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Full Length Research Paper

Potential phytate-degrading enzyme producing bacteria isolated from Malaysian maize plantation

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Phytases catalyze the hydrolysis of phosphomonoester bonds in phytate, thereby releasing lower forms of myo-inositol phosphates and inorganic phosphate. Phytase enzyme preparations have a wide range of applications in animal and human nutrition. The addition of phytate-degrading enzyme can improve the nutritional value of plant-based foods by enhancing protein digestibility and mineral availability through phytate hydrolysis during digestion in stomach or during food processing. 30 strains of potential phytate-degrading enzymes bacteria isolated from Malaysian maize plantation were cultivated in Luria Bertani (LB) and Luria Bertani + Rice Bran (LBRB) media for 5 days and were analyzed for phytase activity. The 6 strains with highest activity were chosen for species identification. Two set of broad-range 16S rRNA PCR primers were used for genotypic identification. ASUIA279 was the strain that had has the highest phytase activity in LBRB followed by ASUIA271, ASUIA138, ASUIA260, ASUIA243 and ASUIA30. The genotypic technique revealed *Pantoea stewartii* ASUIA271, *Enterobacter sakazakii* ASUIA279, *Bacillus cereus* ASUIA260, *Bacillus subtilis* ASUIA243, *P. stewartii* ASUIA138 and *B. cereus* ASUIA30.

Key words: Bacterial phytase, rice bran, production, genotypic.

INTRODUCTION

Phytases (myo-inositol hexakisphosphate phosphohydrolase), also known as phytate-degrading enzymes, hydrolyse phytate to myo-inositol and phosphoric acid in a stepwise manner forming myo-inositol phosphate intermediates (Koneitzny and Greiner, 2002). Phytases are widespread in nature because they can be found in animals, plants and microorganisms. Like filamentous fungi (Pasamontes et al., 1997), phytate-degrading enzymes of yeasts (Nakamura et al., 2000; Vohra and Satyanarayana, 2002) gram-negative (Tambe et al., 1994; Sajidan et al., 2004) and gram-positive bacteria (Kerovuo et al., 1998) were also identified and characterized. Phytate is an abundant plant constituent comprising 1 to 5% (w/w) of edible legumes, cereals, oil seeds, pollens and nuts (Cheryan, 1980). Most foods of plant origin contain 50 to 80% of their total phosphorus as phytate (Harland and Morris, 1995). Phytate-bound phosphorus is poorly utilized by monogastric animals, due to insufficient phytate-degrading activity in the gut (Ravindran et al., 1995; Fandrejewski et al. 1997).

The first commercial phytase product, which was available 10 years ago, offered animal nutritionists the tool to drastically reduce phosphorus excretion of monogastric animals (Haefner, 2005). According to Kornegay (1999), phosphorus excretion can be reduced between 25 and 50% depending on diet, species and level of phytase supplementation. Normally, phytase is incorporated into commercial poultry, swine, and fish diets to improve the availability of phosphorus, minerals, amino acids, and energy.

At present, there is no single phytase that is able to meet the diverse needs for all commercial and environmental applications. Therefore, there is an ongoing

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intrest in screening microorganisms, including bacteria for novel and efficient phytases. Different sources of phytases would have differences in some properties, such as substrate specificity, resistance to proteolysis and catalytic efficiency. The aims of the present study were to investigate and identify the potential bacteria strains as phytase producers isolated from Malaysian maize plantation.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Phytic acid, dodecasodium salt, was purchased from Sigma chemical Co. (St. Louis, Mo.). All other chemicals and media were of analytical grade commercially available. LB broth was prepared from 10 g tryptone, 5 g yeast extract, and 10 g NaCl and LBRB was prepared by adding of 50 g of rice bran in 1 l of LB media. Both media were adjusted to pH 7.0 prior to utilization. 30 bacterial strains showing phytase activities were previously isolated from Malaysian maize plantation (Anis Shobirin et al., 2007) were cultivated in 10 ml LB and LB + 5% rice bran media, and incubated at 37°C and agitated at 200 rpm. Samplings for extra-cellular enzyme activity were performed after 2 and 5 days of growth. Samples for enzyme activity assays were prepared by centrifugation of 1.5 ml bacterial culture at 13,000 rpm for 1 min (Idriss et al., 2002). The cell-free supernatant was separated and tested for phytase activity. Potential phytase producing strains (ASUIA260, ASUIA271 and ASUIA279) were grown in LB + 0.1% sodium phytate to determine the Na-phytate efficacy on the ability of those bacteria in producing phytase.

Standard phytase assay

Phytate-degrading activity was determined at 50°C in 350 µl of 100 mM sodium acetate buffer, pH 5.0 containing 1.03 mM sodium phytate. The enzymatic activity was done by adding 10 µl of enzyme solution to the assay mixture. After incubating for 30 min at 50°C, the liberated phosphate was measured according to ammonium molybdate method (Heinonen and Lahti, 1981) with some modifications. Added to the assay mixture was 1.5 ml of a freshly prepared solution of acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v) and 100 µl acetic acid. Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355 nm. To calculate the enzyme activity, a calibration curve was produced over the range of 5-600 mmol phosphate (ε = 8.7 cm^2/nmol). Activity (units) was expressed as 1 µmol phosphate liberated per min. Blanks were run by addition the ammonium molybdate solution prior to adding the enzyme to the assay mixture (Greiner, 2004).

Species identification

6 strains (ASUIA279, ASUIA271, ASUIA138, ASUIA260, ASUIA243 and ASUIA30) were choosing for species identification based on the high phytase activity. The strains were grown on LB broth and harvested by centrifugation at 10000 rpm. DNA was extracted using DNA isolation kit (Qiagen, Hilden, Germany). The DNA was quantified spectrophotometrically (A260) and purity checked by electrophoresis on a 0.8% agarose gel. 2 sets of 16S rRNA PCR primers, PF3: (5' GAGAGTTTGATCCTGGCTCAG 3'), PR4: (5'CT GTTTGCTCCCCACGCTTTC 3') and PF5; (5' GTAGCGGTGA AATGCGTAGA 3') PR6: (5' CTACGGCTACCTTGTTACGA 3') were used. PCR amplification was performed using an Eppendoff mastercycler (Hamburg, Germany). The PCR reaction mixtures contained 2 µl of dNTPs mixture (1.25 mM each), 1 µl of each primer (20 mM), 2.5 µl of DNA, 1 µl of Taq polymerase (Promega, Southampton, UK) and sterile deionized water to bring the final volume to 100 µl. The mixture was denatured at 94°C for 2 min. The PCR temperature profile consisted of 34 cycles of 1 min denaturation at 94°C, 45 s annealing at 50°C and 1.5 min primer extension at 72°C. A negative control was included to eliminate the possibility of reagent contamination. PCR products were analyzed using agarose (0.8%) gel electrophoresis and visualized using ethidium bromide. The identities of the bacteria detected by the 16S rRNA PCR were revealed by sequencing of the PCR products and compare-son of these sequences to the genbank database using the BLAST program available at the national centre for biotechnology information (http://www.ncbi.nlm.nih.gov).

RESULTS

The addition of 5% rice bran in the LB media showed the significant different ($P < 0.05$) on the phytase production by all the bacterial strains tested. Figure 1 shows the induction of phytase production in the 30 bacterial strains when cultivated in LB + 5% rice bran media compared to LB media. ASUIA279 was the strain that have the highest phytase activity in LBRB followed by ASUIA271, ASUIA 138, ASUIA260, ASUIA243 and ASUIA30. So, they were choosing for species identification using the genotypic technique. Based on their colony morphology, microscopic observation, phytase activity and phenotypic technique result, ASUIA273 and ASUIA261 were assumed as the same species as ASUIA279 whereas, ASUIA136 was the same as ASUIA138. So, they were not included in the potential list. The addition of 0.1% Na-phytate into LB medium resulted (Figure 2) the slightly increment of phytase production by the 3 selected bacteria compared to the LB itself even though the increment were not as much as the addition of rice bran.

Two set of broad-range 16S rRNA PCR primers were used and the PCR product with 800 base pairs in size were referred for gene sequencing analysis. From the comparison of these sequences (Figure 3a-f) to the genbank database using the blast program neighbour-joining trees were obtained. The 6 strains were identified as *P. stewartii* ASUIA 271, *E. sakazakii* ASUIA279*, B. cereus* ASUIA260, *B. subtilis* ASUIA243, *P. stewartii* ASUIA 138 and *B. cereus* ASUIA30.

DISCUSSION

Nutrient source and concentration greatly influence bacterial growth and enzyme production (Vohra and Satyanarayana, 2003). 5% of rice bran was added in one of the cultivation media. Rice bran is rather low in inorganic phosphate and has relatively high phytate content. Therefore, the high phytate content in the medium might trigger the biosynthesis of phytate-degrading enzyme in these soil bacteria.

The increment of phytase production by 3 selected

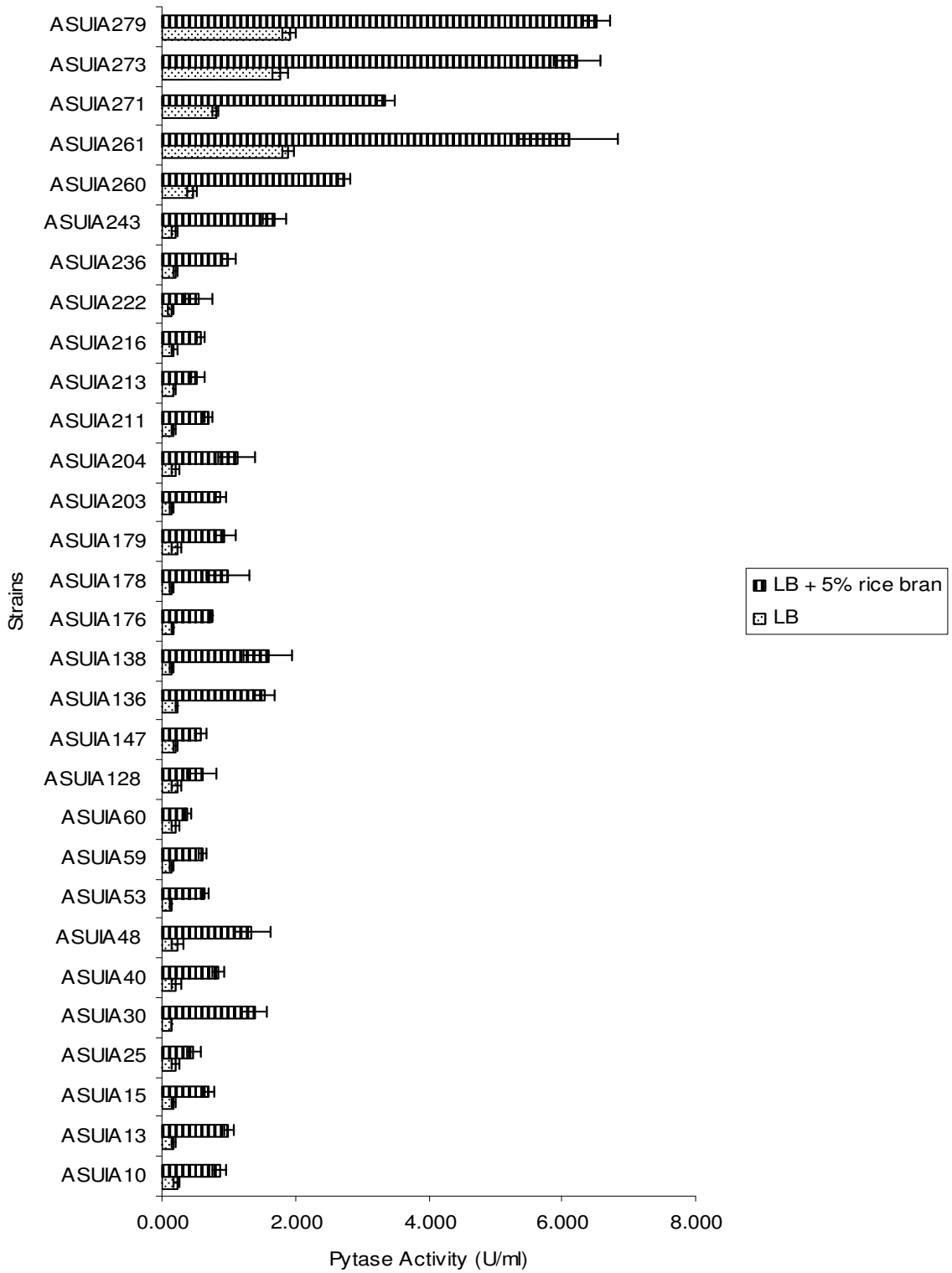

Figure 1. Phytase enzyme activities of 30 soil bacteria strains after 5 days cultivation in LB and LB + 5 % rice bran media. Error bars show mean ± standard deviation.

bacteria when cultivated in 0.1% Na-phytate clarify that the phytate induced the production of phytase in those bacteria (Figure 2). The rice bran has better impact compared to Na-phytate could be due to the different nature of these substrates. This finding was compatible with the study of Lan et al. (2002) who suggested that phytate in

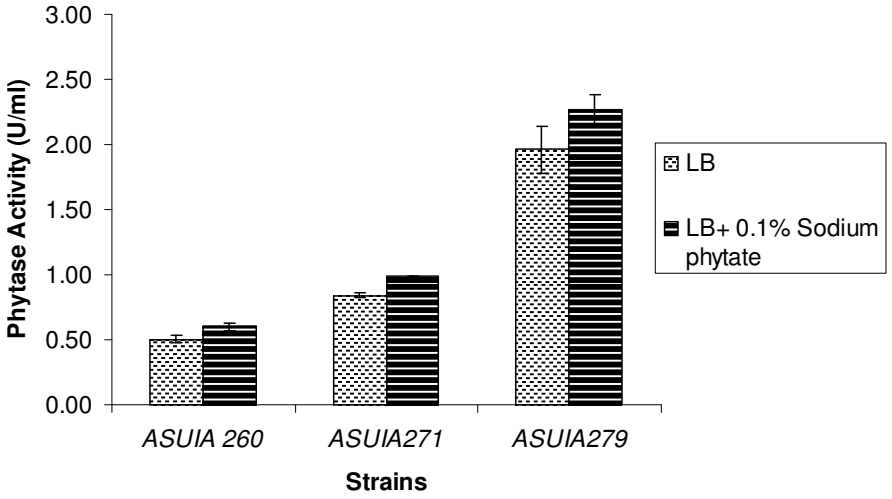

Figure 2. Comparison of phytase production of the bacterial strains in LB and LB + 0.1 % sodium phytate. Error bars show mean ± standard deviation.

GNTGGAGATCCGGTGGCGAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGG
AGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGT
ACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGCGAACTTNNCAGA
GATGCCTTGGTGCCTTCGGGAACGCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT
TGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTC
GGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAA
GTCATCATGGCCCTTACGAGTANGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAG
CGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAATCCGGATCGGANTCTGCAA
CTCGACTCCGTGAANTCGGANTCNCTAGTATCGTGNATCAAAAGCCCCGGGAATACGTTCC
NGGCCTTGTACCCCCCCCGTCCACCATGGGAGTGGTTGCAAAAAAATAGNTACTTAACCTCN
GGAGGGCCTTACCCTTTTGAATNTTGACNGGGGNANCN

Figure 3a. 16S rRNA gene sequence of ASUIA271.

GNTGGANGATNCGGTGGCGAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGG
AGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAG
TACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCAGAGAATCCTGCAG
AGATGCGGGAGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGT
GTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGT
TCGGCCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTC
AAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGA
AGCGACCTCGCGAGAGCAAGCGGACCTCATAAGTGCGTCGTAATCCGGATTGGANTCTGCA
ACTCGACTCNTGAANTCNGAATCNCTAGTATCGTGGATCAAATGCCNCGGGAAAACNTTCCC
GGNCTTGTAACCCCCCCGTCCNCCNTGGGATGGGTTGCAAAAAAATAGGTNCTTAACCTNC
NGGAGGGCCCTNCCNNTTNGNANTTTGACGGGGGAANNCAAAAAAGG

Figure 3b. 16S rRNA gene sequence of ASUIA279.

rice bran occurs as a less soluble potassium-magnesium salt, usually combined with protein, or enclosed by starch and other carbohydrates and the rate of rice bran phytate being hydrolyzed could be slower as compared to Na-phytate. The lower rate of rice bran hydrolysis ensures that phytase production is continuously induced during

GNTGGAGGAANCCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAA
GTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGA
GTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCT
AGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCAT
TAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTC
AAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCT
GCAAGACCGCGAGGTGGAGCTAATCTCATAAANCGTTCTCAGTTCGNATTGTAGNCTGCAAC
TCNCCTACNTGAANCTGGAATCCCTAGTAATCNCGNATCANCATGCCCCGGTGAATACGTTC
CCGGGCCTNGTANCNCCCCCCCCGTCCCCCCCCCGAAAGTTNGTAACCCCNAAANTCNGNNG
GGGTAACCTTTTTGGANCCCCCCCCTAAGGGGGAAAAATATTGGGGGNATCTAAAAAAGGG
TACCCNN

Figure 3c. 16S rRNA gene sequence of ASUIA260.

GTGTGGAGACCCAGTGGCGANGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAG
CGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAG
TGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAG
TACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTA
GAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCAT
TCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTC
AAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCA
GCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCANTCTGCAA
CTCGACTGCGTGAANCTGGAATCNCTAGTAATCNCGNATCAACATGCCNNGGTGAANACGT
TNCCGGGNCNTTTTACCNCCCCCCNGTCCANCCNNGAAAGTTTTTAACCCCCGAANTCGGG
GAGNTANCNTTTNAGAACNCCCCCCCCAAGNGGGAAAAAAATTGGGGGAATNTAAAAAGGNA
NCCCTAAAAAANNNNNNNNNNN

Figure 3d. 16S rRNA gene sequence of ASUIA243.

NTGGANGAATCCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTG
GAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGA
GTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGCGAACTTTCCA
GAGATGGATTGGTGCCTTCGGGAACGCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGT
GTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGAT
TCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTC
AAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAAG
AAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAATCCGGATCGGANTCTG
CAACTCGACTCCNTGAANTCGGAATCNCTAGTAATCGTGGATCAAANTGCCCNGGNGAATAC
GTTCCNGGCCTTGTACCCCCCCCCGTCCCCCNTGGGANTGGGNTNGNAAAAAAATAGTTNC
TTANCCCCNGGAGGGNCTTACNNTTNNNATNCTGACTGGGGNAANNAAAANAGGGNCCCCN
NAAANNNNNNNNNNNNNNNNNNNNN

Figure 3e. 16S rRNA gene sequence of ASUIA138.

the whole fermentation process thus leading to increased on phytase production. This may also explain why the phytase activity was higher in longer fermentation period.

Papagianni et al. (1999) found that when wheat bran (20 g l^{-1}) was included in a semisynthetic medium comprising cornstarch, glucose and peptone, the biomass and phytase production of *Aspergillus niger* increased and they suggested that the increased phytase production might be due to the low release of phosphorus from wheat bran or phytase induction by the presence of phytate. Phytases have been reported in a number of bacteria. Bacterial phytases are mostly cell associated, with the exception of *B. subtilis, Lactobacillus amylovorus*, and *Enterobacter* sp.4 (Vohra and Satyanarayana, 2003). In this study, all the strains produced extra-cellular enzymes where *E. sakazakii* ASUIA279 (4480 mU/ml)

GTTGGNNGACCCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAG
TGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGT
ACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAG
AGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTT
AGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGGATGACGTCA
AATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAANAGCTG
CAANACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGANTTGTAGGCTGCAA
CTCNCCTACATGAANCTGGAATCNCTAGTANTCCCGNATCACCATGCCCCGGTGAAAACNTT
CCCGGGCNTTTAANNCCCCCCCTCCNNCNCNAAAGTTNTTAAACCCCNAATCCGNGGGGGT
AACCTTTTTGANCCCCCCCCTAAGGGGGAAAAANATTGGGGNAANNCNANAANGGNACCCN
NAAAAANNNNNNN

Figure 3f. 16S rRNA gene sequence of ASUIA30.

showed the highest phytase activity, followed by *P. stewartii* ASUIA271 (1570 mU/ml) and *B. cereus* ASUIA260 (1160 mU/ml). There is no report yet on *P. stewartii* as phytase producer. Extra-cellular enzymes are good candidates for mass production and commercialization. Although inherent differences in experimental conditions and purity of enzyme preparations make comparisons difficult, the initial crude levels of activity in these bacteria compare favorably with those reported for *Selemonas ruminantium* (JY35 703 mU/ml) (Yanke et al., 1998) *B. subtilis* (44 mU/ml) (Shimizu, 1992) and *Escherichia coli* (5600 mU/ml) (Greiner et al., 1993).

Conclusion

The measured levels of phytase activity suggest that soil bacteria are a potential source of a phytase that could be developed commercially. Ongoing studies are being conducted to optimize the phytase production and further characterize phytase from selected strains. The phytase genes from these potential strains would be clone and sequence to elucidate its primary structure. And as well, to be overexpress in *E. coli* in order to increase the production level to cost-effective levels and would offer improve properties.

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