Available online freely at www.isisn.org

Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2020 17(SI-1): 134-141. **OPEN ACCESS**

Effects of carbon and nitrogen sources on different strains of phytase-producing bacteria isolated from Malaysia's Hot Spring

Nurul Asma Hasliza Zulkifly, Ainin Sofiya Kholed, Anis Adilah Mustafa, Nurul Izyan Che Mohamood, Siti Nur Dinie Mohd Zailan, and Nadiawati Alias

School of Agriculture Science and Biotechnology, Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin (UniSZA), Besut Campus, Terengganu, **Malaysia.**

***Correspondence:** asmahasliza@unisza.edu.my

The main purpose of this research was to identify the effect of carbon and nitrogen sources to the production of phytase from different potential phytase-producing bacterial strains isolated from different hot springs in Malaysia; which were in Labis, Johor (L3), Dusun Tua, Selangor (RT), Ulu Legong, Kedah (A), and Ranau, Sabah (B9). Nutrient Agar (NA) and modified Phytase Screening Medium (PSM) liquid media were used for enrichment and batch culture optimisation, respectively. Bacteria strains were screened according to their ability to grow and produce clear halo zones in solid PSM media supplemented with sodium phytate as the substrate which indicates positive phytase activity. Optimisation of growth media was studied in batch culture using shake-flask scale in laboratory. Strains growth and enzyme activity were quantitatively measured, involving supplemental parameters of carbon sources (glucose and lactose) and nitrogen sources (yeast extract and peptone). pH of the culture was also measured, where lower pH was indicated as the result of organic acids accumulation due to the process of phytic acid degradation by phytase in producing inorganic phosphate (Pi). Enzyme activity was determined according to the reaction of the phytase with its substrate (sodium phytate) and was expressed in phytase activity units (U/ mL). As for the overall, strain L3 (from Labis, Johor) showed promising rate of inorganic phosphate released in the media with glucose and yeast extract, with optimum phytase activity values of 0.220 U/mL and 0.216 U/mL, respectively. Moreover, L3 strain produced the lowest pH reading (pH 2.9) compared to other bacterial strains when supplemented with glucose and yeast extract. Due to the fact that these bacterial strains were originally taken from several hot springs, further analysis of temperature optimization and addition of other source of nutrients should be carried out. In addition, for the future plan, biochemical research and molecular identification may also be investigated to identify molecular identity of the strains.

Keywords: phytic acid, phytase, myo-inositol, sodium phytate, soybean extract, phytase-screening medium

INTRODUCTION

Chemically, phytate or phytic acid is known as myo-inositol hexakis-dihydrogen-phosphate (IP6), which catalyse the hydrolysis removal of phosphates from phytic acid or its salt phytate to less phosphorylated myo-inositol intermediates and inorganic phosphate (Pi) (Rocky-Salimi et al. 2016). This phytate-degrading enzyme could be recovered in numerous sources such as plants, microorganisms, yeast and certain animal tissues (Gibson and Ullah, 1988). Four classes of phytases have been recently characterized in terrestrial organisms. The class of histidine acid phosphatases (HAPs) is the first and most broadly

studied group of phytases. The other groups of phytase are categorised as b-propeller phosphatase (BPPhy), purple acid phosphatase (PAP) and protein tyrosine phosphatase (PTP (Chu et al. 2004).

The harmful effects of phytate on the availability of phosphates and other nutrients have long been detected. Phytate in the digestive tract of monogastric animals mainly like fish, poultry, and pig is barely available to metabolize phytic acid due to insufficient levels of phytate-degrading enzymes (Singh et al. 2016). Phytase and phosphatase are the enzymes that facilitate the mineralization of phosphate. Phytase produced by the microbes reduce the phosphorus concentration in the water sources that caused the death of aquatic animals (Kusale and Attar, 2017).

Microbial phytases represent a crucial technical enzyme in the feed industry as for their importance in human and animal nutrition (Lei et al. 2013). In recent years, effectiveness of phytase in efficacy trials showed a positive result when tested in food additive of human (Troesch et al. 2011). Using wheat phytase in improving infant formulas from soybean milk and adding *Aspergillus niger* phytase helps in improving bread iron availability as well as escalating iron absorption in human (Sandberg et al. 1996).
Moreover, Pagano et al. (2007) stated that osteoporosis can be treated or prevented when phytase being used alone or in combination with other reagents. Because this phytase are well known, it gives a great potential for industrial applications of large-scale productions such as food processing.

According to Liu et al. (1998), phytase also has been speculated to be important in the pulp and paper industry. During the pulp and paper processing, a thermostable phytase could potentially degrade phytic acid as highly toxic by-products may not produce through this degradation.

Bhavsar and Khire (2014) in their research stated that phytase has its application in agricultural, animal and human nutrition as they improve the availability of phosphate needed by organisms. However, there was no phytase relevant for human food application. There was potential of using bacterial and fungal producing phytase in improvement of phosphorus utilisation in the feed industry.

Optimisation of the culture conditions such as carbon source, nitrogen source, temperature, pH and agitation can influence the high production of phytase. Carbon and nitrogen sources give significant effects to the growth of bacteria and their phytase production (Sreedevi and Reddy, 2012). Glucose is commonly utilised by microorganisms due to simplest carbon source which enhanced biomass along with high production of phytase (Das and Ghosh, 2014) as it is providing faster growth rate compared to other sugars. Glucose is a major contributor of energy to the cells as it is directly induced into glycolysis in the form of glucose 6-phosphate and degraded through the tricarboxylic acid (TCA) cycle. In this study, we used glucose and lactose as the carbon source for optimisation. Previous study showed that lactose is a poor carbon source for enzyme production since it was not inducing phytase activity compared to glucose (Khianngam et al. 2017).

Meanwhile, yeast extract and peptone are used as nitrogen sources for optimisation because nitrogen source is significant in phytase production which can affect the enzyme productivity. The growth and production of phytase will be greatly affected with the absent of nitrogen source in growth medium. Generally, organic nitrogen source such as biopeptone supported the production of cell mass compared to inorganic nitrogen source (Banerjee, 2002). Nitrogen source is important in the growth of organism and the production as the nitrogen source is the secondary energy sources for the organisms. The concentration and nature of the compound being used may stimulate or down modulate the enzyme production (Kumar et al. 2013). For industrial fermentation medium, the choice of appropriate nitrogen sources is crucially important as it act as the utmost precursor for protein synthesis (Kote et al. 2009); which can affect the pH of the medium (Jia et al. 2015), while also affecting the enzyme activity and stability (Bajaj et al. 2014). In this study, yeast extract and peptone being used to identify the effect of nitrogen sources on different strains of phytase producing bacteria isolated from Malaysia's hot spring. There were some reports stating that 1 % of yeast extract resulting in maximum phytase production (Sasirekha, 2012).

MATERIALS AND METHODS

Screening of Phytase-Producing Bacteria

The bacterial strains used in this study were originally isolated from several hot springs in Malaysia; which were L3 (from Labis, Johor), RT (Ranau, Sabah), A (Ulu Legong, Kedah) and B9

(Dusun Tua, Selangor). All of these samples were previously collected by Mohamad et al. (2012) to screen for the phytase positive strains.

Each bacterial strain from the glycerol stocks were grown on Nutrient Agar (NA) plates and incubated for 24 h at 37°C. Under an aseptic condition, inoculation loop was used to pick up a single colony from the streaked plates and inoculate into sterile tube containing Luria Bertani (LB) broth. The mixtures were then incubated at 37°C for at least 18 h in an incubator shaker (200 rpm). All selected single strong colonies were maintained as slant cultures.

Screening for phytase production was accomplished by using phytase screening media (PSM). Samples were taken out after 72 h of incubation and the analysis of phytase activity was performed by detecting the inorganic phosphate released. The bacteria strain that produced the highest phytase activity was selected for subsequent characterization and phytase production.

Growth Conditions

All bacteria strains which have the ability to produce phytase were grew in modified PSM media. The substrate used was soybean meal extract. The cultures were grown at 37°C on a 200-rpm rotary shaker in 500 mL flasks containing 100 mL medium (Alias et al. 2017). The liquid modified PSM was prepared to contain ($g.L^{-1}$) glucose, 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; NH_4NO_3, 5.0; $MnSO_4.H_2O$, 0.1; $CaCl_2.2H_2O$, 5.0; $FeSO_4.H_2O$, 0.1; yeast extract, 1.0 and with 0.1% Tween-80 and 0.1% citric acid in 10% soybean meal extract solution. The pH of 5.5 was adjusted by using NaOH 1 M, and were autoclaved (121ºC for 15 min at 15 psi). Oxoid bacteriological agar (15 $g.L^{-1}$) was added prior to autoclaving. Lactose and peptone were used for the purpose of carbon and nitrogen sources optimisation.

The soybean extract was prepared by soaking 100 g soybean in 1 L distilled water for 8 h. The suspension was boiled for 6 min, cooled to room temperature, filtered through filter cloth and adjusted to the volume of 1 L. These boiled extracts were used as stocks for complex medium preparation (Dharmsthiti et al. 2005).

Screening for Phytate-Degrading Strains

The screening for phytate-degrading strains was carried out according to Alias et al. (2017). A single colony of different bacterial strains from NA plates were taken and streaked on PSM agar. The plates were incubated at 37°C for 72 h. Then, the formation of clear zone around the bacterial growth were measured. The formation of clear zones around the microbial colonies exhibited positive strains (Zulkifly et al. 2010), which indicate phytase activity occurred (Fredrikson at al. 2002) as the colonies capable of hydrolysing Na-phytate showed against an opaque colour of non-hydrolysed medium.

Growth Analysis

A volume of 1.0 mL samples was pipetted out periodically at every hour intervals for the first 8 h and further 24-, 48- and 72-hours period for bacteria growth determined by measuring turbidity (Optical Density, OD) on a Shimadzu UVmini-1240 UV-Vis Spectrophotometer at 600 nm wavelength. The cultivability of the cells was determined by serially diluted 100 µL of the cell cultures in 900 µL sterile PBS up to 10^7 dilutions, plated onto non-selective NA plates, incubated at 30°C for 24 h and measured as CFU (Colony Forming Unit).

Analytical Methods

A volume of 5.0 mL samples was pipetted out aseptically from conical flasks in the incubator shaker after 72 h incubation of the batch culture. Then, the sample was transferred into a falcon tube and spinned at 12,000 rpm (4°C) for 20 min. The supernatant was subsequently used for phytase assay.

Phytase Assay

Activity of the enzyme was determined by quantitatively measuring the released amount of inorganic phosphate during enzymatic reaction of phytase. Each assay was carried out in duplicates, by incubating the reaction mixture consisted of 400 µL of 1.5 mM sodium phytate in 100 mM Tris–HCl buffer (pH 7.0) and 100 µL of crude enzyme at 55°C for 30 min. The reaction was terminated by adding 400 µL of colour reagent solution (1.5:1.5:1) ratio of 0.24% ammonium vanadate, 10% ammonium molybdate and 65% nitric acid. Then, the samples were centrifuged at 12,000 rpm (room temperature) for 10 min (Rocky-Salimi et al. 2016).

The resulting yellow colour developed by phytase activity were measured spectrophotometrically at 415 nm wavelength (Boyce et al. 2004) and compared by using the standard curve prepared from KH_2PO_4. For phytase activity based on Pi release, one unit (U) of phytase activity is defined as the amount of enzyme liberating 1 µmol of inorganic phosphate

per minute under appropriate conditions (Qvirist et al. 2015).

RESULTS

Optimisation of Carbon Sources

In this study, we used glucose and lactose as the carbon source for optimisation. Bacterial strains were grown in PSM media supplemented with lactose have a higher ODs compared to the bacterial cultures grown in glucose as each point of hour showing different values in the ODs. Out of all bacteria strains, strain A (with OD_{600} 9.56) and strain B9 (6.68) are found to be the better strains that grow in the media supplemented with lactose. Between these two carbon sources, all strains showed more favourable growth condition in lactose compared to glucose (Figure 1).

A study was conducted by Cheng et al. (2015) for *Streptococcus suis* fermentation process where the carbon sources used into the fermentation medium were glucose, sucrose, lactose and galactose. The result indicated that when glucose was added, the cell density (0.884) and viable count (1.42×10^9 CFU.mL^{-1}) were the lowest. Meanwhile, in the presence of lactose, the growth yielded an increase in cell density (1.023) and viable count (1.72×10^9 CFU.mL^{-1}).

In contrast for this observation, some of the CFU counts were surprisingly inconsistent with the observed ODs when the bacterial strains were grown in the medium containing lactose. Strains L3, B9 and RT had a lower count of the CFU compared to strain A (2.0×10^9 CFU.mL^{-1}), where it presented the highest value from all strains at two time points (5 and 24 hours). Strains incubated with glucose had the consistency between CFU counts and the observed ODs, where the CFU count of strain A (1.27×10^9 CFU.mL^{-1}) were the highest while strain RT (1.2×10^9 CFU.mL^{-1}) were the lowest.

The phytase activity was assayed according to Rocky-Salimi et al. (2016). After three days of incubation at 37°C, the cultures were harvested to obtain the supernatants which containing inorganic phosphate (Figure 2). Phytase activity was determined by measuring the amount of phosphate released from sodium phytate during enzymatic reaction. The absorbance used to measure the concentration of phosphate was at 415 nm. The phytase activity was expressed in phytase units (U).

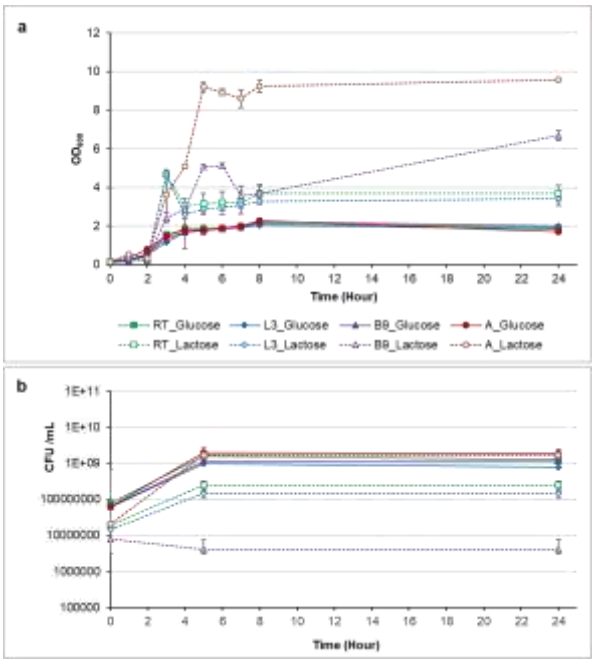

Figure 1: Correlation of all strains growth for the study of carbon sources optimisation for phytase production, evaluated by (a) OD_{600} measurement, and (b) CFU.mL^{-1} counting.

All potential phytase-producing bacterial strains were grown at 37°C (pH 5.5) and supplemented with two different carbon sources; glucose and lactose. The OD_{600} was measured at intervals as shown in Figure 1(a). Serial dilution of culture samples at 0 h, 5 h and 24 h post-inoculation was plated onto NA agar for indication of cell viability as shown in Figure 1(b). All data shown are mean values from replica flasks with error bars are ±1 standard deviation.

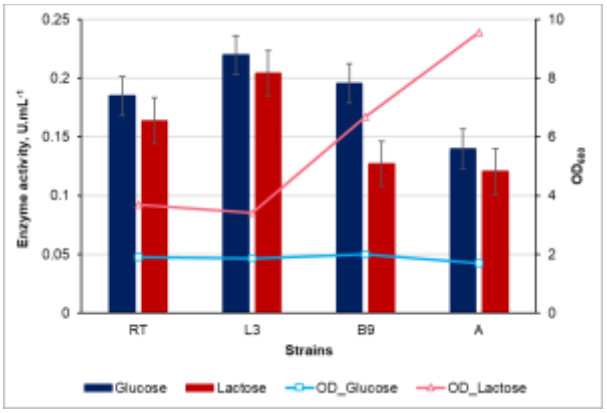

Figure 2: Effect of carbon sources on growth and phytase production by all potential bacterial strains. Each flask was inoculated with 1% inoculum (overnight culture) and incubated at 37°C for 72 h.

[Blue] Optimisation results of glucose as carbon source. [Red] Optimisation results of lactose as carbon source. Samples were taken for bacterial growth measurement and phytase activity. Results are means of two duplicate samples. Bar corresponds to standard deviation.

One unit of phytase activity is defined as the amount of enzyme that releases 1 micromole of inorganic phosphate per mL per minute under the assay condition.

All bacterial strains have shown their ability to produce phytase enzyme. Among two carbon sources investigated, the maximum phytase activity was recorded for all strains in the presence of glucose; L3 (0.220 U.mL^{-1}), RT (0.185 U.mL^{-1}), B9 (0.196 U.mL^{-1}), and A (0.140 U.mL^{-1}). This result has supported the statement regarding strain L3 is the best phytase producer when using glucose as the carbon source by Alias et al. (2017). Meanwhile, the minimum phytase activity was recorded in presence of lactose for most observed strains, where strain A (0.120 U.mL^{-1}) and B9 (0.127 U.mL^{-1}) gives out the lowest value. This indicated that the strains were preferred to produce high phytase in presence of glucose compared to lactose.

At the end of incubation hours, the result of estimated change of pH for the four strains supplemented with glucose showed that all could produce organic acid that leads to decrease in the pH of the medium (Hosseinkhani et al., 2010). The pH of media at first was set at 5.5 and gradually decreased to A (3.62), B9 (2.92), L3 (2.9) and RT (2.91). Meanwhile for the culture medium with lactose, one of the strains obtained an alkaline pH which is A (7.28) while for others, B9 (3.77), L3 (6.06) and RT (5.33) produced an acidic condition after fermentation for three days. Therefore, lower pH indicates higher phytase activity. This was proved by strain A of both carbon sources which had highest pH and resulting in lowest phytase activity.

Optimisation of Nitrogen Sources

The result for batch culture using peptone and yeast extract as the nitrogen sources are shown in Figure 3 below. Based on Figure 3, strain L3 using yeast extract shows the highest phytase production with 0.216 U.mL^{-1} followed by strain B9 with 0.196 U.mL^{-1}, strain RT (0.185 U.mL^{-1}) and the lowest is strain A (0.140 U.mL^{-1}). While in fermentation using peptone as the nitrogen source resulting that strain RT shows the highest phytase production of 0.125 U.mL^{-1}, followed by L3 (0.111 U.mL^{-1}), strain A (0.106 U.mL^{-1}) and the lowest is strain B9 with 0.0257 U.mL^{-1}. Generally, Figure 3 proved that yeast extract acts as a good nitrogen source compared to peptone in producing phytase enzyme during the fermentation.

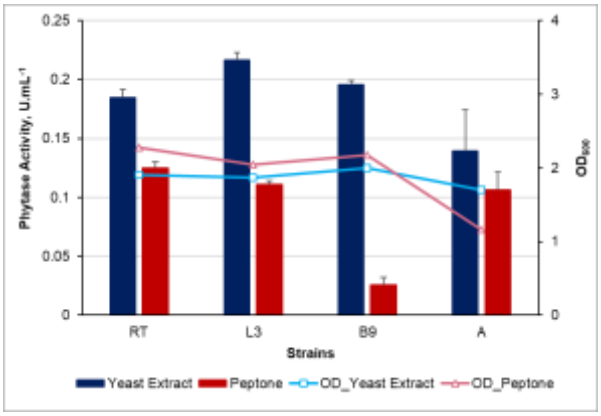

Figure 3: Effect of nitrogen source on growth and production of phytase by all potential bacterial strains. Each flask was inoculated with 1% inoculum (overnight culture) and incubated at 37°C for 72 h.

[Blue] Optimisation result of yeast extract as nitrogen source. [Red] Optimisation result of peptone as nitrogen source. Samples were taken for bacterial growth measurement and phytase activity. Results are means of two duplicate samples. Bar corresponds to standard deviation.

Figure 4 shown effect of nitrogen sources on phytase-producing bacteria growth. Strain A grown in culture media that using yeast extract as nitrogen source showed the highest OD compared to three other strains. However, in batch culture fermentation using peptone as nitrogen source showed that strain RT has the highest OD compared to the three other strains. Strain A showed the best growth among the other strains when fermented with yeast extract meanwhile opposite result obtained from fermentation using peptone in which strain A had the lowest growth compared to other strains.

On the other hand, Figure 3 showed that the growth of cells not directly influenced the phytase activity. This was proven when strain A which had the highest growth among other strains fermented with yeast extract produced the lowest phytase production (0.140 U.mL^{-1}). And also, strain A had the lowest growth among other strains fermented with peptone and produced the third lowest phytase production (0.106 U.mL^{-1}).

The growth of the phytase producing bacteria observed during 5 h after incubation. The highest CFU indicated that the OD value at 5 h has the greatest number of viable bacteria, where rapid growth shown. All bacterial strains using yeast extract as nitrogen source had higher growth than strains in fermentation using peptone as shown in

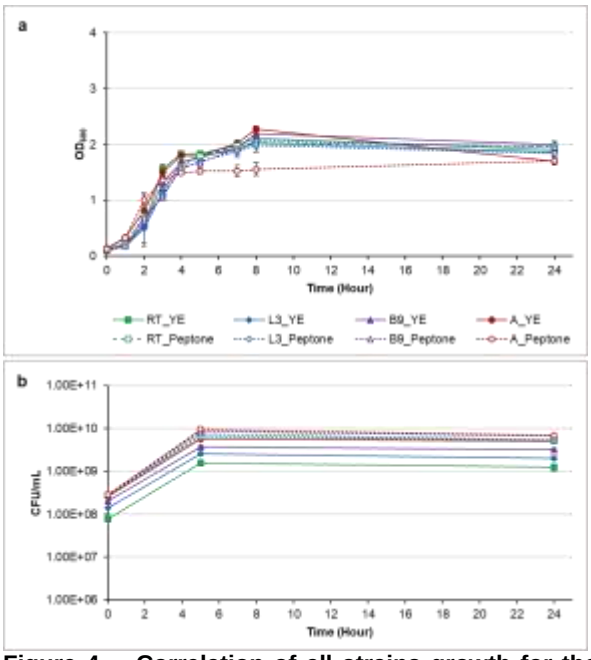

Figure 4: Correlation of all strains growth for the study of nitrogen source optimisation for production of phytase, evaluated by (a) OD_{600} measurement, and (b) CFU.mL^{-1} counting.

All potential phytase-producing bacterial strains were grown at 37 °C (pH 5.5) and supplemented with two different nitrogen sources; yeast extract and peptone. The OD_{600} was measured at intervals as shown in Figure 4(a). Serial dilution of culture samples at 0 h, 5 h and 24 h post-inoculation was plated onto NA agar for indication of cell viability as shown in Figure 4(b). All data shown are mean values from replica flasks with error bars are ±1 of standard deviation.

Figure 4. However, the contrast occurred as all bacterial strains using yeast extract had the lower CFU compared to bacterial strains using peptone despite of their higher growth. The results also showed that the growth of bacterial strain observed in OD measurement did not directly influenced the viable bacteria obtained in CFU.

At the end of the fermentation, the measurement of pH was carried out to determine the culture condition. For media with yeast extract used as nitrogen source, the starting culture conditions which was pH 5.5 changed to acidic medium with strain A (3.62), B9 (2.92), L3 (2.9) and RT (2.91). Meanwhile for the culture media that used peptone as nitrogen source, the media changes slightly into acidic with strain A (4.87), B9 (3.24), L3 (3.19) and RT (3.24). From the degradation of the phytate by phytase enzyme in the culture media, there was production of organic acid as by-products. The pH of the culture media shift to acidic due to the accumulation of organic acid (Hosseinkhani and Emtiazi, 2010).

DISCUSSION

A study conducted by Qasim et al. (2017) for the effect of carbon sources to the phytase production by *Aspergillus tubingensis* SKA showed that addition of glucose produced the highest phytase yield (42.0 U.mL^{-1}) while lactose yielding significantly less phytase. Similar studies reported for *Penicillium purpurogenum*, where among different carbon sources; glucose, corn starch, sucrose, maltose and lactose, the result showed only glucose achieved the maximum phytase productivity (Awad et al. 2014). These findings had led to the frequently used of glucose in the industrial fermentations; as raw material for the most effective sources of carbon and energy.

In culture media incorporated with 0.1 % yeast extract, 10 % soybean extract, 0.1 % Tween 80 and citric acid showed high OD of bacterial cells (6.0) and phytase activity (Dharmsthiti et al. 2005). There were some reports stated that 1% of yeast extract results in maximum phytase production (Sasirekha, 2012). Yeast extract is the best nitrogen source followed by peptone and tryptone for production of better phytase (Singh et al. 2016). From the research conducted by Tungala et al. (2013) they observed that yeast extract showed maximum phytase activity. From the result of the batch culture conducted with all the references stated, yeast extract was supported as optimum nitrogen sources for phytase.

CONCLUSION

In order to select bacteria that produce phytase, the major concern will always be high specificity activity because it is very crucial for feed industrial application. Bacterial phytase have established significant attention as they are essential to maintain of the ecological balance. In addition, it also has been used to determine the mechanism of phytate degradation at biochemical and molecular stages. This study succeeded in identifying the effects of carbon and nitrogen sources on each strain of phytase-producing bacterial. The results obtained from this study show that glucose and yeast extract act as the optimum carbon and nitrogen sources which contribute to maximum phytase production compared to lactose and peptone, as supported by previous studies. To date, there have been numerous reports on the primary structure and function of phytate, but detailed information on

phytase is still lacking. This study is also important to screen bacteria that fundamentally produce phytase at the highest yields in the optimised culture medium. All relevant parameter measurements were applied in this study. Furthermore, this study had also identified several bacterial strains that are useful for phytase production.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

The authors would like to thank Universiti Sultan Zainal Abidin (UniSZA) for their generous support of this research especially in terms of financial and laboratory facilities.

AUTHOR CONTRIBUTIONS

NAHZ designed and supervised the experiments, as well as wrote the manuscript. ASK, AAM, NICM and SNDMZ performed bacterial growth experiments, batch culture optimisation, phytase assay, data analysis and also wrote the manuscript. NA co-supervised and reviewed the manuscript. All authors read and approved the final version.

REFERENCES

Alias N, Shunmugam S, Ong PY, 2017. Isolation and Molecular Characterization of Phytase Producing Bacteria from Malaysia Hot Springs. J Fundam Appl Sci 9(2S): 852-865.

Awad GEA, Helal MMI, Danial EN, Esawy MA, 2014. Optimization of phytase production by *Penicillium purpurogenum* GE1 under solid state fermentation by using Box-Behnken design. Saudi J. Biol. Sci 21(1): 81-88.

Bajaj BK, Singh S, Khullar M, Singh K, Bhardwaj S, 2014. Optimization of Fibrinolytic Protease Production from *Bacillus subtilis* I-2 Using Agro-Residues. Braz Arch Biol Technol 57(5): 653–662.

Banerjee UC, 2002. Studies on the production of phytase by a newly isolated strain of *Aspergillus niger var teigham* obtained from rotten wood-logs. Process Biochem 38: 211-217.

Bhavsar K, Khire JM, 2014. Current research and future perspectives of phytase bioprocessing. RSC Adv 4(51): 2667-2669.

Boyce A, Casey A, Walsh G, 2004. A Phytase Enzyme-based Biochemistry Practical. IUBMB 32(5): 336-340.

Cheng L, Wang J, Fu Q, Miao L, Yang X, Li S, Shen Z, 2015. Optimization of carbon and nitrogen sources and substrate feeding strategy to increase the cell density of *Streptococcus suis*. Biotechnol. Biotechnol. Equip 29(4): 779-785.

Chu HM, Guo RT, Lin TW, Chou CC, Shr HL, Lai HL, Wang AHJ, 2004. Structures of *Selenomonas ruminantium* phytase in complex with persulfated phytate: DSP phytase fold and mechanism for sequential substrate hydrolysis. Structure 12(11): 2015-2024.

Das S, and Ghosh U, 2014. Effect of nutritional supplementation of solid-state fermentation medium on biosynthesis of phytase from *Aspergillus niger* NCIM 612. J. Sci. Ind. Res 73: 593-597.

Dharmsthiti S, Chalermpornpaisarn S, Kiatiyajarn M, Chanpokapaiboon A, Klongsithidej Y, Techawiparut J, 2005. Phytase production from *Pseudomonas putida* harbouring *Escherichia coli* appA. Process Biochem 40(2): 789-793.

Fredrikson M, Andlid T, Haikara A, Sandberg AS, 2002. Phytate degradation by microorganisms in synthetic media and pea flour. J. Appl. Microbiol 93(2): 197-204.

Gibson DM, Ullah AH, 1988. Purification and characterization of phytase from cotyledons of germinating soybean seeds. Arch Biochem Biophys 260(2): 503-513.

Hosseinkhani B, Emtiazi G, 2010. Analysis of phytase producing bacteria *Pseudomonas sp* from poultry faeces and optimization of this enzyme production. Afr. J. Biotechnol 8: 2-6.

Jia J, Yang X, Wu Z, Zhang Q, Lin Z, Guo H, Lin CSK, Wang J, Wang Y, 2015. Optimization of Fermentation Medium for Extracellular Lipase Production from *Aspergillus niger* Using Response Surface Methodology. Biomed Res. Int 2015: 1-8.

Khianngam S, Pootaeng-on Y, Sonloy A, Kajornaroonkij J, Tanasupawat S, 2017. Characterization and comparison of phytase production by *Bacillus*. Malays J Microbiol 13(Dec): 318-325.

Kote NV, Patil AGG, Mulimani VH, 2009. Optimization of the Production of Thermostable endo-β-1,4 Mannanases from a Newly Isolated *Aspergillus niger gr* and *Aspergillus flavus gr*. Appl Biochem Biotechnol 152: 213–223.

Kumar D, Rajesh S, Balashanmugam P, Rebecca LJ, Kalaichelvan PT, 2013. Screening, Optimization and Application of Extracellular Phytase from *Bacillus megaterium* Isolated from Poultry Waste. Journal of Modern Biotechnology 2(2): 46-52.

Kusale SP, Attar YC, 2017. Optimization and Production of Phytase by *Bacillus subtilis* and its Agricultural Application. IJRASET 5: 1595-1602.

Lei XG, Weaver JD, Mullaney E, Ullah AH, Azain MJ, 2013. Phytase, a New Life for an 'Old' Enzyme. Annu. Rev. Anim. Biosci 1(1): 283-309.

Liu BL, Rafiq A, Tzeng YM, Rob A, 1998. The induction and characterization of phytase and beyond. Enzyme Microb Technol 22(5): 415-424.

Mohamad NM, Ali AM, Salleh HM, 2012. Carbon Sources Utilization Profiles of Thermophilic Phytase Producing Bacteria Isolated from Hot-spring in Malaysia. World Acad Sci Eng Technol 64(4): 304-307.

Pagano AR, Yasuda K, Roneker KR, Crenshaw TD, Lei XG, 2007. Supplemental *Escherichia coli* Phytase and Strontium Enhance Bone Strength of Young Pigs Fed a Phosphorus-Adequate Diet. J Nutr 137(7): 1795-1801.

Qasim SS, Shakir KA, Al-Shaibani AB, Walsh MK, 2017. Optimization of Culture Conditions to Produce Phytase from *Aspergillus tubingensis* SKA. Food Sci Nutr 8(7): 733-745.

Qvirist L, Carlsson N, Andlid T, 2015. Assessing phytase activity - Methods, Definitions and Pitfalls. J. Biol. Methods 2(1): 1-7.

Rocky-Salimi K, Hashemi M, Safari M, Mousivand M, 2016. A novel phytase characterized by thermostability and high pH tolerance from rice phyllosphere isolated *Bacillus subtilis* B.S.46. J. Adv. Res 7(3): 381-390.

Sandberg AS, Hulthén LR, Türk M, 1996. Dietary *Aspergillus niger* phytase increases iron absorption in humans. J. Nutr 126(2): 476-480.

Sasirekha B, 2012. Optimization and partial purification of extracellular phytase from *Pseudomonas aeruginosa* p6. Eur. J. Exp. Biol 1: 95-104.

Singh NK, Joshi DK, Gupta RK, 2016. Isolation of Phytase Producing Bacteria and Optimization of Phytase Production Parameters. Jundishapur J.Microbiol 6(5).

Sreedevi S, Reddy BN, 2012. Isolation, Screening and Optimization of Phytases Production from Newly Isolated *Bacillus sp* C43. Int. J. Pharm. Biol. Sci 2: 218-231.

Tungala A, Anantha NK, Muthuraman MS, 2013. Isolation of phytase producing bacteria from poultry faeces and optimization of culture conditions for enhanced phytase production. Int J Pharm Pharm Sci 5(4): 264-269.

Troesch B, van Stuijvenberg ME, Smuts CM, Kruger HS, Biebinger R, Hurrell RF, Baumgartner J, Zimmermann MB, 2011. A micronutrient powder with low doses of highly absorbable iron and zinc reduces iron and zinc deficiency and improves weight-for-age Z-scores in South African children. J. Nutr 141(2): 237-242.

Zulkifly AH, Roslan DD, Hamid AAA, Hamdan S, Huyop F, 2010. Biodegradation of Low Concentration of Monochloroacetic Acid-Degrading *Bacillus sp.* TW1 Isolated from Terengganu Water Treatment and Distribution Plant. J Appl Sci 10(22): 2940-2944.