JOURNAL OF AGROBIOTECHNOLOGY 2020, VOL 11(1S):79-91
e-ISSN: 2180-1983
http://dx.doi.org/10.37231/jab.2020.11.1S.237
https://journal.unisza.edu.my/agrobiotechnology/index.php/agrobiotechnology/index

Effect of pH on Different Strains of Phytase Producing Bacteria from Malaysia's Hot Spring

Ainin Sofiya Kholed, Nurul Asma Hasliza Zulkifly*, Afnani Alwi@Ali, Tajul Afif Abdullah and Nadiawati Alias

School of Agriculture Science and Biotechnology, Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Besut Campus, 22200 Besut, Terengganu, MALAYSIA

***Corresponding author: asmahasliza@unisza.edu.my**

Received: 16/08/2020, Accepted: 23/10/2020, Available Online: 31/10/2020

ABSTRACT

In the recent research, the optimisation of culture condition for phytase production rarely done for *Acetinobacter baumanii*. The optimisation of the phytase production from the bacterial strains largely contributed by *Bacillus sp*. The study on the phytase originated from hot spring are limited and the species that identified from the hot spring samples are not in the same species from the previous study and mainly the species isolated from *Bacillus sp*. In this study, four potential strains of bacteria producing phytase isolated from hot spring in several regions in Malaysia. For enrichment of the bacterial, Nutrient Agar was used, meanwhile for batch culture optimisation, the bacteria producing phytase grown in modified liquid Phytase Screening Media with soy extract as agro residual substrate as a replacement for sodium phytate, the chemical substrate. The bacteria were screened for their ability to produce clear zone in solid PSM with sodium phytate as substrate. Optimisation of media through its physical factor that is pH of the media carried out using shake flask scale in laboratory. The growth of the bacterial strains and phytase activity measured quantitatively through the two different pH of media at pH 5.5 and pH 7. The analysis of colony-forming unit and pH determination after fermentation was carried out in this study. From the study, bacterial strain L3 from Labis, Johor has the highest phytase activity in the two parameters studied where the inorganic phosphate released at pH 5.5 (0.21953 U/mL) and pH 7 (0.2047 U/mL). Optimisation carried out through manipulating the culture condition that is pH of the media to determine at which condition has the highest phytase production. Several effects on enzyme activity caused by culture conditions identified. The optimisation of the fermentation medium able to contribute to the less cost production of the industrial enzyme as phytase has potential production for feed additives for poultry feeding. In the future research, molecular identification of the bacterial strains from the better-quality bacteria producing phytase grown in optimised culture media to investigate the molecular identity of the bacterial.

Keywords: Phytase, phytate, bacteria producing phytase, sodium phytate, soybean extract

INTRODUCTION

Phytic acid (myo-inositol, 1, 2, 3, 4, 5, 6-hexakis (dihydrogen phosphate) or phytate in the nature are the storage form of phosphate in mixed cation salts of phytic acid nature (Jorquera et al., 2008). In the mechanism of

nutrient absorption of animal digestive system, phytic acid chelates the micronutrient absorption. (Singh et al., 2013). As phytate are negatively charged, they able to chelates the positively charged nutrients (Dermikan et al., 2014). Phytic acid forms complex with the chelated nutrient as it has high phosphate content with high negative charge that able to chelate the positive charged nutrients. The complex formed affected the digestion by the monogastric animals as the inhibition of the other enzyme such as amylase, trypsin, acid phosphatase and tyrosinase (Jongbloed et al., 2000). Phytate causes environmental problems when high concentration of phytate release by the animal after feeding as manure, the excessive phosphorus in the manure causes water pollution (Coban et al., 2014). The high concentration of phosphorus in water favours for algal bloom in the surface of water that caused the sunlight unable to penetrate the water source caused the aquatic plant unable to carry out their mechanism for feeding (Kumar et al., 2012).

Phytase or myo-inositol hexakis phosphate phosphohydrolase are a group of enzymes that hydrolyse phytic acid or phytate releasing lower inositol phosphates and inorganic phosphate (Vats & Banerjee, 2004). Phytase may originated in plants, animal tissues, some microorganisms. Phytase supplemented in animal feed to resolve the phosphorus bound in phytic acid that incorporate in the animal feed that usually source from legumes crops for efficient absorption in the intestine of monogastric animals as they lack of the enzyme to catalyze phosphate originate from the feed source from leguminous plant (Hill et al., 2009).

Phytase produced by the microbes reduce the phosphorus concentration in the water sources that caused the death of aquatic animals (Kusale & Attar, 2017). Phytase has its application in agricultural, animal and human nutrition as they improve the availability of phosphate needed by organisms (Bhavsar & Khire, 2014). The application of biotechnological using the microorganism such as fungus and bacteria applied in environmental protection to avoid the water pollution. Phytase as an animal feed additive has impact in animal feed industries (Jorquera et al., 2008).

The range of pH at 5.0 to 7.0 is the optimum pH for phytase production by most of the bacteria and fungi (Vohra & Satyanarayana, 2003). The favourability of the bacterial towards certain pH influence the production of phytase. According to Singh et al. (2013) when pH is lower or higher than the optimum pH it will affect the phytase production as the bacteria producing phytase sensitive towards the changes in the environment. The production of phytase influence the degradability of phytate. pH 5.5 was optimum pH set at culture media by Alias et al. (2017) and Mohamad et al. (2010), while another parameter of pH set as pH 7 as there were bacteria producing phytase that native from hot spring in Tarabalu, Orissa India grown in pH 7 (Khuntia et al., 2010). In this study, pH of the media optimised in two condition that are pH 5.5 and pH 7 to observe the maximum phytase production in different conditions.

MATERIALS AND METHODS

Bacterial sample

Bacteria producing phytase isolated from hotspring of several regions in Malaysia such as Labis, Johor for bacteria strain L3, Ulu Lenggong, Kedah for bacteria strain A, Ranau, Sabah for bacteria strain RT and Dusun Tua, Selangor for bacteria strain B9. The samples of bacteria collected by Mohamad et al. (2012) and screened for their phytate degradability on solid PSM by Alias et al. (2017). The transparent clear zone produced by the strains indicated positive results where the bacterial have the ability to degrade sodium phytate that supplemented in the PSM media (Zulkifly et al., 2010).

Revival of Phytase producing bacteria strains

For the revival of the bacterial strains in glycerol stocks streaked on Nutrient Agar (NA) and incubated at 37°C for 24 hours. The single colony grown after 24 hours was used as inoculum for overnight culture in Luria Bertani (LB) broth proceed with incubation in incubator shaker at 180 rpm, 37°C for at least 18 hours.

The overnight culture prepared for inoculation in Phytase Screening Media in optimum growth curve and batch culture later.

Bacterial growth curve

The bacterial grown in liquid modified Phytase Screening Media (PSM) with the substrate used was 10% soybean extract. The cultures grown in 500 mL flasks with 100 mL of inoculated PSM medium at 200 rpm rotary shaker at 37°C for 72 hours. The liquid modified PSM prepared with (g/L) glucose, 1.0; 0.5 KCl, 0.5; $MgSO_4.7H_2O$, 0.5; NH_4NO_3, 5.0; $MnSO_4.7H_2O$, 0.1; $CaCl_2.2H_2O$, 5.0; $FeSO_4.H_2O$, 0.1; yeast extract, 1.0; 0.5% Tween 80 and 0.1% of citric acid. The media set at the designated parameter at pH 5.5 and pH 7 with 1 M NaOH before autoclaved for 15 minutes at 15 psi. The preparation of soybean extract by soaking 100 g of soybean in 1 L of distilled water for 8 hours before the suspension boiled at 6 minutes then the extract left to cool in room temperature before it was filtered using muslin cloth and adjusted to a volume of 1 L (Lan et al., 2002).

Growth analysis

1 mL sample pipetted at hourly interval for the first 8 hours and further with 24 hours period to determine the biomass of the sample through turbidity measurement on a Shimadzu UV mini-1240 UV-Vis Spectrophotometer at 600 nm wavelength. The dilution of the media using PBS carried out when the OD exceed the reading of 0.8 for the purpose for accurate measurement of the turbidity of the sample. At interval time of 0 hour, 5 hours and 24 hours after incubation where the 0 hour of incubation is the starting point of the bacterial growth where the bacterial grown in LB broth inoculated in the PSM media. 5 hours after incubation, the bacterial is massively dividing in the media as they entered log phase and last point of sampling at 24 hours the bacterial entered death phase. The sample then diluted with sterile PBS was plated on nutrient agar and left incubated for 24 hours for bacterial colonies grow for colony forming unit analysis in unit of colony forming unit per 1 mL.

Phytase assay

Phytase activity exhibited by the strains measured by using the supernatant from the sample of 72 hours after incubation of the batch culture which the crude enzyme harvested through centrifugation at 12,000 rpm (4°C) for 20 minutes. The crude enzyme from the supernatant used for phytase assay.

The activity of enzyme measured by the released of inorganic phosphate during enzymatic reaction through ammonium molybdate method by Engelen et al. (2001). Each assay for the parameters carried out in duplicate through incubation of 400 µL of 1.5 mM sodium phytate in Tris-HCL buffer at pH 7 with 100 µL of crude enzyme harvested from the sample. The reaction mixture was incubated at 55°C for 30 minutes. The reaction terminated with 400 µL of stop reaction mixture at ratio of 1.5: 1.5: 1 of 10% ammonium molybdate, 0.24% ammonium vanadate and 65% of nitric acid. The mixture was centrifuged at room temperature for 10 minutes at 15,000 × g. From the reaction, yellow colour developed and measured spectrophotometrically at 415 nm and compared using standard curve generated from of KH_2PO_4 at different dilutions. Under assay conditions, the liberated inorganic phosphorus at 1 µmol by an amount of enzyme defined as one unit of phytase activity (Rocky et al., 2016). In other word, 1.0 U of phytase activity is the amount of the enzyme catalyze the production of inorganic phosphate per minute in assay condition (Boyce et al., 2004).

Determination of pH of the culture media after incubation

The pH of culture media was measured using pH meter to identify the pH at 0 hour, 24 hours and 72 hours of incubation at the end of fermentation. The production of inorganic phosphate from degradation of phytic acid source from the substrate, soy extract. The pH of the media that was initially adjusted using NaOH affected by the bacterial growth in the media or phytase activity. The degradation of phytic acid in the media by phytase

contributed to the pH changed after the fermentation. The growth of the bacteria or phytase activity proven through the change of the media as pH of the media.

Statistical analysis

The experiments were conducted using two different pH of culture media, made in duplicates of the replicate flasks. Data were recorded and analysed using Microsoft Excel Software 2016; tested using T-test to determine the *P* value. The group means were considered significantly different at $P<0.05$.

RESULTS AND DISCUSSION

Bacterial growth curve

The standard growth curve was determined through optimum condition for fermentation. The optimum condition for fermentation at temperature of 37°C and pH of media set at 5.5 with agitation of 200 rpm using glucose and yeast extract as carbon and nitrogen source in the culture media. From the sample drawn at 1 ml for every hour, the absorbance at 600 nm obtained and the readings used to plot optimum growth curve.

The growth curve exhibited the maximum growth at 1 hour until 8 hours after incubation and agitation, the bacterial growth enters log phase where the bacteria highly dividing in the media. The growth curve pattern identified for each strain determined the time point to sample for analysis in batch culture later where there were manipulated conditions of the media.

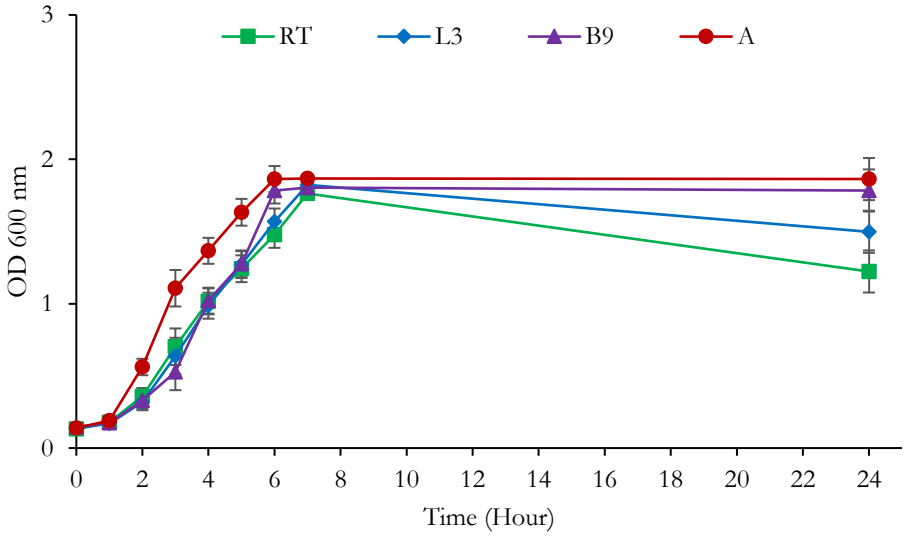

Fig. 1. Optimum growth curve generated through determination of culture medium sample biomass using optical density measurement carried out through hourly sampling. All data are mean values from replica flasks, error bars are ±1 standard deviation.

Optimisation of batch culture at pH 5.5

In this study, the phytase screening media set as pH 5.5 with 1M NaOH and autoclaved to ensure sterility of the media. Media at pH 5.5 was optimum for *Acetinobacter baumanii* identified from bacteria strain L3 (Alias et al., 2017). Other bacterial strains that have optimum growth in media at pH 5.5 are *Bacillus subtilis* BR6 isolated from soil sample from poultry farms (Singh et al., 2013), *Pseudomonas sp.* isolated from poultry faeces

(Selvamohan et al., 2012) and *Enterobacter sp.* isolated from soil in the area surrounding root of leguminous plant (Yoon et al., 1996).

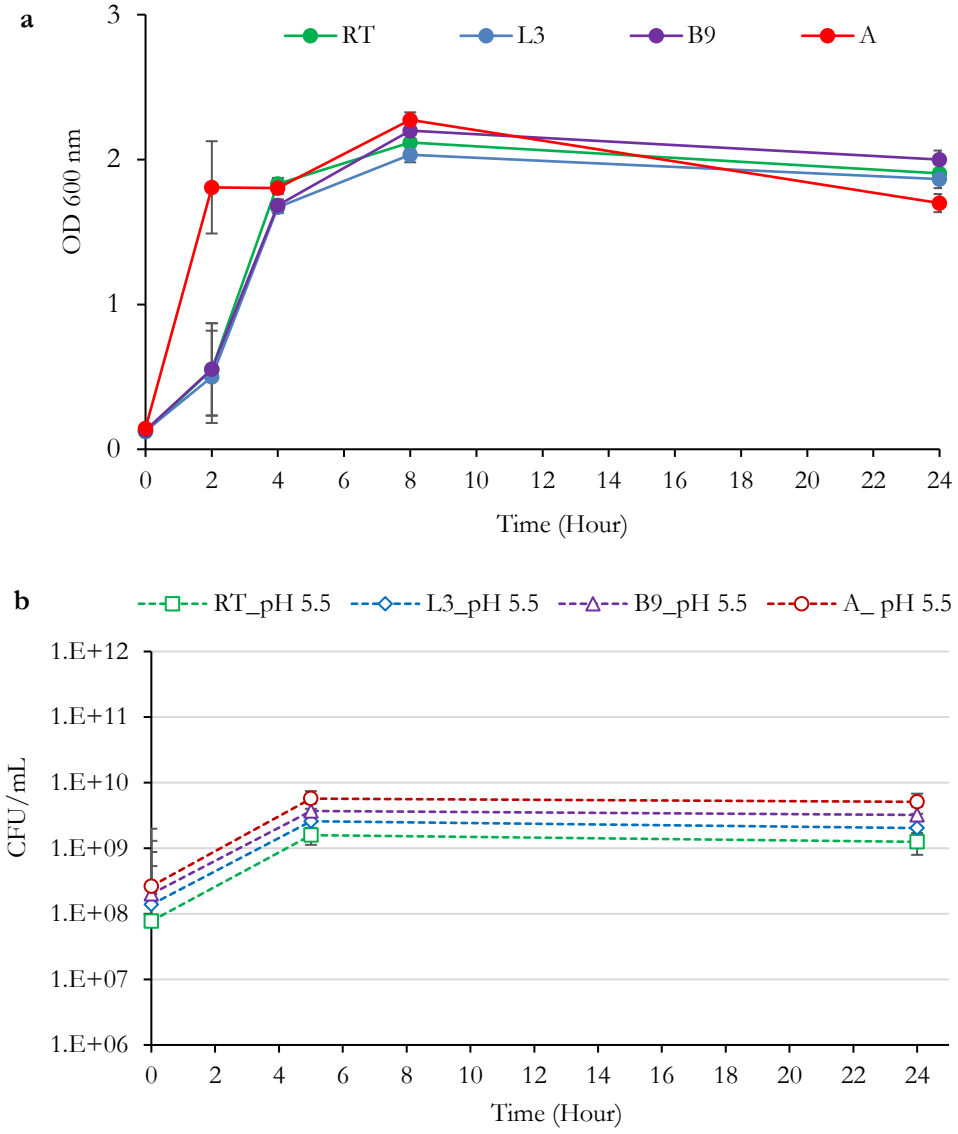

Fig.2. Correlation of all strains for the study of pH optimisation at pH 5.5 for phytase production evaluated by Optical Density at 600 nm and CFU/mL. The potential bacteria producing phytase grown at 37°C with pH of the culture media manipulated at pH 5.5. (a) The OD_{600} nm measured at intervals. (b) Serial dilution of culture samples at 0 hour, 5 hours and 24 hours after inoculation plated on NA plate for determination of cell viability. All data are mean values from replica flasks, error bars are ±1 standard deviation.

The favourability of bacteria towards certain pH influence the production of phytase. Acidic phytase beneficial for application in feed additives as they able to release phosphate in acidic conditions of digestive system in animals. From Fig. 2(a), the bacterial entered lag phase for an hour and started to massively dividing. The bacterial strains entered log phase after one hour of incubation until 4 hours after incubation. Their stationary phase lasts from 5 hours of incubation until 24 hours of incubation.

The pattern of the bacterial growth among the strains are relatively similar and only the bacterial strain A showing rapid growth in their log phase before entering the stationary phase along with other strains even though the bacteria strain A enter death phase rapidly from the other strains at 24 hours after incubation. The pattern curve for the bacteria strain RT, L3, B9 and A differs after 2 hours of incubation that the condition continued for two hours before the curve pattern of bacterial growth become uniform. Through sampling for determination of phytase in the sample at designated times, Shobirin et al. (2010) concluded that during the stationary phase of bacterial growth by bacteria producing phytase, it was the phase that phytase produced continuously. The reduction of bacterial forming linear with the phytase production decrement. From the data obtained through their research, phytase produced when the cultures entered stationary phase and gradually increased until they reach maximum production. The limitation of the nutrients in the culture medium caused the biomass reduction in the sample. The measurement of sample turbidity through optical density requires further analysis for identification of viable bacteria in the sample.

For analysis of colony forming unit counting in media at pH 5.5 as illustrated in Fig. 2(b), the highest CFU counting obtained by strain A (2.01 x 10^9 CFU/mL) when the bacterial strains grown at pH 5.5 obtained from sample culture media of 5 hours after incubation. Meanwhile, the other bacterial strains such as strain L3 (9.98 x 10^8 CFU/mL), RT (1.59 x 10^9 CFU/mL) and B9 (1.12 x 10^9 CFU/mL). The higher viable bacteria obtained in the culture media sample after 5 hours incubation due to strains entered the log phase as the strains enter the log phase at two hours of media incubation. The bacterial cells doubling observed in 5 hours of incubation that showing that the bacterial growth is in log phase. The bacteria may already lose their viability during CFU analysis (Jordan & Jacob, 1947). The higher viable bacteria obtained from strain A linear with the highest optical measured from bacteria strain A.

Table 1. Effect of pH of media at pH 5.5 on growth and phytase production by all bacterial strains. Each flask inoculated with 1% inoculum (overnight culture) and incubated at 37 °C for 72 hours

Strains	Phytase Activity (U/mL)	OD_{600} nm
RT	0.185	1.136
L3	0.220	1.372
B9	0.196	1.255
A	0.140	1.497

Samples from the culture media at pH 5.5 were taken after 72 hours of incubation for phytase activity and biomass measurement. Results are mean from two duplicate samples. At the end fermentation for 72 hours, 0.1 mL of sample withdrawn from each flask and used for enzyme extraction and used to determine the inorganic phosphate release in the mixture. The phytase activity does not directly influenced by OD measurement at 600 nm as shown in Table 1.

Bacteria strain A has highest OD at 1.497 but the phytase activity exhibited by the strain is the lowest among the strain at 0.140 U/mL. Bacteria strain L3 has highest phytase activity at 0.220 U/mL proven the results obtained by Alias et al. (2017) that identified L3 as the best phytase producing bacteria strain grown in media with pH 5.5 and grown at temperature 37°C in the condition of the nitrogen sources manipulated with the presence and absent of peptone. Bacteria strain L3 may has highest phytase activity but there was no correlation with optical density at 1.372.

Optimisation of batch culture at pH 7.0

From Fig. 3(a), bacteria producing phytase strain A has the highest optical density indicating high bacterial activity. The other three strains that are RT, L3 and B9 has the same pattern of growth curve. The lag phase lasts for an hour before entered log phase for 4 hours. From 4 hours to 8 hours after incubation, the bacteria remained in stationary phase and entered death phase. Bacteria strain A entered death phase after 7 hours of incubation. All the bacteria strain entered death phase after 24 hours of incubation.

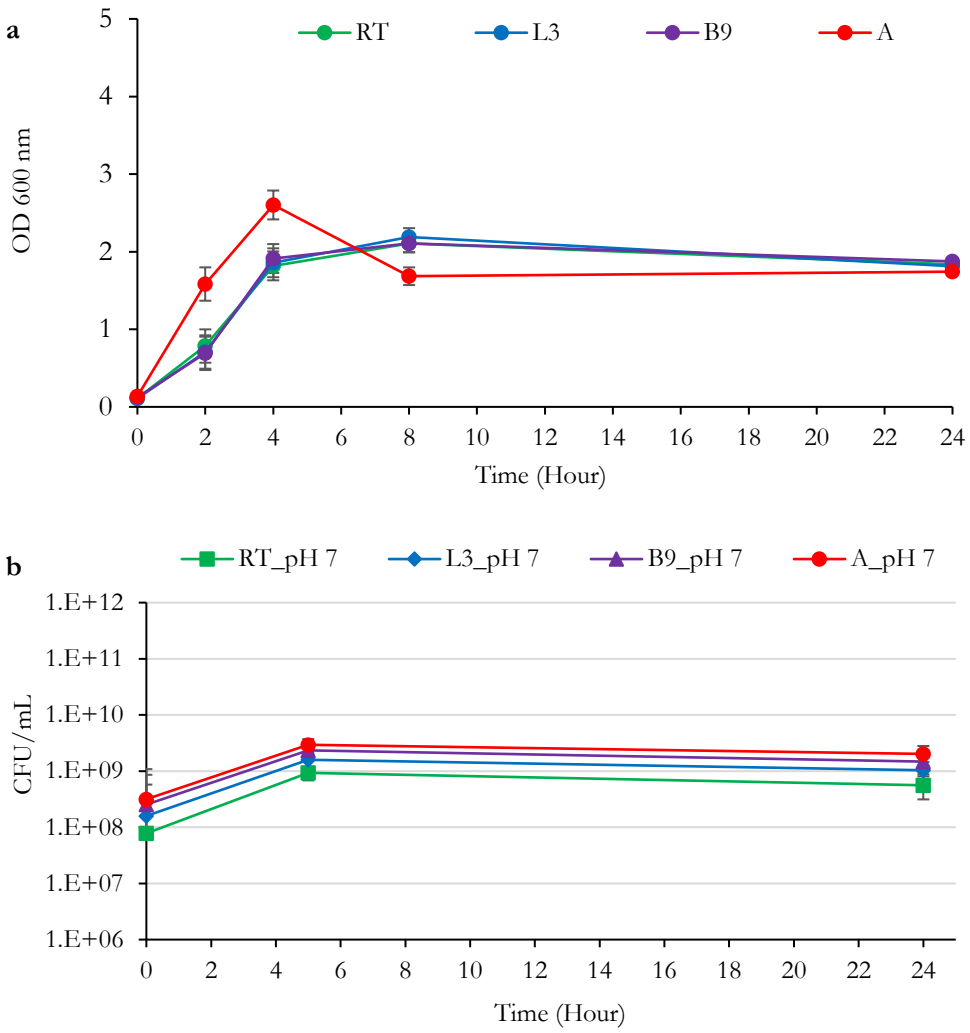

Fig. 3. Correlation of all strains for the study of pH optimisation at pH 7 for phytase production evaluated by Optical Density at 600 nm and CFU/mL. The potential bacteria producing phytase grown at 37°C with pH of the culture media manipulated at pH 7. (a) The OD600 measured at intervals. (b) Serial dilution of culture samples at 0 hour, 5 hours and 24 hours after inoculation plated on NA plate for determination of cell viability. All data are mean values from replica flasks, error bars are ±1 standard deviation.

For quantification of viable bacteria in the sample of the culture media illustrated in Fig. 3(b), at 5 hours the bacterial cells enter log phase where rapid growth exhibited. The highest counting of bacteria obtained from strain A. From the growth shown by the strains in the batch culture, the higher cell density measured through spectrophotometric was not linear with the viable cells in the culture media during the colony-forming unit analysis carried out on designated time. During 5 hours of incubation, the predicted time for highest cell doubling from the pattern obtained from growth curve, the optical density from the batch culture proven through the analysis.

Table 2. Effect of pH of culture media at pH 7 on growth and phytase production by all bacterial strains. Each flask inoculated with 1 % inoculum (overnight culture) and incubated at 37°C for 72 hours.

Strains	Phytase Activity (U/mL)	OD_{600} nm
RT	0.180	1.372
L3	0.205	4.225
B9	0.191	3.843
A	0.126	1.516

Phytase activity in pH 7 correlates the bacterial growths with exception to bacteria strain RT and strain A shown in the Table 2. The highest phytase activity at 0.205 U/mL exhibited by bacteria strain L3 in pH 7 with highest optical density value of 4.225. The lowest phytase activity exhibited by strain A does not correlate with the optical density value as the value of optical density is lower at strain RT with the Strain A has phytase activity at value of 0.126 and optical density value of 1.516.

Comparison between pH 5.5 and pH 7.0

The comparison from the analysis of bacterial growth (OD), CFU and phytase activity from the designated pH at pH 5.5 and pH 7 determined the physical culture condition that influenced the bacterial producing phytase. The figures presented in Fig. 4(a) and (b) used to determination of the optimum conditions for pH of the PSM media between pH 5.5 and pH 7. For comparison between the parameter includes all the analysis in the batch culture. From Fig. 4(a), the consistency of bacteria growth observed in culture media at pH 5.5 although bacteria highly dividing in the media at pH 7. The pattern of growth curve also observable for each strain when grown in media at pH 5.5 rather than pH 7. Bacterial strain A has different pattern of growth curve during log phase when grown in pH 7.

The favourability of the bacterial towards certain range of pH influence the production of phytase. According to Singh et al. (2013) when pH is lower or higher than the optimum pH it will affect the phytase production as the bacteria producing phytase sensitive towards the pH changes in the environment. The production of phytase influence the degradability of phytate. The acidic phytase has optimum pH in the range of 3.5 to 6.0 (Singh et al., 2013). Acidic phytase are desirable for application in feed additives as they are able to release phosphate in the livestock feed in acidic conditions during digestive system in animals.

Viable bacteria obtained from bacterial strain A has the highest CFU counting at pH 5.5 (2.01×10^9 CFU/mL) while at pH 7, it was 6.0×10^8 CFU/mL. The highest viable bacteria were at pH 5.5. From the result obtained, all strains showed no significant difference in their CFU with *P* value of A (*P*=0.584), L3 (*P*=0.437), RT (*P*=0.274) and B9 (*P*=0.228). The CFU counting proves that the rapid growth shown in the 5 hours after incubation of the batch culture have high viable bacteria in the culture medium.

The analysis of the OD may only measure the turbidity of the sample, but with CFU counting, it would be complemented to the bacteria growth analysis. The lowest CFU counting obtained by pH 7.0 resulted from the less favourability of the bacteria towards the neutral pH. From the biomass measurement through OD (600 nm), it is observable that the bacterial has higher biomass in the sample but the viability of the bacterial in the sample was lower. Between both pH 5.5 and pH 7.0, all strains showed no significant difference in their OD reading with *P* value of L3 (*P*=0.378), RT (*P*=0.498), B9 (*P*= 0.449) and A (*P*=0.366).

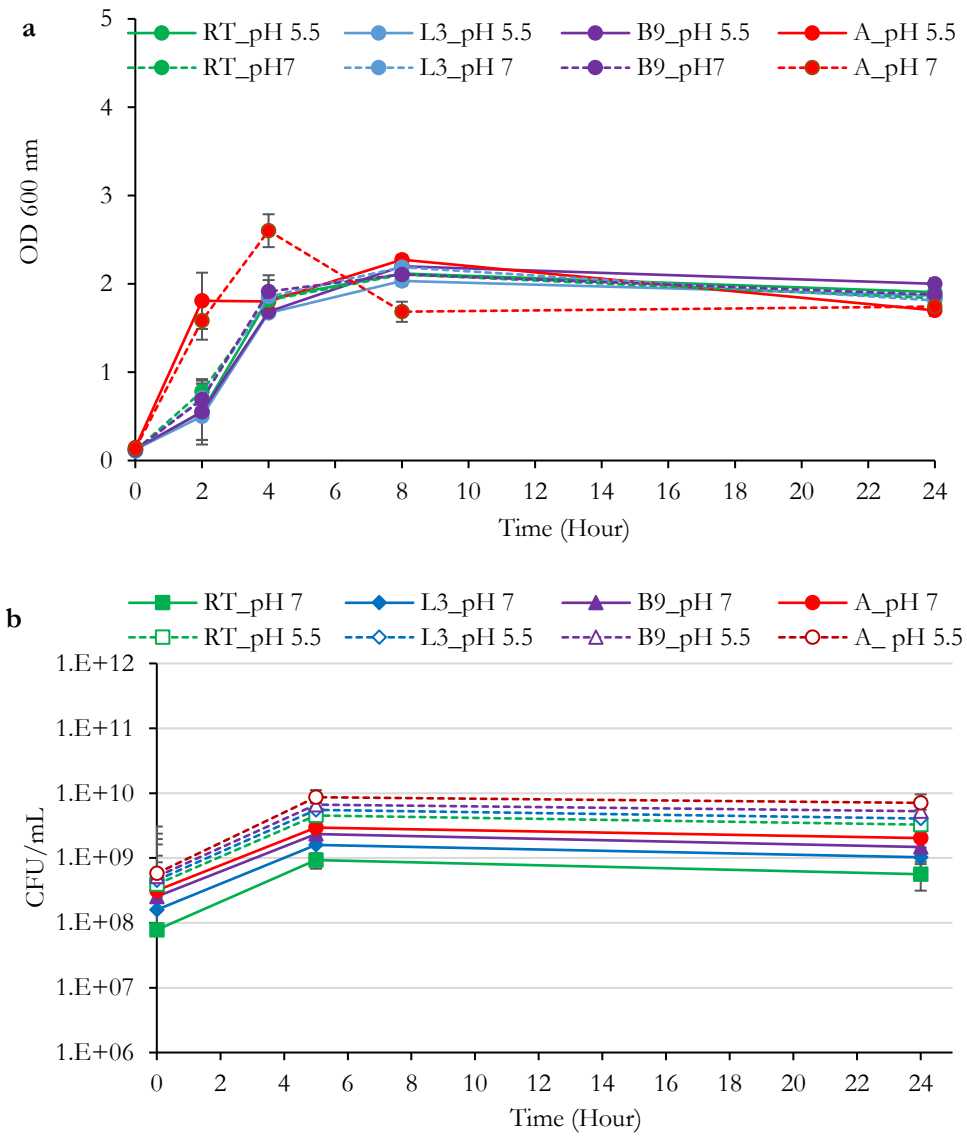

Fig. 4. Correlation of all strains for the study of pH optimisation at pH 7 for phytase production evaluated by (a) Optical Density at 600 nm and (b) Colony Forming Counting (CFU/mL). Results are mean from two duplicate samples, error bars are ±1 standard deviation.

The correlations between the OD and phytase activity showed that the growth of the bacteria does not influence the phytase production. Fig. 5 showed the highest phytase activity was obtained from media with pH 5.5 (Strain L3) while the lowest phytase activity showed by Strain A (with pH 7.0). From the result obtained, there was no significant difference for enzyme activity between both pH for all bacterial strains, with *P* value of RT (P=0.463), L3 (P=0.070), B9 (P=0.442) and A (P=0.110). From a study by Akter et al. (2018) to determine the optimum pH for phytase production, lower capability of phytate degradation at pH 7 leads to lower phytase activity. The higher pH caused growth and enzyme production reduced (Akter et al., 2018). Thus, the phytase activity reduced and less amount of inorganic phosphate liberated in the media. For efficient phytase activity that hydrolyse the phytic acid source from feed in the gastrointestinal tract of the monogastric animals, there were research suggested that the alkaline and acid phytase could be combined (Simon & Igbasan, 2002). The production of biomass occurred simultaneously with the maximum phytase activity (Staden et al., 2007). Phytase activity was

reported to be the highest during stationary phase than log phase and there was potential of phytase production in pH 7.0 with some improvement (Roy et al., 2012).

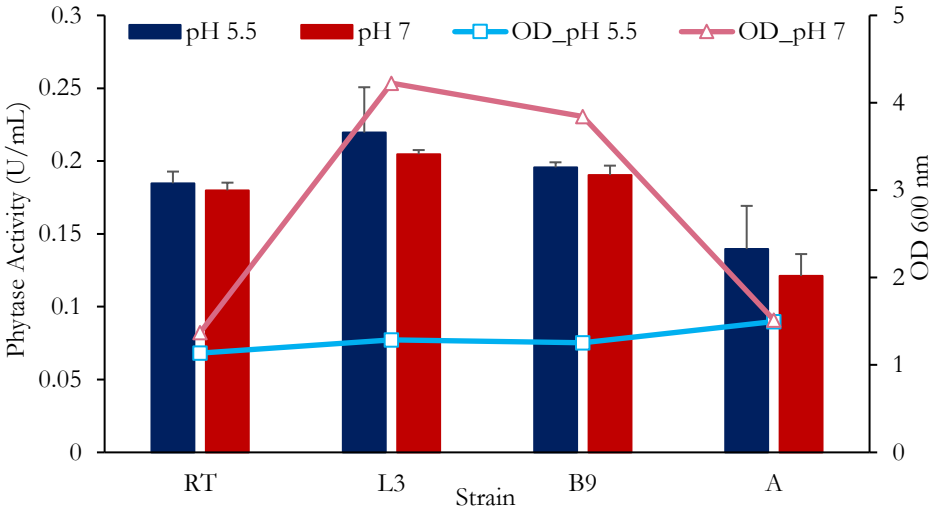

Fig. 5. (Blue) Optimisation result for pH 5.5. (Red) Optimisation result for pH 7. Samples taken after 72 hours of incubation for phytase activity and biomass. Results are mean from two duplicate samples. Bar corresponds to standard deviation.

Determination of pH after incubation at pH 5.5 and pH 7.0

After 72 hours of incubation for media with initial pH 5.5, there were reduction of pH observed as shown in Fig. 6. The bacterial strain L3 has the lowest pH reduction of 2.90 followed by strain RT (2.91), B9 (2.92) and A (3.62). The reduction of pH indicated that there was presence of the phosphate solubilizing bacteria in the culture medium that solubilizes the phytic acid into inorganic phosphate and producing the organic acids in the medium and resulting in medium acidification (Hosseinkhani & Emtiazi, 2010). While for the incubation of pH 7.0, almost all pH values in flasks declined after 72 hours, however strain RT had slightly increased to pH 8.46 at 24 hours of incubation. According to the results, the values of the pH are not significantly different between pH 5.5 and pH 7.0 as the *P* value for each strain are RT (*P*=0.264), L3 (P=0.658), B9 (*P*=0.646) and A (*P*=0.662).

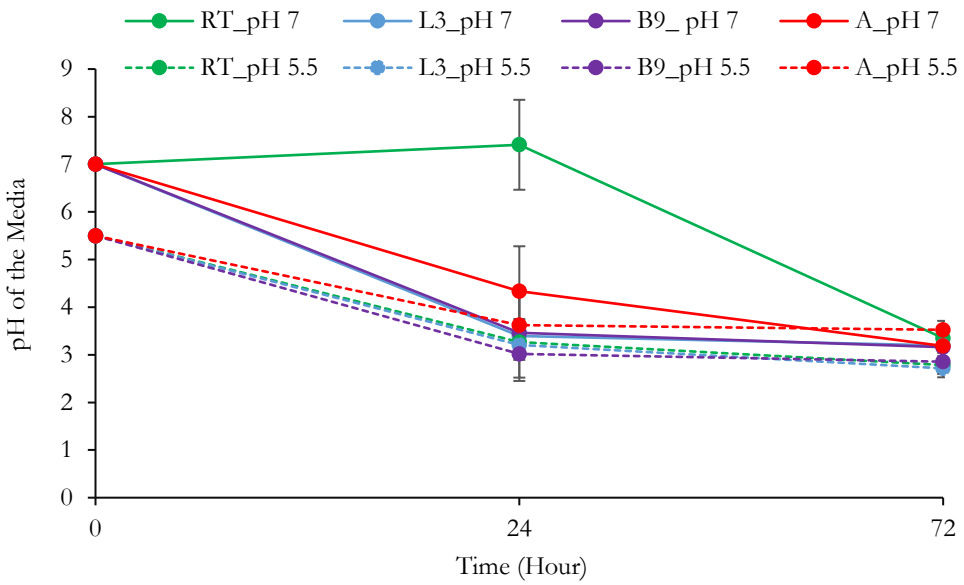

Fig. 6. pH of the media after 24 hours and 72 hours of incubation Results are mean from two duplicate samples, error bars are ±1 standard deviation.

CONCLUSION

Bacteria strains in this study the optimum condition at pH 5.5 has better bacterial growth and phytase production than media at pH 7. The analysis pH after fermentation shown that the bacterial strain L3 was utilized the substrate producing phytic acid higher than other strains. The bacteria producing phytase with high specificity activity are the main concern as they have contribution to the feed industry and the enzyme of interest, phytase has significant role in maintaining ecology. From the study, the fundamental of the bacteria in producing phytase as the desirable enzyme with high yield in the optimised culture medium determined.

ACKNOWLEDGEMENTS

I would like to thank Universiti Sultan Zainal Abidin (UniSZA) for their generous support for this research project, especially for laboratory facilities and chemicals needed in this study.

REFERENCES

Akter, D., Khan, M. M., Mian, M. M., Khan, S. N., & Hoq, M. M. (2018). Phytase Production from a Novel *Klebsiella sp.* on Wheat Bran for Animal Feed Digestion. *Microbial Bioactives*, *1*(1), 14–21.

Alias, N., Shunmugam, S., & Ong, P. Y. (2017). Isolation and molecular characterization of phytase producing bacteria from Malaysia hot springs. *Journal of Fundamental and Applied Science*. *9*(2S), 852-865.

Bhavsar, K., & Khire, J. M. (2014). Current research and future perspectives of phytase bioprocessing. *Royal Society of Chemistry Advances*, *4*(51), 26677–26691.

Boyce, A., Casey, A., & Walsh, G. (2004). A phytase enzyme-based biochemistry practical particularly suited to students undertaking courses in biotechnology and environmental science. *Biochemistry and Molecular Biology Education*, *32*(5), 336–340.

Coban, H. B., & Demirci, A. (2014). Screening of phytase producers and optimization of culture conditions for submerged fermentation. *Bioprocess Biosystem Engineering*, 609–616.

Demirkan, E., Baygın, E., & Usta, A. (2014). Screening of phytate hydrolysis *Bacillus sp.* isolated from soil and optimization of the certain nutritional and physical parameters on the production of phytase. *Turkish Journal of Biochemistry*, 206–214.

Engelen, A. J., Heeft F. C., Randsdorp P. H., Somers W. A., Schaefer J., & Van Der Vat B. J. (2001). Determination of phytase activity in feed by a colorimetric enzymatic method: collaborative interlaboratory study. *Journal of AOAC International*, *84*(3), 629–633.

Hill, B. E., Sutton, A. L., & Richert, B. T. (2009). Effects of low-phytic acid corn, low-phytic acid soybean meal and phytase on nutrient digestibility and excretion in growing pigs. *Journal of Animal Science*, 1518–1527.

Hosseinkhani, B., & Emtiazi, G. (2010). Analysis of phytase producing bacteria (*Pseudomonas sp.*) from poultry faeces and optimization of this enzyme production. *African Journal Biotechnology*, 2–6.

Jongbloed, A. W., Mroz, Z., van der Weij-Jongbloed R., & Kemme, P.A. (2000). The effects of microbial phytase, organic acids and their interaction in diets for growing pigs. *Livestock Production Science*, *67*, 113-122.

Jorquera, M., Oscar M., Fumito M., Petra M., & Maria. D. L. L. M. (2008). Minireview Current and Future Biotechnological Applications of Bacterial Phytases and Phytase-Producing Bacteria. *Microbes and Environment*, *23*(3), 182–191.

Khuntia, H. K., Samal, S., Sahoo, R. K., Kar, S. K., Pal, B. B., & Bihari, K. M. (2010). Isolation and characterization of thermophilic bacteria from hot spring in Orissa, India. *Biosciences, Biotechnology Research Asia*, *7*(1), 449–451.

Kumar, P., Chamoli, S., & Agrawal, S. (2012). Enhanced phytase production from *Achromobacter sp.* PB-01 using wheat bran as substrate: Prospective application for animal feed. *Biotechnology Progress*, *28*(6), 1432–1442.

Kusale, S. P., & Attar, Y. C. (2017). Optimization and Production of Phytase by *Bacillus subtilis* and its Agricultural Application. *International Journal for Research in Applied Science and Engineering Technology*, *5*, 1595–1602.

Lan, G. Q., Abdullah, N., Jalaludin, S., & Ho, Y. W. (2002). Optimization of carbon and nitrogen sources for phytase production by *Mitsuokella jalaludinii*, a new rumen bacterial species. *Letters in Applied Microbiology*, *35*(2), 157–161.

Mohamad, N. M., Ali, A. M., & Salleh, H. M. (2012). Carbon Sources Utilization Profiles of Thermophilic Phytase Producing Bacteria Isolated from Hotspring in Malaysia. *World Academy of Science, Engineering and Technology*, *64*(4), 304–307.

Rocky-salimi, K., Hashemi, M., & Safari, M. (2016). A novel phytase characterized by thermostability and high pH tolerance from rice phyllosphere isolated *Bacillus subtilis* B.S. 46. *Journal of Advance Research*, 381–390.

Roy, M. P., Poddar, M., Singh, K. K., & Ghosh, S. (2012). Purification, characterization and properties of phytase from *Shigella sp.* CD2. *Indian Journal of Biochemistry and Biophysics*, *49*, 266–271.

Selvamohan, T., Ramadas, V., & Rejibeula, M. (2012). Optimization of Phytase Production by *Pseudomonas sp.* Isolated from Poultry Faces. *International Journal of Modern Engineering Research*, *2*(3), 1326–1330.

Shobirin, A., Hussin, M., Farouk, A., Ali, A. M., & Greiner, R. (2010). Production of Phytate-Degrading Enzyme from Malaysian Soil Bacteria Using Rice Bran Containing Media. *Journal of Agrobiotechnology*, 1, 17–28.

Simon, O., & Igbasan, F. (2002). In vitro properties of phytases from various microbial origins. *International Journal of Food Science and Technology*, *37*, 813–822.

Singh, N. K., Joshi, D. K., & Gupta, R. K. (2013). Isolation of phytase producing bacteria and optimization of phytase production parameters. *Jundishapur Journal of Microbiology*, *6*(5), 1–7.

Staden, J. V., Haan, R. D., Zyl, W. H. V., Botha, A., & Viljoen-bloom, M. (2007). Phytase activity in *Cryptococcus laurentii ABO 510*. *7*, 442–448.

Vats, P., & Banerjee, U. C. (2004). Production studies and catalytic properties of phytases an overview. *Enzyme and Microbial Technology*, 35, 3–14.

Vohra, A., & Satyanarayana, T. (2016). Phytases: Microbial Sources, Production, Purification, and Potential Biotechnological Applications, *Critical Review in Biotechnology*, *23*(1), 29-60.

Yoon, S. J., Choi, Y. J., Ki, H., Kwang, M., Cho, K., Kim, J. W., & Jung, Y. H. (1996). Isolation and identification of properties of phytase enzyme. *Enzyme and Microbial Technology*, *0229* (95), 449–454.

Zulkifly, A. H., Roslan D. D., Hamid A. A. A., Hamdan S., & Huyop, F. (2010). Biodegradation of Low Concentration of Monochloroacetic Acid-Degrading Bacillus sp. TW1 Isolated from Terengganu Water Treatment and Distribution Plant. *Journal of Applied Science*, *10*(22), 2940-2944.

How to cite this paper:
Kholed, A. S., Zulkifly, A. H., Alwi, A., Abdullah, T. A. & Alias, N. (2020). Effect of pH on different strains of bacteria producing phytase isolated from Malaysia's hot spring. Journal of Agrobiotechnology, *11*(1S), 79-91.