JOURNAL OF AGROBIOTECHNOLOGY 2020, VOL 11(1S):56-68
e-ISSN: 2180-1983
http://dx.doi.org/10.37231/jab.2020.11.1S.235
https://journal.unisza.edu.my/agrobiotechnology/index.php/agrobiotechnology/index

Comparison on Effects of Temperature on Different Strains of Phytase Producing Bacteria Isolated from Malaysia's Hot Spring

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Received: 17/08/2020, Accepted: 23/10/2020, Available Online: 31/10/2020

ABSTRACT

The main purpose of this research was to find the best growth curve for bacterial growth and the optimum temperature for the production of phytase from different potential phytase producing bacterial strains. A total of four strains used were originally isolated from 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 the culture enrichment while optimisation was carried out through batch culture method using a shake-flask scale. Strains growth and enzyme activity were quantitatively measured at different temperatures at (30°C and 37°C) values. Enzyme activity was determined according to the reaction of the phytase with its substrate (sodium phytate) and expressed in units of phytase activity (U/mL). As for the overall, strain L3 (from Labis, Johor) exhibit promising rate of Pi released in the media at 30°C and 37°C, with optimum phytase activity values of 0.2047 U/mL and 0.2195 U/mL, respectively. The pH of the cultures was also measured, where it shows that strains grown in cultures at 37°C produced a higher phytase activity and resulting a lower reading of pH compared when grown at 30°C. All around, L3 strains has the lowest value of pH when cultured at 30°C and 37°C, with the pH value of 3.62 and 2.37, respectively. From the result obtained, the lower pH indicates the process of phytic acid degradation take place by the phytase in producing inorganic phosphate (Pi) due to the accumulation of organic acid. Since these bacterial strains were originally taken from Hot Springs, further analysis of temperature optimization using 55°C and even 60°C should be carried out. In the future, biochemical research and molecular identification may also be carried out to identify molecular identity in the strains.

Keywords: phytic acid, phytase, myo-inositol, inorganic phosphate, soybean extract

INTRODUCTION

Phytate or phytic acid is known as myo-inositol hexakis-dihydrogen-phosphate (IP6), while phytase (myo-inositol hexakisphosphate phosphohydrolase) is a group of enzymes which catalyse the hydrolysis to remove the phosphates from phytic acid or its salt phytate. The hydrolysis resulting less phosphorylated myo-inositol

intermediates and inorganic phosphate (Rocky-Salimi et al., 2016). This phytate-degrading enzyme could be recovered in numerous sources such as plants, microorganisms, yeast and certain animal tissues (Adeola & Cowiesonf, 2011). 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 the pig is barely available to metabolize phytic acid due to insufficient levels of phytate-degrading enzymes (Singh et al., 2013). Microbial phytases represent a crucial technical enzyme in the feed industry as for their importance in human and animal nutrition. Not only act as a feed supplement in monogastric animals, but these enzymes also being proposed to be potential feed supplementation for aquaculture, human nutrition and plant nutrition (Lei et al., 2013; Troesch et al., 2013).

The development of thermostable phytase has emerged for new possibilities biotechnological applications especially from thermophilic bacteria which can survive at high temperature. Thus, this has led to a number of phytase research nowadays (Demirjian et al., 2001). When it comes to food and feed processing, the major restriction involved in the low thermal endurance of the phytases (Jorquera et al., 2018). Thermal stability is one of the important characteristics that should be possessed by phytase. This is to let the enzyme to stay alive in the brief period of upraised temperature during the process of palletisation and to reduce the substantial activity loss during manufacturing (Mullaney et al., 2000).

Optimisation of the culture conditions such as carbon source, nitrogen source, temperature, pH and agitation play an important role in the bacterial growth and also to the phytase production. *Aspergillus niger*, mostly used in commercial microbial phytase has a temperature optimum of 55°C and their activity decreased when the temperature reached at 80°C. This problem associates with enzyme denaturation especially during pelleting process in the making of feed supplementation (Bhavsar & Khire, 2014). To some extent, the heat destruction of the enzyme can be overcome by the help of protected formulation or chemical coating by giving a thermo-protective coating for the phytases (Lei, 2001; Slominski et al., 2007; Lei et al., 2013; Nuge et al., 2014; Bhavsar & Khire, 2014; Rebello et al., 2017; Jorquera et al., 2018). However, in industrial purposes, thermostable phytases will be the priority for the usage (Roland et al., 2001) as phytases that withstand elevated temperatures would definitely be the selected choice for feed supplements (Nuge et al., 2014). Also, the range of temperatures at which they exist depend on aspects such as species and the history of individual ancestral (Kumar et al., 2019). Moreover, the optimum temperature of the enzyme depends on the source and type of the enzyme (Hafsan et al., 2018). Therefore, further studies are required to generate more than one phytase from the isolates (Konietzny & Greiner, 2002) and optimizing their stability in temperature were determined as it can be used as an approach in order to obtain a better - improved enzyme for the desired biotechnological applications.

Out of all strains used in this study, L3 strain was identified 99% similarity to *Acinetobacter baumannii* based on BLAST homology of 16S rRNA gene sequence conducted by Alias et al. (2017). During their study, 37°C resulted as the optimum temperature for *A. baumannii* to produce phytase in PSM media when compared to 45°C, 50°C and 55°C. However, there is no further study conducted on the *A. baumannii* characteristics in producing phytase as until now, the phytase production was studied in some detail only in *Escherichia coli* and *Raoultella terrigena* on several biological characteristics including substrate specificity (Zamudio et al., 2002).

MATERIALS AND METHODS

Revival of bacteria and stock preparation

The bacterial strains used in this study were previously collected by Mohamad et al. (2012) from different hot springs in Malaysia to screen for the phytase positive strains. Each bacterial strain from the glycerol stocks was grown on NA plates and incubated for 24 hours at 37°C. Under an aseptic condition, inoculation loop was used to pick up a single colony from the streaked plate and inoculate into a sterile tube containing Luria Bertani (LB) broth. The mixture was then incubated for 16 hours to 18 hours at 37°C in an incubator shaker (200 rpm). All single strong colonies on the plate were selected and kept as slant cultures.

Growth conditions

All bacteria strains which have the ability to produce phytase were grown 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. The liquid modified PSM was prepared to contain (g/L) 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 30°C were used for temperature optimisation. The soybean extract was prepared by soaking 100 g soybean in 1 L distilled water for 8 hours. The suspension was boiled for 6 minutes, 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).

Growth curve

A volume of 1.0 mL samples was pipetted out periodically at every hour intervals until the reading become static and further 24-, 48- and 72-hours period. The growth pattern of the bacterial strains determined by measuring turbidity (Optical Density, OD) on a Shimadzu UVmini-1240 UV-Vis Spectrophotometer at 600 nm wavelength. Samples were diluted using phosphate – buffered (PBS) when the reading was larger than 0.8. It was necessary to measure the reading within the linear range of the spectrophotometer, and zeroing the machine was done with ddH_2O (Meyers et al., 2018). The growth sampling was done in a total of 3 days incubation. A growth curve was plotted using absorbance and time for each sample collected with OD measurements.

Temperature optimisation analysis

Growth analysis

A volume of 1.0 mL samples was pipetted out periodically at every hour intervals for the first 8 hours and further at 24 hours period. The bacteria growth was determined by measuring turbidity 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 dilution, plated onto non-selective NA plates, incubated at 30°C for 24 hours and measured as CFU (Colony Forming Unit).

Phytase assay

This phytase activity was assayed according to ammonium molybdate blue method designed by Engelen et al. (2001) and modified by Rocky-Salimi et al. (2016). The activity of the enzyme was determined by quantitatively measuring the released amount of inorganic phosphate during the enzymatic reaction of phytase. A volume of 5.0 mL samples was pipetted out aseptically from conical flasks in the incubator shaker after 72 hours incubation of the batch culture. Then, the sample was transferred into a Falcon tube and spinned at 12,000 rpm (4°C) for 20 minutes. The supernatant was subsequently used for phytase assay. 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 the crude enzyme at 55°C for 30 minutes. 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, 65% nitric acid. Then, the samples were centrifuged at 15,000 × g for 10 minutes at room temperature. The yellow colour developed due to phytase activity was measured spectrophotometrically at 415 nm wavelength (Boyce et al., 2004) and compared by using the standard curve prepared from standard solution of KH_2PO_4. This solution was prepared by initially making a 1 mM stock solution of KH_2PO_4 in water. The stock solution was then diluted to prepare a range of phosphate standard concentrations ranging from 200 to 1000 nmol/mL phosphate. For phytase activity based on Pi release, one unit of phytase activity is defined as the amount of enzyme liberating 1 µmol of Pi per minute under assay conditions (Qvirist et al., 2015).

pH analysis

The pH reading of the culture was taken at 3 points of time which begin at 0 hour, 24 hours and also at the end of incubation, 72 hours. The reading obtained using a pH meter can indicate whether the level of phytate is reduced during the fermentation. Also, indicating the process of degradation the phytic acid takes place by the phytase in producing inorganic phosphate (Pi).

Statistical analysis

All experimental conditions in this study were made in duplicates of the replica flasks. Statistical data were analysed using Microsoft Excel Software 2019 (Microsoft 365) and calculated as the mean of the measurements. The results were presented with ± standard deviation. The difference between the means was tested via T-test and the means of the group were considered significantly different at $P<0.05$.

RESULTS AND DISCUSSION

Bacterial growth curve

Bacteria normally reproduce by an asexual process referred to as binary fission, which forming two equal- sized progeny cells and thus doubling the number of bacterial cells (Desiree et al., 2000). This doubling of time is the time required for the cell population to divide where it varies with environment and organism. Most for under ideal conditions, the bacterium growth can range from 20 minutes, until several hours (Nassif, 2004). Therefore, it is important to identify the curve pattern of each strain and to decide the best time-point to sample for analysis. The growth curve obtained by measuring the OD of the bacteria represented in Fig. 1. It shows the characteristics of the growth curve of four distinct phases during the batch culture: lag phase, log phase, stationary phase, and the death phase.

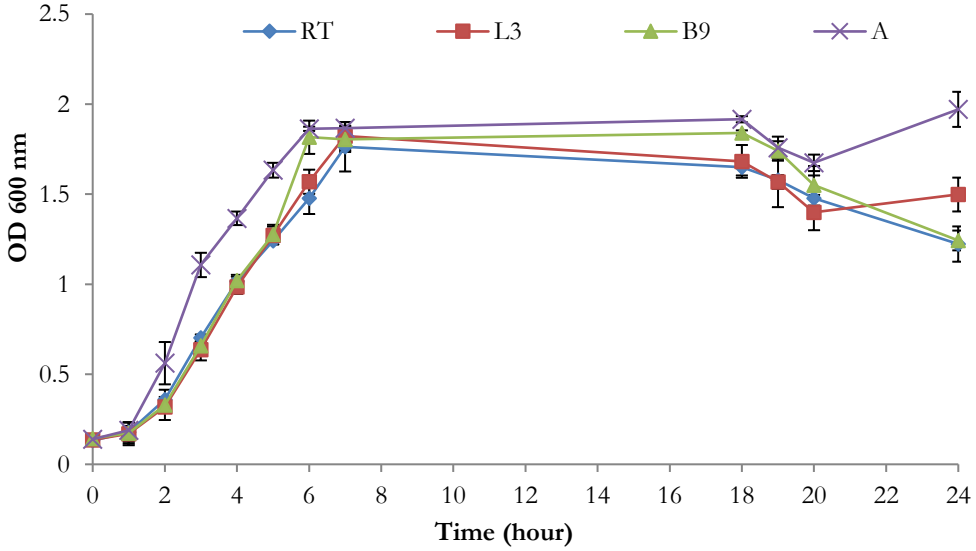

Fig. 1. Bacterial growth curve at 24 hours. The bacterial curves for 4 different strains grown in PSM media, using soy extract as the substrate. The horizontal axes represent time (in hours), while the vertical axes show optical density illustrating bacterial growth by measuring OD at 600 nm. All data shown are mean values from replica flasks, error bars are ±1 standard deviation.

In this growth system, glucose and yeast extract were supplemented in the PSM media to generate a reproducible and robust lag phase in a regulated environment of 37°C, pH 5.5 and on a 200-rpm rotary shaker. For the first 1 hour of incubation, all of the bacterial strains were able to adapt to the environment before they start massively dividing. The bacterial strains survived in the log phase for 8 hours before they reach the stationary phase. At the point of 9 hours, the conditions become unfavourable for the bacterial growth and no further replication. Then, the bacterial strains undergo death phase at 17 hours, except for strain L3 and A where they still showed some growth until reach at the point of 24 hours. Overall, all of the bacterial strains having a 24 hours life time before most of them die.

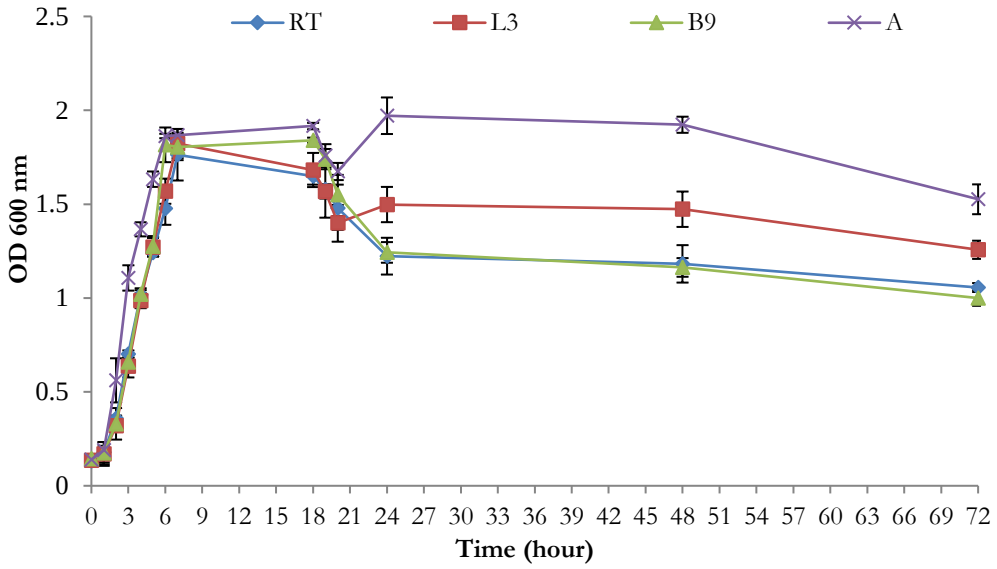

Fig. 2. Bacterial growth curve at 72 hours. The bacterial curves for 4 different strains grown in PSM media, using soy extract as a substrate. The horizontal axes represent time in hours, while the vertical axes show optical density illustrating bacterial growth by measuring OD at 600 nm. All data shown are mean values from replica flasks, error bars are ±1 standard deviation.

From the growth curve above (Fig. 2), all of the strains having a declined in their OD starting at the point of 24 hours until 72 hours. This is where the cells lose their viability, and finally long–term stationary phase (Finkel, 2006). The result shows that at the end of the batch culture at 24 hours, most of the bacterial growth incline. This indicated that the bacteria entering the death phase. Bacterial phytase is known to be an inducible enzyme in which the expression is regulated in a complex manner, but the production of phytase uniformly controlled between different bacteria (Konietzny & Greiner, 2004). According to Jain and Singh (2016), enzyme synthesis is known to take place in the stationary phase with either energy or nutrient limitation. For instance, a study on *E. coli* and *R. terrigena* was observed that the suppression for phytase production occurred during exponential phase and was restored when the strains entered the stationary phase (Konietzny & Greiner, 2004).

Temperature optimisation

Growth analysis

In this study, the temperature of 30°C and 37°C were used for optimisation. The growth curve for both 30°C and 37°C shows that all strains in both conditions having the same pattern of growth. For each strain, the value of the ODs and CFU differentiate their level of growth for different temperature. Between the two temperatures, all of the strain shows more favourable growth condition at 37°C compared to 30°C as bacterial strains grown under 37°C have a higher ODs compared to the bacterial cultures grown at 30°C (Fig. 3). In 2012, Mohamad et al. have used the same strains, grew them at 55°C on NA agar to obtain single colony of the

bacteria. Also, the strains were incubated at 55°C on PSM agar media to screen for the extracellular phytase production. From the previous used of 55°C temperature, it explains why these strains favour to grow at 37°C compared to 30°C since these strains were originally isolated from the Hot Springs (Mohamad et al., 2012).

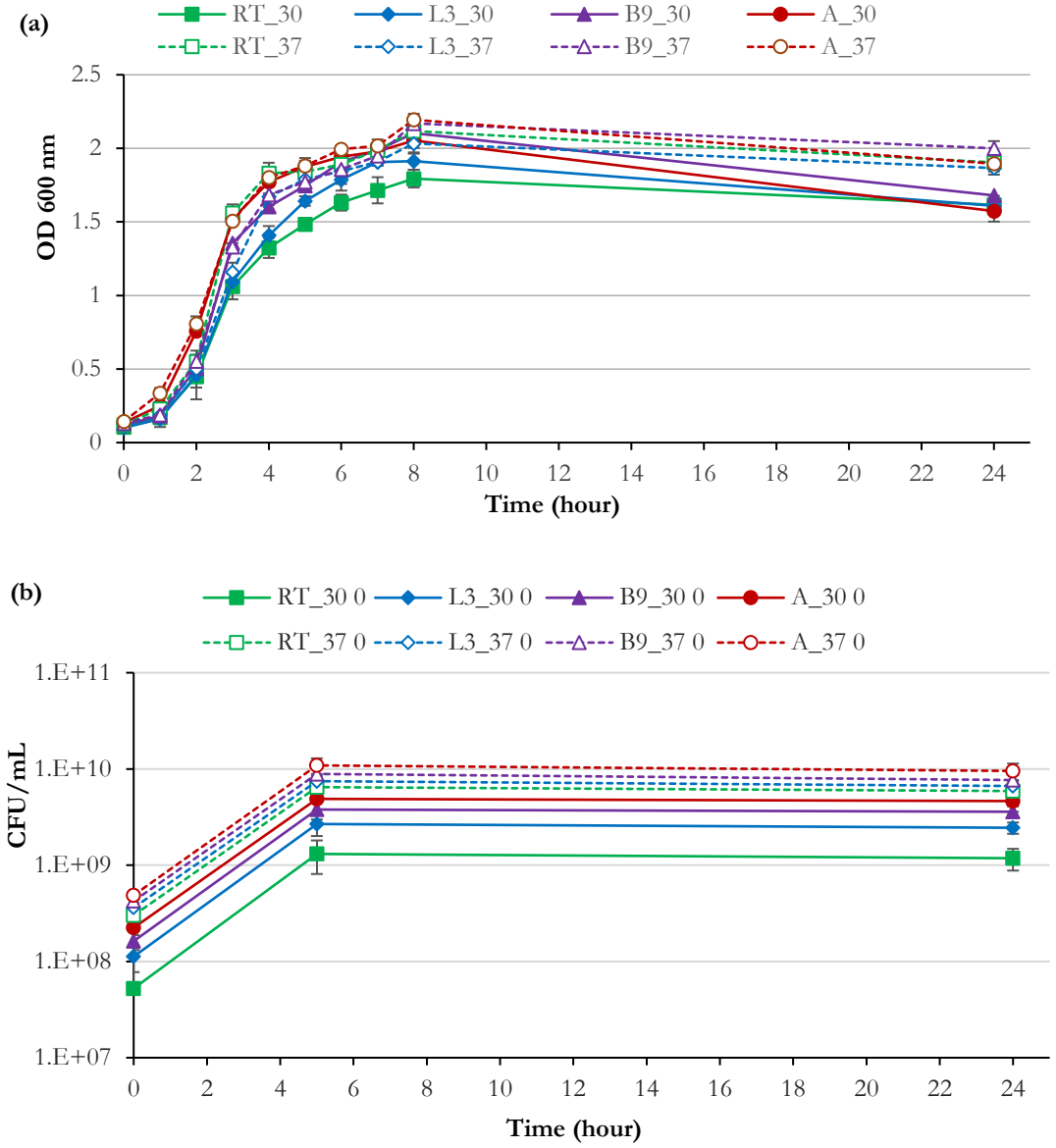

Fig. 3. Correlation of all strains growth for the study of temperature optimisation for phytases production, evaluated by (a) OD_{600} measurement at 24 hours and (b) CFU/mL counting. All potential phytase–producing bacterial strains were grown at pH 5.5, supplemented with yeast extract and glucose as nitrogen and carbon source, with two different temperature conditions; 30°C and 37°C. The OD 600 nm was measured at intervals. Serial dilution of culture samples at 0 hour, 5 hours and 24 hours post-inoculation was plated onto NA agar for an indication of cell viability. The dashed lines (----) represent the optimisation result of temperature condition at 37°C and the straight lines represent the optimisation result of temperature condition at 30°C. All data shown are mean values from replica flasks, error bars are ±1 standard deviation.

Values of the OD were not significantly different between 30°C and 37°C for all strains according to the least significant difference at $P<0.05$. From the result obtained, strain A ($P=0.504$) and B9 ($P=0.446$) were found to be the superior strains that grew in the PSM media for both temperature condition, while strain L3 ($P=0.383$) and RT ($P=0.208$) showed a slower rate of growth. At 8 hours of incubation, the OD reading reached the

maximum value when incubated at 37°C, where strain A (with OD_{600} 2.194), B9 (2.1695), L3 (2.033) and RT (2.1175), while at 30°C, strain A (2.053), B9 (2.103), L3 (1.913) and RT (1.794). This result explained that the bacterial strains are favourable to grow at 37°C. Then at the point of 24 hours, all of the strains show a decline in their ODs and eventually meet death phase at 72 hours.

In 1997, a study conducted by Duffy & Sheridan discovered that the total of viable counts obtained from cultures was affected by changes in temperatures when grown at 25°C, 30°C and 37°C. According to Tortorella (2014), determining the number of bacterial cells using colony counter is one of the procedures where only viable cells were involved. At a cellular level, what determines living and dead is not absolute (Davey & Kell, 1996). However, it is clear that a cell with an intact membrane can be considered alive, actively respiring and capable of undergoing cell division, and this is the basis of the CFU counts (Wyre, 2014). Although these approaches are time–consuming and labour–intensive, these methods are reliable for indicating the cell viability (Ishii et al., 2009). For this observation, the CFU counts are consistent with the observed ODs of the bacterial strains grown at both 30°C and 37°C. Strains L3, B9 and RT have a lower count of the CFU compared to strain A where it presented the highest numbers for both temperatures, around; 30°C (1.59×10^9 CFU.mL $^{-1}$) and 37°C (2.0×10^9 CFU.mL $^{-1}$) counts at 5 hours. Meanwhile strain RT have the lowest CFU counts, of about 1.1×10^9 CFU.mL $^{-1}$ and 1.7×10^9 CFU.mL $^{-1}$ for both 30°C and 37°C, respectively. Between both temperature conditions, all strains showed no significant difference in their CFU with *P* value of L3 (*P*=0.299), RT (*P*=0.424), B9 (*P*= 0.457) and A (*P*=0.234).

At the point of 5 hours incubation, it shows the correlation between the ODs and CFU count where quantification of the viable cell was high and from the growth curve, the bacterial strains are massively growing. At this point in time, all of the bacterial strains are in the log phase, where it can be seen in Fig. 3 showing that the ODs and CFU count are increasing as they are rapidly replicating. Also, from the obtained overall results, it can be said that factors like temperature are affecting the bacterial growth, which explained by an increase in cell viability and cell turbidity when the temperature was set at 37°C.

Phytase assay

The phytase activity was assayed according to Rocky-Salimi et al. (2016) method. After 3 days of incubation at 30°C and 37°C, the cultures were harvested to obtain the supernatants containing inorganic phosphate. Phytase activity was determined by measuring the amount of phosphate released from sodium phytate during the enzymatic reaction. The absorbance used to measure the concentration of phosphate was at 415 nm and the phytase activity is expressed in phytase units (U). One unit of phytase activity is defined as the amount of enzyme that releases 1 μmol of Pi per mL per min under the assay condition (Fig. 4). From the result obtained, there was no significant difference for enzyme activity between the two conditions of temperature for all bacterial strains, with *P* value of RT (*P*=0.292), L3 (*P*=0.188), B9 (*P*=0.266) and A (*P*=0.352).

Fig. 4 shows the correlation between the growth of potential phytase producing bacterial strains with phytase released at 30°C and 37°C for 72 hours. All bacterial strains have shown their ability to produce phytase enzyme. Among two parameters of temperature investigated, the maximum phytase activity was recorded for all strains when incubated at 37°C; L3 (0.2196 U / mL), RT (0.1847 U/mL), B9 (0.1956 U/mL), and A (0.1396 U/mL). This study has proved the statement made by Alias et al. (2017) whose stated that strain L3 is the best phytase producer at 37°C. Meanwhile, the minimum phytase activity was recorded at 30°C for most observed strains, where strain A (0.1212 U/mL), RT (0.1799 U/mL) and B9 (0.1904 U/mL) gives out a lower value. The temperature changes resulted in difference for the phytase activity where most of the strains showed increased activity when up to 37°C. This indicated that the strains are preferred to produce high phytase when grown at 37°C compared to 30°C.

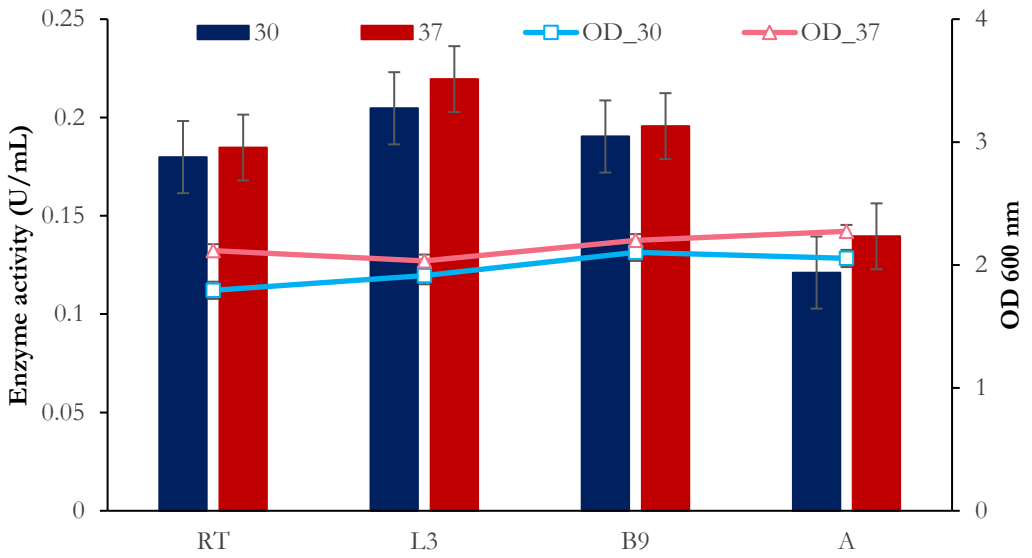

Fig. 4. Effect of temperature on growth and phytase production by all potential bacterial strains. Each flask was inoculated with 1% inoculum (overnight culture) and incubated at 30°C and 37°C for 72 hours. (Red) Optimisation result of temperature condition at 37°C. (Blue) Optimisation result of temperature condition at 30°C. Samples were taken for phytase activity and biomass. Results are means of two duplicate samples and bar corresponds to the standard deviation.

However, the result showed that the phytase activity is not directly influenced by the growth of the cells. Strain A has the highest growth among all of the strains at 37°C (with OD_{600} 2.27) but strain L3 produced the highest phytase activity (0.2195 U/mL). Meanwhile at 30°C, strain L3 also gives out the highest phytase activity with (0.2047 U/mL) while having the OD max of (2.10) from strain B9. Moreover, with the used of soy extract in the PSM media, it shows a positive result where, the phytase activity produced are much higher compared to the chemical substrate (sodium phytate) used in PSM media. In 2017, Alias et al. used the same strain for performing phytase activity with of sodium phytate as a substrate and strain L3 resulting the maximum phytase activity (0.16 U/mL), while when soy extract was used, the strain L3 produced phytase activity of (0.2195 U/mL) at 37°C. It shows that this modified PSM media using an agro-product (soya extract) can be a good substitute of an established substrate (sodium phytate) for the hydrolysing of phytic acid as it has shown a positive result in the growth of the bacterial strains as well as to the production of phytase.

pH analysis

At the end of incubation hours, the result of an estimated change of pH for the four strains incubated at 37°C and 30°C showed that all could produce organic acid that leads to decrease in the pH of the medium (Hosseinkhani et al., 2009). The pH of the cultures was gradually decrease at point incubation of 24 hours and 72 hours (Fig. 5). According to the results, the values of the pH are not significantly different between 30°C and 37°C as the *P* value for each strain are RT (*P*=0.235), L3 (P=0.177), B9 (*P*=0.214) and A (*P*=0.333).

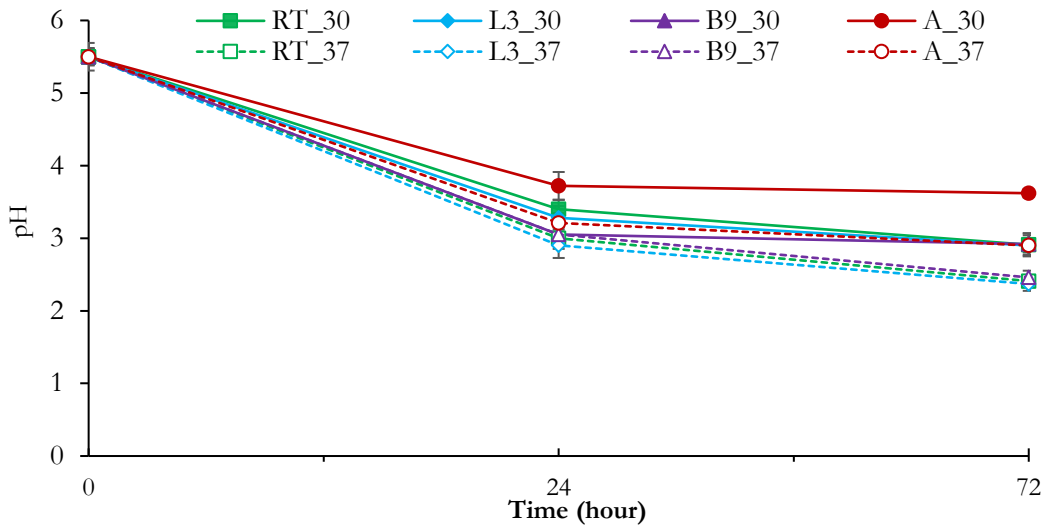

Fig. 5. Effect of temperature on the pH values by all potential bacterial strains. Each flask was inoculated with 1% inoculum (overnight culture) and incubated at 30°C and 37°C for 72 hours. The horizontal axes represent time in hours, while the vertical axes show pH reading illustrating the hydrolysis of phytic acid by phytase produced by bacterial strains into inorganic phosphate. The dashed lines (---) represent the optimisation result of temperature condition at 37°C and the straight lines represent the optimisation result of temperature condition at 30°C.

The pH of media at first was set at 5.5 and gradually decreased to acidic medium. At 37°C of incubation temperature, the pH of strains; A (2.90), B9 (2.46), L3 (2.37) and RT (2.41). At 30°C incubation temperature, the pH of strains; A (3.62), B9 (2.92), L3 (2.90), and RT (2.91). At 72 hours of fermentation, the pH of the culture media shifted to acidic due to the accumulation of organic acid (Hosseinkhani & Emtiazi, 2010). The result obtained indicating the phytase activity produced by the bacterial strains.

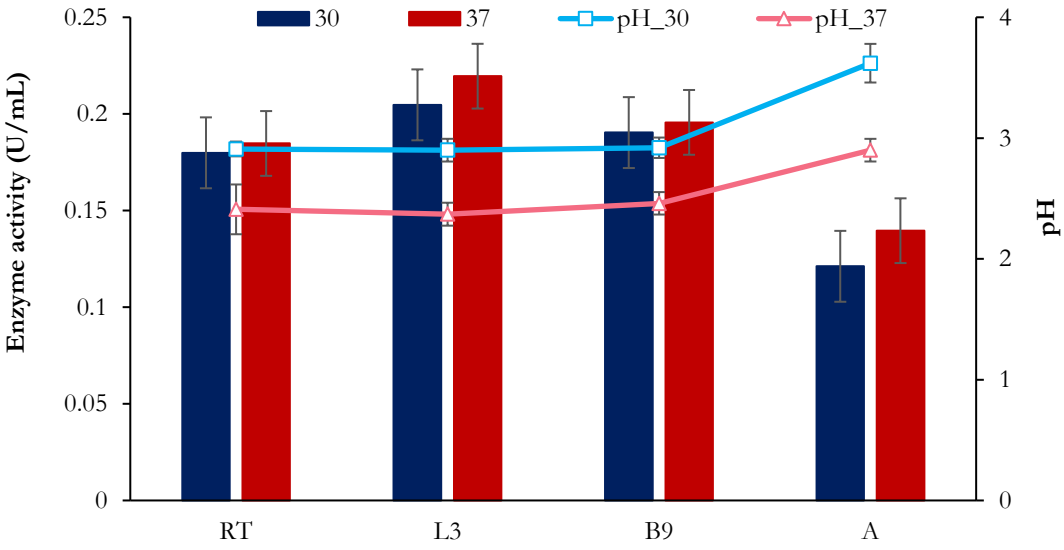

Fig. 6. Effect of temperature on pH cultures and phytase production by all potential bacterial strains. Each flask was inoculated with 1% inoculum (overnight culture) and incubated at 30°C and 37°C for 72 hours. (Red) Optimisation result of temperature condition at 37°C. (Blue) Optimisation result of temperature condition at 30°C. Samples were taken for phytase activity and pH reading. Results are means of two duplicate samples. Bar corresponds to standard deviation.

Fig. 6 shows the correlation between pH values of the culture medium from the bacterial strains with phytase released at 30°C and 37°C for 72 hours. From the result obtained, it shows that strains grown in cultures at 37°C produced a higher phytase activity and resulting a lower reading of pH compared when grown at 30°C. At 37°C, L3 strains produced the highest phytase activity (0.2195 U/mL) with the lowest reading of pH 2.37 while the lowest value of phytase activity (0.1396 U/mL) produced by strain A had the highest reading of pH 2.90 after 72 hours of incubation. The result obtained is same when grown at 30°C, where strain L3 is the highest phytase producer (0.2047 U/mL) with having the lowest value of pH 2.90 while strain A produced the least phytase activity (0.1216 U/mL) with the highest pH reading of 3.62. Therefore, the lower pH of the culture produced the higher phytase activity indicating the process of phytic acid degradation takes place by the phytase in producing Pi. This has been proved by strain A of both temperatures which have the highest reading of pH resulting in the lowest phytase activity.

CONCLUSION

All the conducted analysis for bacterial growth, phytase activity and the pH reading of the cultures were affected by the temperature of 30°C and 37°C. From the ODs obtained, the growth curve of different bacterial strains was successfully identified. The result obtained can conclude that all of the potential bacterial strains shows a favourable growth when incubated in cultures at 37°C in a PSM media supplemented with yeast extract, glucose, pH 5.5, agitated at 200 rpm and using soy extract as a substrate in the media. The maximum phytase activity was recorded at 37°C. From all the bacterial strains, bacteria strain L3 showed the maximum phytase activity in both 30°C and 37°C. Since phytase is reported in numerous applications for phytate degradation, high specificity activity will always be the major concern in selecting bacteria in phytase production. Thus, this study is important to screen bacteria that fundamentally produce phytase at the highest yields in the optimised condition. Although most of the bacterial strains show positive feedback when grown at 37°C, further investigation of the temperature optimisation using 55°C and even 60°C should be done as these bacterial strains were originally taken from Hot Springs. Moreover, biochemical test and molecular identification to identify molecular identity in the strains can be performed in the future.

ACKNOWLEDGEMENTS

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

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How to cite this paper:
Mustafa, A.A., Zulkifly, A.H., Alwi, A., Abdullah, T.A & Alias, N. (2020). Comparison on effects of temperature on different strains of phytase producing bacteria isolated from Malaysia's hot spring. Journal of Agrobiotechnology, *11*(1s), 56-68.