potential probiotic (data not published) for further physicochemical optimization.

Different studies have reported 35-37°C as optimum temperature range for phytase production (Rani and Ghosh, 2011). Present study reported 35°C as optimum temperature for phytate hydrolysis that is similar to phytase production by *Bacillus spp.* as reported elsewhere (Demirkan *et al.*, 2014). In contrast *Lactobacillus amylovorus* and *Lactobacillus sanfrancesis* has previously been reported to show maximum phytase production at 45°C (Sreeramulu *et al.*, 1996; De Angelis *et al.*, 2003).

Optimum acid tolerance range of microbial phytases reported till date is 2.5-7.5. Phytases having optimal activity at pH 2.5 to 5.5 are more common (Oh *et al.*, 2004). Isolates PDP10, PDP24 and FYP38 exhibited best activity at acidic pH (5.0) as previously reported (De Angelis *et al.*, 2003). Few studies have also reported phytase production at alkaline pH 6.0 to 7.0 (Sreedevi and Reddy, 2013).

In present study, results depicted the decrease in phytase production with increase in osmotic pressure. NaCl (1%) has also been reported as optimum osmotic pressure for phytase production previously (Aziz *et al.*, 2015).

Glucose was reported as optimum carbon source among most of the microorganisms (for phytase production (Vohra and Satyanarayana, 2002). Likewise, isolates PDP10, PDP24 and FYP38 exhibited maximum phytase activity in the presence of 2% glucose as compared to lactose and sucrose. Similarly, *Bacillus subtilis* has shown maximum phytase activity in presence of 2% glucose as carbon source (Kerovuo *et al.*, 1998). In contrast different studies reported 1% glucose, wheat bran and myo-inositol as sole source of carbon (Sreeramulu *et al.*, 1996).

Nitrogen is another important factor affecting enzyme production. Different organic forms of nitrogen like peptone, tryptone and urea are widely used for phytase production. This study revealed 0.1 % peptone as optimal nitrogen source for PDP10 and PDP24. Similar nitrogen source has been reported as optimum for phytase production by *Aerobactor aerogenes*. FYP38 yielded optimum results in presence of 0.1% tryptone as reported earlier (Aziz *et al.*, 2015). Malt extract and 1% yeast has also been reported as optimum nitrogen source (El-Toukhy *et al.*, 2013). A previous study has reported that tryptone, beef extract and peptone had no effect on phytase production (Gulati *et al.*, 2007). Bile salt tolerance is one of the important properties required by probiotic bacteria for survival in small intestine. A previous study showed the higher bile salts tolerance (0.3-2.0%) in phytase producing isolates as compared to those which cannot produce phytase (Sharma and Trivedi, 2015) . Likewise, PDP10, PDP24 and FYP38, isolates selected in this study, showed the significantly higher phytase activity in presence of 0.3% bile salt as compared to 1% and 2% bile salt concentrations.

Conclusions: It is concluded that *Lactobacillus gallinarum* PDP10, *Lactobacillus reutri* PDP24 and *Lactobacillus fermentum* FYP38 have phytate solubilizing ability and these strains may have probiotic potential to be used to enhance phosphorus availability in poultry.

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Authors contribution: MN and KM conceived and designed the project. AA and AM executed the experiment. MN, MR and SI analyzed the data. AM and MRY prepared the Manuscript. All authors critically revised the manuscript for important intellectual contents and approved the final version.

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